Biochemische karakterisering en structuur-functie verwantschap van
de vier recombinante Old Yellow Enzyme homologen afkomstig van
Shewanella oneidensis

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in de Wetenschappen : Biochemie
Promotor: Prof. Dr. J. Van Beeumen
Biochemical characterisation and structure-function relationship of the four recombinant Old Yellow Enzyme homologues from *Shewanella oneidensis*
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NEDERLANDSE SAMENVATTING
DANKWOORD

Hoewel het vreemd mag klinken, vind ik het schrijven van een dankwoord een van de moeilijkste taken bij het schrijven van een thesis. Het is niet dat ik niet dankbaar ben voor de hulp van anderen, want zonder hen zou deze thesis niet eens bestaan, maar het voelt raar voor me om dit zo op papier te zetten, daarom geen ronkende woorden en hoogdravende zinnen, maar een kort en welgemeend woord van dank.

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Daarom,

dank je wel iedereen,

Debbie van den Hemel
Old Yellow Enzyme (OYE) was first isolated from brewers yeast in 1932 by Christian and Warburg. A few years later Theorell purified the enzyme. In addition he showed the enzyme to be composed of a colourless apoprotein and a yellow dye, flavine mononucleotide (FMN), necessary for catalysis. OYE became a model for flavoprotein study, and many insights in this protein have been gained through the years. OYE homologues (OYEs) can be found in different organisms. The original OYEs were found in the yeasts *Saccharomyces carlsbergensis* and *S. cerevisiae*; later OYE homologues were shown to be present in the yeasts *Kluyveromyces lactis* and *Hansenula polymorpha*. In plants, an enzyme catalysing a key step of the jasmonic acid pathway was recognised as an OYE homologue and quite surprisingly bacterial enzymes able to reduce nitro-esters turned out to be OYE homologues. Very recently, an OYE homologue was found to catalyse a step of the prostaglandin pathway in the parasite *Trypanosoma cruzi*. Many more OYE homologues do exist, and most are found nowadays by genome sequencing projects.

OYEs are type I α,β-barrel flavoproteins, that have a common structure, composed of an eight stranded α,β-barrel with one non-covalently bound FMN group per monomer. In addition to the barrel, the OYE enzymes display a β-hairpin that closes of the bottom of the barrel, and a capping subdomain on top of the barrel. This subdomain is composed of α-helices and/or β-strands and the exact fold differs between the OYE family members. In addition, the loops on top of the barrel define the shape of an active site tunnel that reaches from the surface of the enzyme to the *si*-face of the flavin.

Catalytically, OYE exhibits a Ping-pong mechanism. The FMN cofactor of OYE is first reduced by NAD(P)H, then a substrate binds the active site and is in its turn reduced. The substrates that can be reduced by OYEs are diverse; they range from simple α,β-unsaturated aldehydes and ketones, over steroids to alkaloids, from nitro-olefins over nitro-esters to nitro-aromatics and even to inter-protein disulfide bridges. Not all OYE homologues can catalyse all reactions; the range of substrates depends on the size and shape of the active site tunnel. Some reactions of OYEs are employed for biotechnological applications or investigated for use in bioremediation. It concerns the production of hydromorphon, the production of actinol and the possible reduction of explosives such as TNT. Although the history of OYE dates back to 1932, its physiological function is still not unravelled, and no biological substrate has been determined. However, recent indications for a physiological function exist. The plant and *Trypanosoma* homologues catalyse reactions of the jasmonic acid and prostaglandin pathway respectively. The bakers’ yeast OY2 is indicated to play a role in protection of the actin cytoskeleton against oxidative stress and the yeast OYE3 homologue is upregulated by hydrogen peroxide and indicated for a function in Bax-induced programmed cell death. In addition, the OYE homologue of the Gram-positive bacterium *Bacillus subtilis* has been shown to be induced under conditions of oxidative stress. The role of these homologues is, however, not entirely clear.

*Shewanella oneidensis* is a Gram-negative bacterium with a very diverse metabolism. A BLAST analysis of its genome indicated four OYE homologues to be present. We named these homologues the *Shewanella Yellow Enzymes* (SYE), in analogy to the *Hansenula* (HYE) and *Kluyveromyces* (KYE) enzymes. Since it is exceptional for an organism to possess as many as four OYE homologues with high homology to yeast OYE1, we proceeded in
characterising these SYE enzymes by a comparative analysis. Different characteristics for these enzymes might indicate a difference in function.
Three different approaches were employed: biochemical, structural and functional characterisation.
The general outline of this thesis is as follows:

In Chapter one, a general introduction to both *S. oneidensis* and the OYE family is given. Chapter two and three describe the production and purification of recombinant SYE, followed by the biochemical characterisation of some properties specific for flavoproteins (e.g. photoreduction) and a property specific for OYE homologues (the titration of an OYE homologue with phenolic ligands). Then, the substrate specificity of the different enzymes was determined and the catalytic parameters for these substrates were compared. In Chapter four, we proceeded to the structural approach: theoretical models of the four SYE enzymes were calculated to see if the observations made during the biochemical characterisation were reflected in the structures of these enzymes. In addition, we were able to obtain protein crystals for SYE1, and the X-ray structure of this homologue was determined. Some novel features were observed for the binding of phenolic ligands in the active site. In the third part of this thesis (Chapter five), induction studies were performed to analyse the expression of the SYE proteins in *S. oneidensis* under different growth conditions. In addition a partner search was performed for SYE1 and SYE4 by ProteinChip technology and pull-down assays. Finally, some preliminary studies were performed, giving an onset for future studies (Chapter six).
ABBREVIATIONS

Abs    absorbance
ACN    acetonitrile
ADNT   amino dinitrotoluene
Ala (A) alanine
AMP    adenosine monophosphate
AOC    allene oxide cyclase
AOS    allene oxide synthase
Arg (R) arginine
Asn (N) asparagin
At     Arabidopsis thaliana
B.     Bacillus
bp     base pair

CHP    cumene hydroperoxide
CSDP   Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-
        (5'chloro)tricyclo[3.3.1.13,7]decan}-4-yl)phenyl phosphate
        (a chemiluminescent substrate of alkaline phosphatase)
CT     charge transfer
Cys (C) cysteine

Da     Dalton
DIG    digoxigenine
DLS    dynamic light scattering
D_T   translational diffusion coefficient

DTT    dithiotreitol

E.     Escherichia
EAM    energy absorbing molecule
EDTA   ethylenediaminetetraacetic acid
e.g.   for example
EGDN   ethylene glycol dinitrate
EPA    eicosapentaenoic acid
et al. and others

FAD    flavin adenine dinucleotide
FF     fast flow
Fig.   figure
FMN    flavin mononucleotide

GDN    glycerol dinitrate
Gln (Q) glutamine
Gly (G) glycine
GMN    glycerol mononitrate
GSH    glutathione
GSSG   glutathione disulfide
GST  Glutathione S-Transferase
GTN  glycerol trinitrate
HADNT  hydroxylamino dinitrotoluene
HEPES  4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (buffer)
His (H)  histidine
H-Meisenheimer  hydride-Meisenheimer
HMX  high melting point explosive or octahydro-1,3,5,7-tetranitro tetrazocine
HPLC  high-performance liquid chromatography
13-HPOT  13-hydroperoxy-octadecatrienoic acid
HYE  *Hansenula* Yellow Enzyme
IMAC  immobilised metal affinity chromatography
IPN  isopropyl nitrate
IPTG  isopropylthiolgalactoside
ISDN  isosorbide dinitrate
JMT  S-adenosyl-L-methionine: jasmonic acid carboxyl methyltransferase
KYE  *Klyveromyces* Yellow Enzyme
LB  Luria-Bertani
Le  *Lycopersicon esculentum* (tomato)
Leu (L)  leucine
MeJA  methyl-jasmonic acid
Mes  4-morpholineethanesulfonic acid (buffer)
Met (M)  methionine
NADH  nicotinamide adenine dinucleotide
NADPH  nicotinamide adenine dinucleotide phosphate
NEM  N-ethylmaleimide
NMR  nuclear magnetic resonance
NTA  nitrilotriacetic acid
OD  optical density
OPC-8:0  3-oxo-2-(2′(Z)-pentenyl)-cyclopentane-1-octanoic acid
OPDA  12-oxophytodienoic acid
OPR  OPDA Reductase
OYE  Old Yellow Enzyme

*P.*  *Pseudomonas*
PA  polyacrylamide
pACE  p-hydroxyacetophenone
PAGE  polyacrylamide gel electrophoresis
PBS  phosphate buffer saline
PCR  polymerase chain reaction
PDB  Protein Data Bank
PDN  propylene dinitrate
PEG  polyethylene glycol
PETN  pentaerytritol tetranitrate
PG  prostaglandin
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGDN</td>
<td>propylene glycol dinitrate</td>
</tr>
<tr>
<td>pHBA</td>
<td>p-hydroxybenzaldehyde</td>
</tr>
<tr>
<td>Phe (F)</td>
<td>phenylalanine</td>
</tr>
<tr>
<td>Pro (P)</td>
<td>proline</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>RDX</td>
<td>royal demolition explosive or hexahydro-1,3,5-triazine</td>
</tr>
<tr>
<td>rmsd</td>
<td>root mean square distance</td>
</tr>
<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>S.</td>
<td><em>Shewanella</em> or <em>Saccharomyces</em>, according to context</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SELDI</td>
<td>surface enhanced laser desorption/ionization</td>
</tr>
<tr>
<td>Ser (S)</td>
<td>serine</td>
</tr>
<tr>
<td>sp.</td>
<td>species</td>
</tr>
<tr>
<td>SPA</td>
<td>sinapinic acid</td>
</tr>
<tr>
<td>SSC</td>
<td>sodium chloride/sodium citrate buffer or Structure Screen according to context</td>
</tr>
<tr>
<td>SYE</td>
<td><em>Shewanella</em> Yellow Enzyme</td>
</tr>
<tr>
<td>t-BOOH</td>
<td>tert-butyl hydroperoxide</td>
</tr>
<tr>
<td>Tc</td>
<td><em>Trypanosoma cruzi</em></td>
</tr>
<tr>
<td>TETRYL</td>
<td>trinitrophenylmethylnitramine or N-methyl-N-2,4,6-tetranitroanaline (explosive)</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>Thr (T)</td>
<td>threonine</td>
</tr>
<tr>
<td>TIGR</td>
<td>The Institute for Genomic Research</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TNT</td>
<td>trinitrotoluene</td>
</tr>
<tr>
<td>TOF</td>
<td>time-of-flight</td>
</tr>
<tr>
<td>TRIS</td>
<td>(hydroxymethyl)-aminomethane (buffer)</td>
</tr>
<tr>
<td>Trp (W)</td>
<td>tryptophane</td>
</tr>
<tr>
<td>TST</td>
<td>transition state theory</td>
</tr>
<tr>
<td>Tyr (Y)</td>
<td>tyrosine</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>ultraviolet-visible</td>
</tr>
<tr>
<td>V.</td>
<td><em>Vibrio</em></td>
</tr>
<tr>
<td>Val (V)</td>
<td>valine</td>
</tr>
<tr>
<td>VpYE</td>
<td><em>Vibrio parahaemoliticus</em> Yellow Enzyme</td>
</tr>
<tr>
<td>VvYE</td>
<td><em>Vibrio vulnificus</em> Yellow Enzyme</td>
</tr>
<tr>
<td>VYE</td>
<td><em>Vibrio</em> Yellow Enzyme</td>
</tr>
<tr>
<td>wt/vol</td>
<td>weight/volume</td>
</tr>
<tr>
<td>Xen</td>
<td>Xenobiotic</td>
</tr>
</tbody>
</table>
CHAPTER ONE :
GENERAL INTRODUCTION

I. Shewanella oneidensis

I.A. Classification and characterisation of Shewanellae

Shewanella species are Gram-negative, highly versatile, facultative anaerobic bacteria, belonging to the group of the γ-proteobacteria. Shewanellae are predominantly organisms of aquatic habitats and sediments. They play important roles in the cycling of particulate iron and organic matter, are associated with the spoilage of proteaceous foods, and they can also be opportunistic pathogens. Shewanella putrefaciens was the first species recognised, originally isolated from rancid butter by Derby and Hammer (1931) who designated it ‘Achromobacter putrefaciens’. In 1960 Shewan et al. reassigned Achromobacter putrefaciens to the genus Pseudomonas. Differences in the DNA G+C content between typical members of the genus Pseudomonas and Pseudomonas putrefaciens prompted Baumann et al. (1972) to reclassify this species as Alteromonas putrefaciens. The species Alteromonas putrefaciens remained the sole member of this genus until the introduction of the bioluminescent bacterium Alteromonas hanedai (Jensen et al., 1980). In 1985 Mc Donell and Colwell argued for the reclassification of these organisms into a new genus based on 5S rRNA sequence data and they proposed the name Shewanella. So the genus Shewanella, including the species S. putrefaciens, S. bentica and S. hanedai was created following 5S rRNA analysis.

Most Shewanella species are capable of dissimilatory reduction1 of a wide range of electron acceptors, including metal oxides (e.g. Fe (III) and Mn (IV)) and a variety of other substrates (e.g. nitrate, nitrite, fumarate, elemental sulphur, trimethylamine-N-oxide and dimethyl sulfoxide). Its diverse respiratory capabilities are conferred in part by multicomponent, branched electron transport systems. The potential of these organisms to mediate the co-metabolic bioremediation of halogenated organic pollutants as well as the destructive souring of crude petroleum have also been considered (Venkateswaran et al., 1999). Because of these characteristics, Shewanellae are of environmental significance, and they are presently under investigation in different research groups. Shewanella oneidensis was chosen as the model organism for bioremediation studies worldwide and its genome has been sequenced by the Institute for Genomic Research (www.tigr.org) (Heidelberg et al., 2002).

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1 Dissimilatory reduction of … : coupling the production of energy to the reduction of …
I.B. Bioremediatory possibilities of *Shewanella oneidensis* : Old Yellow Enzyme homologues

As noted above, *Shewanella* species are, because of their diverse respiratory capabilities, under investigation for possible bioremediatory purposes. In a search for enzymes with such properties, the bacterial Old Yellow Enzyme (OYE) homologues caught our attention. The OYE homologues of *Enterobacter cloacae* (French *et al.*, 1996), *Agrobacterium radiobacter* (Snape *et al.*, 1997), and different *Pseudomonas* species (Blehert *et al.*, 1999), were noted in the reduction of different xenobiotics, especially explosive nitro-compounds [glycerol trinitrate (GTN), trinitrotoluene (TNT)], but were said to have a much broader substrate specificity. This was demonstrated by the ability of one *Pseudomonas putida* OYE homologue to reduce the alkaloid morphinone (French and Bruce, 1994). A BLAST search was conducted on the *Shewanella oneidensis* genome (http://tigrblast.tigr.org/cmr-blast/), using OYE1 of *Saccharomyces carlsbergensis* as the probe; four OYE homologues were identified having quite different primary structures. The goal of this project was to characterise these enzymes biochemically and structurally. In the course of the project, after characterisation of the enzymes, we directed our focus towards the determination of their physiological functions.

II. Old Yellow Enzyme homologues

II.A. An introduction to the Old Yellow Enzyme family

II.A.1. Genealogy

Old Yellow Enzyme (EC 1.6.99.1) is an enzyme of great historical importance. Its history starts in 1932, when German scientists (Warburg and Christian, 1932) isolated a yellow coloured protein from brewer’s bottom yeast (*Saccharomyces carlsbergensis*), during attempts to elucidate the nature of biological oxidations. They called it ‘das gelbe Ferment’ or ‘Yellow Enzyme’. In 1935, Theorell purified this ‘gelbe Ferment’ and showed that it comprised of a colourless apoprotein and a yellow dye, both essential for enzyme activity. The yellow dye was shown to be similar to vitamin B<sub>2</sub> (riboflavin). This finding revolutionised the whole field of enzymatic catalysis, highlighting the importance of small vitamin-derived molecules that act as enzymatic cofactors or prosthetic groups. In 1955, Theorell could demonstrate the exact identity of the vitamin B<sub>2</sub> analogue to be riboflavin 5’-phosphate or flavin mononucleotide (FMN). Since then, OYE has been characterised in great detail, acting as a model for flavoprotein study, and considerable knowledge about the mechanism of the enzyme has been gained. It was until 1986, when Miura *et al.* separated a supposedly pure OYE sample in five different peaks by high-performance liquid chromatography (HPLC), before it dawned that multiple OYE homologues were present in yeast. In 1991, Saito *et al.* cloned a first gene for OYE (*OYE1*) from brewer’s bottom yeast and they showed by Southern analysis that at least one other OYE gene was present. It has been noted that the genome of *S. carlsbergensis* is in fact composed of two types of genomes: one derived from *S. cerevisiae* and the other similar

---

2 The name ‘Old’ Yellow Enzyme arose with the discovery of a second ‘new’ yellow enzyme form yeast by Haas in 1938.
to that of *S. bayanus* and *S. monacensis*; therefore the authors proposed that the other, unknown OYE gene originated from the *S. cerevisiae* genome. In 1993, Stott *et al.* cloned an OYE (*OYE2*) gene from baker’s yeast (*S. cerevisiae*). It was different from OYE1 and the unknown *S. carlsbergensis* gene; in addition, they showed that at least one other OYE gene was present in *S. cerevisiae*, thus counting two OYE genes in *S. carlsbergensis* and two genes in *S. cerevisiae*. This second *S. cerevisiae* gene was cloned in 1995 by Niino *et al.*

Within the last three decades, not only the OYE genes of *Saccharomyces* sp. were cloned, but OYE homologues proteins were found in other yeasts. An enzyme catalysing a key step in the jasmonic acid synthesis in plants was recognised as an OYE homologue and even nitro-ester reducing enzymes of bacteria were shown to be members of the OYE family.

A short summary of the best known homologues, the organism of origin and the mode of discovery is given in Table 1.1.

<table>
<thead>
<tr>
<th>Name</th>
<th>Organism of origin</th>
<th>Reference</th>
<th>Mode of discovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>OYE1</td>
<td><em>S. carlsbergensis</em></td>
<td>Saito <em>et al.</em>, 1991</td>
<td>PCR with degenerated primers based on purified protein</td>
</tr>
<tr>
<td>OYE2</td>
<td><em>S. cerevisiae</em></td>
<td>Stott <em>et al.</em>, 1993</td>
<td>Screening of a genomic library with OYE1 as probe</td>
</tr>
<tr>
<td>OYE3</td>
<td><em>S. cerevisiae</em></td>
<td>Niino <em>et al.</em>, 1995</td>
<td>PCR with degenerated primers based on purified protein</td>
</tr>
<tr>
<td>KYE</td>
<td><em>Kluyveromyces lactis</em></td>
<td>Miranda <em>et al.</em>, 1995</td>
<td>Sequencing of a genomic DNA fragment</td>
</tr>
<tr>
<td>HYE1-3</td>
<td><em>Hansenula polymorpha</em></td>
<td>Komduur <em>et al.</em>, 2002</td>
<td>Confers resistance against allyl alcohol</td>
</tr>
<tr>
<td>OYE</td>
<td><em>Candida macedoniensis</em></td>
<td>Kataoka <em>et al.</em>, 2004</td>
<td>PCR with degenerated primers based on purified protein</td>
</tr>
<tr>
<td><strong>AtOPR1</strong></td>
<td><em>Arabidopsis thaliana</em></td>
<td>Schaller and Weiler, 1997</td>
<td>PCR with degenerated primers based on purified protein</td>
</tr>
<tr>
<td><strong>AtOPR2</strong></td>
<td></td>
<td>Biesgen and Weiler, 1999</td>
<td>Genome sequencing</td>
</tr>
<tr>
<td><strong>AtOPR3</strong></td>
<td></td>
<td>Sanders <em>et al.</em>, 2000</td>
<td>Male sterile mutant of <em>A. thaliana</em></td>
</tr>
<tr>
<td>LeOPR</td>
<td><em>Lycopersicon esculentum</em> (tomato)</td>
<td>Straľner <em>et al.</em>, 1999</td>
<td>A cDNA of a tomato shoot cDNA library</td>
</tr>
<tr>
<td><strong>PETN reductase</strong></td>
<td><em>Enterobacter cloacae PB2</em></td>
<td>French <em>et al.</em>, 1996</td>
<td>Bacteria isolated for growth with nitro-esters as the sole source of nitrogen</td>
</tr>
<tr>
<td><strong>GTN reductase</strong></td>
<td><em>Agrobacterium radiobacter</em></td>
<td>Snape <em>et al.</em>, 1997</td>
<td></td>
</tr>
<tr>
<td><strong>XenA and XenB reductase</strong></td>
<td><em>Pseudomonas putida II-B</em></td>
<td>Blehert <em>et al.</em>, 1999</td>
<td></td>
</tr>
<tr>
<td><strong>Morphinone reductase</strong></td>
<td><em>Pseudomonas putida</em></td>
<td>French <em>et al.</em>, 1994</td>
<td>Bacteria isolated for growth on morphine as sole source of carbon</td>
</tr>
<tr>
<td><strong>YqjM</strong></td>
<td><em>Bacillus subtilis</em></td>
<td>Fitzpatrick <em>et al.</em>, 2003</td>
<td>Genome sequencing project</td>
</tr>
<tr>
<td><strong>NemA</strong></td>
<td><em>Escherichia coli</em></td>
<td>Miura <em>et al.</em>, 1997b</td>
<td>Gene mapping membrane technique</td>
</tr>
<tr>
<td><strong>TcOYE</strong></td>
<td><em>Trypanosoma cruzi</em></td>
<td>Kubata <em>et al.</em>, 2002</td>
<td>Searching for a prostaglandin synthase homologue</td>
</tr>
</tbody>
</table>

Table 1.1. An overview of some well known OYE homologues.
This list is far from complete. Many more OYE homologues are nowadays being discovered, mostly through genome sequencing projects. *Shewanella oneidensis*, for example, discussed in this thesis, contains four OYE homologues, identified during a BLAST search of the genome. And, although OYE and its homologues have been under investigation for more than 70 years, they are still a hot topic, due to recent indications for a physiological function (Reekmans *et al.*, 2005; Fitzpatrick *et al.*, 2003; Haarer and Amberg, 2004), investigations in new catalytic mechanisms (proton tunnelling) (Basran *et al.*, 2003) and the discovery of a new type of OYE homologue (YqjM) (Kitzing *et al.*, 2005). In the following sections the characteristics of OYE homologues in general, the catalytic mechanism(s), the possible physiological functions and the known biotechnological applications will be discussed.

II.A.2. Characteristics of OYE enzymes

Structure

The first crystal structure of an OYE homologue was reported in 1994 by Fox and Karplus. It concerned OYE1 from *Saccharomyces carlsbergensis* itself. This structure indicated that OYE belongs to the family of the Class I, eight stranded α,β-barrel flavoproteins. The α,β-barrel is one of the most commonly observed protein structures, comprising about 10% of all structures known (Scrutton, 1993) (Fig.1.1).

![Fig.1.1. Side (left) and top view (right) of the (α,β)_8-barrel of PETN reductase. Note the β-hairpin that closes off the barrel, which is made up by the 8 β-strands that are surrounded by 8 α-helices and the capping subdomain. Pictures were generated with Pymol (DeLano Scientific, San Carlos, CA, USA).](image)

Besides the eight α-helices and the eight β-strands, other structural elements make up a typical OYE barrel (Fig.1.1, right panel). The N-terminus is folded into a short β-hairpin (Fig.1.1, left panel), which closes off the bottom of the barrel. The helices and strands of the barrel are connected by loops; the loops at the bottom of the barrel are short, these on top of the barrel are long and adopt different secondary structures. However, some regularities are observed. Loop 4 and Loop 8 (the loops between β4 and α4, and between β8 and α8 respectively) both form an α-helix. Loop 4 is thought to aid in substrate recognition and Loop 8 is necessary for binding of the flavin. Loop 3 is very long, and forms a sort of capping subdomain on top of the barrel (Fig.1.1, left panel). Thus far, three different conformations have been observed for this loop : an α-β-α-β-motif (Fox and Karplus, 1994), a β-sheet composed of 2 x 2 β-strands
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(Breithaupt et al., 2001) and a β-sheet composed of 2 β-strands connected by a hairpin loop and preceded by a very short α-helix (Barna et al., 2001; Fig. 1.2).

Fig. 1.2. Topology diagram for PETN reductase and morphinone reductase. The cylinders represent the α-helices, the arrows the β-strands. Loop 3 (the capping subdomain) is folded into a β-sheet preceded by a short α-helix (Barna et al., 2001; Barna et al., 2002).

Thus far, five structures of OYE homologues have been published, each having a different quaternary structure. The barrel can exist as a monomer [PETN reductase (Barna et al., 2001) and OPDA reductase (Breithaupt et al., 2001)], as a homo/heterodimer [OYE1 (Fox and Karplus, 1994) and morphinone reductase (Barna et al., 2002)], or even as a tetramer [YqiM (Kitzing et al., 2005)]. A monomeric structure is presented in Figure 1.6, the dimer and tetramer interaction of morphinone reductase and YqiM, respectively, are shown in Figure 1.3.

Fig. 1.3. (left) Morphinone reductase, interaction of the subunits that make up the dimer (Barna et al., 2002); (right) YqiM, tetrameric structure composed of two dimers AB and CD (Kitzing et al., 2005). The molecule represented in the centre of the barrels is FMN.
Flavin

The yellow colour of the enzyme is brought about by the presence of a riboflavin-derived cofactor. This cofactor is a non-covalently bound flavin mononucleotide (FMN) molecule. Two different types of flavin cofactor can occur in flavoproteins; both are derived from riboflavin. FMN is formed by the addition of a phosphate to riboflavin through the action of a flavokinase, flavin adenine dinucleotide (FAD) is subsequently formed from FMN by addition of adenosine monophosphate (AMP) by a pyrophosphorylase (Fig.1.4). Riboflavin is not known to occur as a protein prosthetic group (Methods in Molecular Biology, Vol. 131, 1999).

An oxidised flavin molecule has a characteristic UV-Vis spectrum (Fig.1.5) due to the conjugated double bonds of the ring system. Little difference exists between the spectra of free riboflavin, FMN and FAD. The peak maxima of the spectra that are of interest are the ones at 360 and 450 nm (Methods in Molecular Biology, Vol. 131, 1999). When bound in the active site of a protein, the absorbance properties of the flavin molecule are modulated and the peaks at 360 and 450 nm are red or blue shifted.
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Fig. 1.5. UV-Vis spectrum of riboflavin (http://omlc.ogi.edu/spectra/PhotochemCAD/html/riboflavin.html). The values of the vertical axis (Molar Extinction Coefficient) are presented in M⁻¹ cm⁻¹.

FMN, in OYE homologues, is always bound in a similar fashion on top of the barrel (Fig. 1.6) and is anchored into its structure by an extensive hydrogen bonding network between the side chains and backbones of various conserved amino acids and the flavin itself.

Fig. 1.6. FMN (orange) bound on top of the barrel in PETN reductase. Picture was generated with Pymol.

These conserved amino acids are represented in yellow in the sequence alignment presented in Figure 1.7. Indicated in green are amino acids involved in substrate binding and enzyme catalysis.
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Fig. 1.7. Sequence alignment of MorB (morphinone reductase), OnR (PETN reductase), OPR (OPDA reductase), OYE, and YqjM generated by the program ClustalW. Shown in yellow are residues involved in FMN binding, in green are catalytically important residues. Capital letters: conserved in all 5 sequences, small letters: conserved in 4 sequences, *: conservative substitution in at least 4 sequences. The secondary structure elements are noted—-as reported for OPR1 (Breithaupt et al., 2001)—-below the sequences, with 'a' representing the α-helices and ‘b’ the β-strands.

Pro35, Thr37, Gly/Ala68, and Gln110 (OPR1 numbering) make contacts with the ring system of the flavin and Arg239 with the first two hydroxyl groups of the ribityl tail (Fig.1.8). The phosphate oxygens are hydrogen bonded by a lesser conserved recognition motif; however, Gly308 and Arg331 are conserved throughout the OYE family. It is to note that the amino acids that make contacts with FMN by their amino acid side chains show a better conservation throughout the OYE family than those that contact it by their backbone.

Fig.1.8. FMN bound in the active site of OPR (Breithaupt et al., 2001). Note the conserved amino acids Pro35, Thr37, Ala68, Gln110, Arg239, Gly308 and Arg331.
Ligand binding

One of the most characteristic features of OYE homologues is the ability to bind phenolic ligands with the formation of long wavelength charge transfer (CT) complexes (Fig.1.9; Abramovitz and Massey, 1976a and b).

![Fig.1.9. Spectral changes in Old Yellow Enzyme upon titration with p-chlorophenol (Abramovitz and Massey, 1976b). The spectra numbered 1-7 were recorded for increasing ligand concentrations.](image)

The binding results in long wavelength optical transitions between 500 and 800 nm and in reciprocal perturbations of the oxidised flavin absorption spectrum (350 to 500 nm region). In order to form the CT bands, the ligands must have a parasubstituted ionisable –OH or -SH group, since the broad long wavelength band arises from a charge transfer interaction between the phenolate (electron donor) and oxidised FMN (electron acceptor). It is the result of the transition of the flavin-phenolate complex from its ground state to an excited state upon irradiation by light of the appropriate energy. The hypothesis that the phenolate is the electron donor was confirmed by the fact that (1) reduced (electron rich) FMN does not bind phenolic compounds, (2) the dissociation constant rises with the pKₐ of the ligand and (3) the energy of the charge transfer band (the reciprocal of the wavelength of the CT peak maximum) and the Hammett para constant of the p-substituted phenolic compounds are positively correlated (Abramovitz and Massey, 1976b). The wavelength of the CT peak maximum differs between proteins and is thought to be correlated with the redox potential of the flavin (Stewart and Massey, 1985). Structural studies with p-hydroxybenzaldehyde bound in the active site of OYE showed that the aromatic ring is held parallel to the si-face of the flavin by π overlap, and the phenolate oxygen is hydrogen bonded to Asn191 and His194 (Fox and Karplus, 1994).

II.A.3. The catalytic mechanism

OYE catalyses quite different enzymatic reactions: denitrification of nitro-esters and nitro-aromatics, reduction of the aromatic ring of nitro-aromatics, and reduction of the unsaturated α,β-bond in simple and complex aldehydes and ketones. Some examples are depicted in Figure 1.10.
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Fig.1.10. Different reactions catalysed by OYE homologues. A and B: denitrification of pentaerythritol tetranitrate and glycerol trinitrate, C: reduction of the aromatic ring of TNT, D: reduction of morphinone; E, F and G: reduction of cyclohexenone, trans-2-hexenal and nitrocyclohexene to cyclohexanone, hexanal and nitrocyclohexane respectively (Williams et al., 2004).

Not all enzymes are capable of catalysing all reactions; the reduction of cyclohexenone however is observed for all known homologues. The preference for the different substrates is conferred by the shape of the active site, which has the form of a tunnel, spanning from the top of the barrel to the $\textit{si}$-face of the flavin. Whereas the shape of the cavity just above the flavin is quite rigid and lined with conserved amino acids, the shape of the distal part of the tunnel differs between homologues and little homology can be found between the amino acids that make up its lining. For example, morphinone reductase has a very wide access to the active site, which allows for the bulky alkaloids morphinone and codeinone to enter (Barna et al., 2002).

And, although the substrates are very different, the mechanisms by which the reactions proceed differ little and are discussed below.

Mutational studies have confirmed the importance of different amino acids in some or all reactions. Among these amino acids are Thr32$^3$ (Xu et al., 1999), which modulates the redox potential of the FMN, His186 and Asn189, which are involved in substrate binding (Brown et al., 1998) and Tyr191, which acts as a general acid (Kohli and Massey, 1998), protonating the substrate after hydride transfer from the FMN group (green, Fig.1.5).

OYE$^s$ operate by a Ping-Pong mechanism: the flavin is first reduced by nicotinamide adenine dinucleotide (phosphate) or NAD(P)H and, the substrate then binds into the same active site and becomes reduced. We call these two steps the ‘reductive’ (p11, top) and the ‘oxidative’ half reaction (p11, bottom).

The subject of quantum tunnelling, recently indicated for morphinone reductase and PETN reductase will be discussed briefly (p14).

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$^3$ Unless otherwise noted, numbering according to morphinone reductase is used.
The reductive half reaction

If an OYE enzyme is isolated in an oxidising environment, the flavin is oxidised and needs to be reduced before any substrate can be converted to product. This reduction of the flavin is accomplished by NADH or NADPH. It is possible, by stopped flow measurements, to discern two kinetically distinguishable steps by which the reduction reaction proceeds. At first a complex is formed by NAD(P)H and the oxidised enzyme, then the reduction of the flavin is observed and NAD(P)⁺ is released (Khan et al., 2002).

Most OYE homologues prefer NADPH over NADH, although they can accept both. Only some [e.g. morphinone reductase (French and Bruce, 1994) and GTN reductase (Blehert et al., 1997)] are known to prefer NADH.

The mechanism by which the enzymes discern between the two nucleotide species is not known. No structures of enzyme complexed with NAD(P)H have been determined yet. When NAD(P)H is soaked into crystals of oxidised enzyme, a decolouration is observed, indicating a reduction of the flavin, but the structural data obtained from these crystals show no presence of NAD(P)⁻ in the active site (Barna et al., 2002). It is believed that the affinity of NAD(P)⁻ for reduced enzyme is very low, indicating direct release of the NAD(P)⁻ after reduction. It has thus not been possible to see the mode of binding of NAD(P)H in the active site and to determine the amino acids that make up the cofactor preference. However, some assumptions have been made regarding possible determinants for cofactor preference. It is believed that the presence of positively or negatively charged residues in the Loop 3 capping subdomain determines the preference for NADPH or NADH (Barna et al., 2002).

However, other amino acids that influence the reductive half reaction have been determined by mutational studies. The amino acids His186 and Asn189 (Brown et al., 1998), involved in binding the substrate in the active site, are also involved in cofactor binding and when the conserved tyrosine356 is mutated to phenylalanine, the affinity for NADH is lowered tenfold in morphinone reductase (Messiha et al., 2005a).

The oxidative half reaction

The oxidative half reaction comprises the reduction of different types of substrate. Depending on the substrate used, different steps can be observed. E.g. in morphinone reductase three kinetically resolvable steps are observed when codeinone is reduced; the first being the formation of a charge-transfer complex between enzyme and codeinone, followed by flavin reoxidation and, finally, the release of hydrocodon from the oxidised enzyme (Craig et al., 1998).

The oxidative half reaction has been examined in much detail for the different substrates, and comparisons have been made between different homologues. Among the reactions we will discuss are: the reduction of α,β-unsaturated carbonyl compounds (p11) and nitro-olefins (p12), demitirification of nitro-esters (p13) and the dismutase reaction (p14). The reduction of TNT will be discussed in section II.C.2.

One of the first experiments conducted regarding the activity of OYE consisted of an elaborate screen of different α,β-unsaturated carbonyls (Vaz et al., 1995). Different aldehydes, ketones, esters, amides, nitriles and acids were used, and only ketones and aldehydes were shown to be reduced by OYE. This difference can be ascribed to the fact that ketone and aldehyde carbonyl groups are far more basic than the corresponding ester and amide carbonyl and cyano groups, making it possible for His186 to protonate the carbonyl oxygen and so to increase the electrophilicity of the β-carbon for hydride transfer.
In addition, the effects of substitutions to the α- and β-carbon were evaluated. It was shown that the size and the hydrophobicity of the substituent at the β-carbon is relevant for substrate recognition (Straβner et al., 1999) and may cause steric hindrance. No such relationship could be detected for substituents at the α-carbon. Figure 1.11 depicts the reduction of cyclohexenone.

![Reduction of cyclohexenone](image)

Fig.1.11. Reduction of cyclohexenone. The C=C double bond is polarised by binding to His186 and Asn189 (a), enabling hydride transfer from flavin (b), followed by proton addition from Tyr191(c) (Messiha et al., 2005a).

Other investigations involve the nitro-olefins⁴ (Meah and Massey, 2000). Unsaturated nitro compounds are similar to α,β-unsaturated carbonyls in that the nitro group, just like the carbonyl group, exerts a strong electron attracting influence within the molecule, enhancing the acidity of the hydrogen atoms attached to the α-carbon. This electron attracting influence is further enhanced by the binding of the molecule to His186, allowing hydride transfer from the flavin to the C2 atom to proceed, thereby reducing the double bond. Hydride transfer generates a stable intermediate: a nitronate species. This nitronate binds weakly in the active site and can be released in the solvent, where it is degraded slowly. Alternatively, it can be protonated by Tyr191, generating the corresponding nitrocyclohexane. The Y191F mutant in OYE catalyses the hydride transfer without hinder, but the protonation activity is completely abolished, thus establishing unequivocally the role of Y191 as a general acid in this reaction (Kohli and Massey, 1998). The reaction depicted in Figure 1.12 shows the reduction of nitrocyclohexene to nitrocyclohexane.

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⁴ The nitro-group is bound to a carbon atom.
The third type of substrate considered is the **nitro-ester**, a man-made condensation product of alcohol and nitric acid. Nitro-esters are strong oxidising agents, their oxidising strength depending on the number of nitro-groups present in the compound. The model substrate considered is glycerol trinitrate (GTN), the most prevalent nitro-ester explosive (GTN is a component of dynamite). One (GTN reductase, Snape *et al.*, 1997) or two nitro groups (PETN reductase, Binks *et al.*, 1996) are readily removed by OYE homologues, thus generating glycerol dinitrate (GDN) and glycerol mononitrate (GMN) and one or two nitrite molecules. The rate constant for the removal of the subsequent nitro-groups diminishes, consistent with the lower oxidising capacity of glycerol dinitrate compared to GTN. A Y191F mutation showed that Tyr191 is not required to protonate the substrate (Meah *et al.*, 2001). This is illustrated by that fact that GTN can as readily be reduced by free flavin as by OYE.

Two possible mechanisms are proposed to describe the denitrification reaction (Figure 1.13). In the first case (A) a hydride is transferred directly from the flavin to the nitro-group. This is followed by or concerted with electron redistribution and removal of nitrite. In the second case (B) an electron and a proton are transferred separately. The experimental data, however, were not sufficient to discern between the two mechanisms (Meah *et al.*, 2001).

**Fig. 1.12.** Reduction of nitrocyclohexene. The nitrocyclohexene is first reduced to the nitronate species, which is subsequently protonated by tyrosine to yield nitrocyclohexane (Messiha *et al.*, 2005b).

**Fig. 1.13.** Two possible reactions for denitrification of nitro-esters. A depicts the direct transfer of hydride to the flavin. B shows the reaction in which proton and electron are transferred separately (Meah *et al.*, 2001).
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A special reaction is the **dismutation** reaction (Vaz et al., 1995). A single substrate is capable of both oxidising and reducing the enzyme, thus generating two different products. Neither NADPH nor NADH is needed in these reactions. The reaction was recognised for cyclohexenone and similar substrates by the fact that long-wavelength transition bands were observed similar to those of phenolic ligands. In fact, cyclohexenone is oxidised, thus generating phenol, and subsequently another molecule of cyclohexenone is reduced to cyclohexanone. The phenol can inhibit the reaction by binding strongly in the active site.

**Quantum tunnelling**

Quantum tunnelling (Sutcliffe and Scrutton, 2002) is a recent theory designed to overcome the limits of the traditional transition state theory (TST). The transition state theory ascribes the catalytic activity of enzymes to the lowering of energy required to surmount the barrier between reactants and products. It has been used to depict enzyme-catalysed reactions during the last 50 years.

The first indications that the transition state theory cannot be applied indiscriminately to all reactions came in the late 1980’s- 1990’s. At that time, a modified form of the transition state theory, incorporating a quantum tunnelling correction, was sufficient to cope with the observed abnormalities. In 1996, however, one observed such large deviations from classical transition state theory behaviour that the tunnelling correction alone was not sufficient to explain the discrepancies. It was shown that some reactions proceed solely by quantum tunnelling. Quantum tunnelling is driven by thermal vibrations of the enzyme-substrate complex, which serve to increase the tunnelling probability. It is said to be most probable for reactions with a very high and narrow reaction barrier, where the chance of the reaction going ‘over the hill’ is less probable. The probability of tunnelling also decreases with the molecular weight of the tunnelling component (for example deuterium will have more difficulties in tunnelling than hydrogen). The exact understanding of this theory is beyond the scope of this thesis. Quantum tunnelling has already been shown to play a role in quinoprotein and flavoprotein amine dehydrogenases and in the OYE homologues PETN reductase and morphinone reductase (Basran et al., 2003)

**II.B. Studies investigating the physiological functions of OYE homologues**

The reduction of nitro-esters or other xenobiotic compounds is not thought to be the physiological action of OYEs, but, the answer to the question : 'What is the ‘real’ function of OYE?’ remains unanswered. It is possible that there is no single substrate that can be assigned the title of ‘physiological’ substrate, or it is possible that people just haven’t found the right one yet.

The search for the physiological function of OYEs is complicated by the broad substrate specificity displayed by many homologues. In addition, in the first study of an OYE deletion mutant (OYE2, Stott et al., 1993) no ‘true’ phenotype was observed. Later, it was shown by Haarer and Amberg (2004) that the OYE2 mutant did have a phenotype, but that the ‘right’ conditions to test it were not recognised at the time. This might also be the case for other OYE homologues.

For some homologues, however, a function is do known. It concerns OPR3 of *Arabidopsis thaliana* and *TcOYE* of *Trypanosoma cruzi*. Their functions will be discussed in the sections II.B.1 and II.B.2 respectively.
In addition we will discuss what is known about the physiological function of OYEs in yeasts and bacteria. Since, although the exact function is not yet known, some indications for function exist. In the yeast *Saccharomyces cerevisiae*, both OYEs are indicated to fulfil a role in the oxidative stress response, but each one is involved in a different aspect of this response (II.B.3). A similar conclusion was drawn when studying the expression profile of the OYE homologue YqjM in the Gram-positive bacterium *Bacillus subtilis*: the enzyme might have a role in the oxidative stress response (II.B.4).

To conclude, in Section II.B.5, we discuss ‘how’ the OYE homologues might play a function in this oxidative stress response.

**II.B.1. The jasmonic acid pathway**

The function of the plant OYE homologue OPR3 in the jasmonic acid pathway will be described for *Arabidopsis thaliana* (Turner *et al.*, 2002), as little differences are known for other plants. Jasmonic acid is produced in plants as a response to wounding and stress, but also regulates developmental processes, like that of stamen. Jasmonic acid is not the only functional compound of this pathway. The intermediate oxophytodienoic acid (OPDA) and the derivates Z-jasmone and methyl jasmonic acid are also functional. These molecules are called the ‘jasmonates’. The different jasmonates are synthesised in different cellular compartments: the first steps producing OPDA from linolenic acid occur in the chloroplast. OPDA is converted to jasmonic acid and Z-jasmone in the peroxisomes and the methylation of jasmonic acid occurs in the cytosol.

We will describe the pathway in more detail using Figure 1.14.

The first steps in the biosynthetic pathway occur in the chloroplast, which contains an abundance of α-linolenic acid esterified in glycerolipid and phospholipids. α-Linolenic acid is liberated from these glycerol- and phospholipids by a phospholipase A. The fatty acid is then oxygenated to the hydroxyperoxy derivative 13-hydroperoxy-octadecatricenoic acid (13-HPOT) by the action of a lipoxigenase. AOS (allene oxide synthase) catalyses the subsequent dehydration of 13-HPOT to an unstable epoxide, which is in a stereospecific reaction cyclised by AOC (allene oxide cyclase) to (9S,13S)-12-oxo-(10,15Z)-phytodienoic acid (cis- (+)-OPDA). OPDA is the first compound of the so called ‘jasmonates’. It has its own functions that are different from jasmonic acid. OPDA is now transferred from the chloroplast to the peroxisome. There it will be reduced by the OYE homologue OPR3 to 3-oxo-2-(2'(Z)-pentenyl)-cyclopentane-1-octanoic acid, or OPC-8:0 for short. Thus OPR3 catalyses a key step in the jasmonate pathway: it controls the production of jasmonic acid from OPDA and hence controls the OPDA pool.

OPC-8:0 then undergoes three rounds of β-oxidation to generate jasmonic acid, the second ‘jasmonate’. Jasmonic acid can undergo a further round of β-oxidation to produce (Z)-jasmone, or it can be transferred to the cytosol where it can be methylated by the enzyme S-adenosyl-L-methionine: jasmonic acid carboxyl methyltransferase (or JMT), producing methyl-jasmonic acid (MeJA).
There are two other OYE homologues present in Arabidopsis thaliana: OPR1 and OPR2. They have different preferences for the OPDA stereo-isomers (Fig. 1.15) (Schaller et al., 1998). OPR1 uses the 9R,13R and 9S,13R isomers, with a preference for the first, but not the natural 9S,13S isomer. OPR2 accepts all isomers. The relevance of OPR1 and OPR2 in Arabidopsis is not yet known.

Fig. 1.15. The four stereo-isomers of OPDA. (Schaller et al., 1998).
II.B.2. The prostaglandin pathway

The prostaglandin pathway is similar to the jasmonic acid pathway (Kubata et al., 2002). It exists in mammals and in parasites such as worms (cestodes, trematodes and nematodes) and protozoa (e.g. *Trypanosoma* sp.). The prostaglandins (PGs) mediate inflammatory responses, the blood pressure, the sleep/wake cycle, etc. Their biosynthetic pathway is presented in Figure 1.16. Here the starting point is not linolenic acid (18:3) but arachidonic acid (20:4). Arachidonic acid is liberated from the phospholipids of the cell membranes by a phospholipase (A or C). In mammals, the subsequent peroxidase and cyclooxygenase steps of plants, are performed by a single enzyme: a cyclooxygenase.

![Fig.1.16. The prostaglandin pathway (Kubata et al., 2002). Indicated is PGF$_{2\alpha}$ synthase; the enzyme for which the *Trypanosoma cruzi* OYE homologue substitutes.](http://www.emdbiosciences.com/product/538927)

In *Trypanosoma cruzi* (Kubata et al., 2002), an OYE homologue is found that catalyses the reduction of PGH$_2$ to PGF$_{2\alpha}$. This was quite surprising since other *Trypanosoma* sp. were noted to use an aldo/keto reductase enzyme to catalyse this reaction. The similarity of the prostaglandin and the jasmonic acid pathway is reflected by the fact that TcOYE can convert OPDA and that OPR3 accepts PGJ$_2$ as a substrate (Costa et al., 2000). PGJ$_2$ is presented in Figure 1.17; note the similar structure to OPDA presented in Figure 1.15.

![Fig.1.17. PGJ$_2$ (http://www.emdbiosciences.com/product/538927).](http://www.emdbiosciences.com/product/538927)
II.B.3. Physiological functions of OYE in *Saccharomyces*

The study related to the physiological function of OYE in yeast has known the longest history of all OYE homologues. In 1993, Stott *et al.* constructed an OYE2 mutant; based on their observations they argued the OYE2 homologue to be non-essential. The mutant was, however, only tested for impaired growth under ‘normal’ conditions. In 1995, Vaz *et al.* speculated about an involvement in sterol metabolism, since OYE1 was shown able to reduce nortestosterone to β-estradiol. Kohli and Massey proposed in 1998 a function for OYE in the degradation of lipid peroxidation products. More recent studies proposed a role in the oxidative stress response (Lee *et al.*, 1999).

Recent studies however indicate two distinct functions for the OYE homologues (OYE2 and OYE3) of *Saccharoyces cerevisiae*.

OYE2 responds in a yeast-two-hybrid screen with actin. Actin filaments make up the cytoskeleton of higher cells. They contain two conserved cysteines that can form a disulfide bridge by oxidation. Cross-linking of the actin filaments makes the skeleton more rigid. This cross-linking can be wanted or unwanted. In the wanted reaction, the cross linking controls the plasticity of the cytoskeleton, in the unwanted reaction the cross-linking occurs due to oxidative damage and makes the cytoskeleton too rigid. Investigations indicate that OYE2 reduces the disulfide bridge between the two conserved cysteine residues C285 and C374 (Haarer and Amberg, 2004) and thus controls the plasticity of the cytoskeleton and protects it against oxidative damage. This is supported by the fact that knock-out mutants of OYE2 are more susceptible to damage of the cytoskeleton by hydrogen peroxide, menadione and diamide than wild type cells. The damage by these substances is of a different nature: hydrogen peroxide reacts with metal ions such as Fe$^{2+}$ in the Fenton reaction to produce hydroxyl radicals, menadione produces a semiquinone radical that is a highly thiol-reactive electrophile and that can further react with oxygen to make a superoxide anion (Thor *et al.*, 1982). The reactive oxygen species (ROS) produced by hydrogen peroxide or menadione will react with the sulfhydryls of the cysteine residues to initiate disulfide bond formation.

Diamide treatment, on the other hand, is not correlated with the production of ROS but reacts directly with thiols to produce disulfides. This can cause oxidative stress in two ways, either indirectly by depletion of reduced glutathione or directly by inducing disulfide bonds within or between proteins (Kosower and Kosower, 1995). In addition, in expression array experiments OYE2 is strongly induced by hydrogen peroxide, menadione and diamide treatment, and clusters with other oxidative stress responsive genes (Gasch *et al.*, 2000). Besides reducing the disulfide bridge between actin filaments, it is possible that OYE2 has other protein disulfide substrates (Haarer and Amberg, 2004).

The second OYE of *S. cerevisiae*, OYE3, has been shown to be under the control of the hydrogen peroxide responsive transcriptional regulators Yap1 and Skn7 (Lee *et al.*, 1999). A later study (Reekmans *et al.*, 2005), confirmed the induction of OYE3 by hydrogen peroxide. An OYE3 knock-out mutant was shown to be more susceptible to hydrogen peroxide induced cell death, possibly because the cells are no longer able to detoxify the breakdown products of lipid peroxidation, hence sensitising the cells to oxidative damage. In addition, Reekmans *et al.* demonstrated an involvement of the protein in Bax-mediated programmed cell death.

Apoptotic cell death is linked to NADPH consumption and lipid peroxidation. Nevertheless, in both cases the OYE proteins are induced under conditions of oxidative stress and are able to either detoxify the breakdown products of lipid peroxidation or to reduce disulfide bridges formed by oxidative damage.
II.B.4. Physiological functions in bacteria

For the Gram-positive bacterium *Bacillus subtilis* it was determined by Western analysis under which conditions the OYE homologous YqjM protein is expressed (Fitzpatrick et al., 2003). Under conditions of normal growth, the enzyme appears when the cells enter the stationary phase. The expression can, however, be induced by addition of the substrates glycerol trinitrate and TNT to the culture medium. Since these substrates are able to indirectly exert oxidative stress on cells by the induction of NO radicals and superoxide, it was subsequently investigated whether the induction was caused by oxidative stress or by the substrates themselves. Induction was therefore assayed by the addition of hydrogen peroxide, t-butyl hydroperoxide, and the superoxide generating substance paraquat (producing direct oxidative stress) to the culture medium. Induction was observed in all cases, indicating that induction occurred in response to oxidative stress. In addition, induction of YqjM was observed when a heat shock was applied to the cells. Heat shock induces stresses similar to those of oxidative stress.

So, as previously determined for yeasts, the bacterial OYE homologue YqjM is also involved in an oxidative stress response. Current studies focus on the search for an interaction partner.

II.B.5. A function for yeast and bacterial OYE homologues in protecting cells against oxidative stress?

**Detoxification of lipid peroxidation degradation products**

Lipid peroxidation is a physiological process where, under the influence of oxygen, lipid acids are degraded into smaller parts like α,β-unsaturated aldehydes. This process can be unwanted, for example during oxidative stress, or wanted, producing bactericidal agents in wounded plants (Croft et al., 1993). Some well known peroxidation degradation products are acrolein and trans-2-hexenal. These compounds are toxic to the cell by targeting the DNA and free sulfhydryl groups of proteins. They can be detoxified by conjugation reactions with glutathione, the general redox protector of cells.

Lipid peroxidation predominantly occurs in eukaryotic cells, since poly-unsaturated lipids are the starting point of the peroxidation cascade. Few prokaryotic organisms possess poly-unsaturated lipids, its presence being restricted to predominantly psychrophilic, marine organisms (Allen and Bartlett, 2002; Yano et al., 1994; Gentile et al., 2003). However, α,β-unsaturated carboxyls are good substrates of OYE homologues and the resulting –anals are less toxic than the original -enals (Kubo et al., 2004). In several articles it is speculated that OYEs might have a role in detoxifying these compounds (Komduur et al., 2002, detoxification of acrolein), or are linked to a phenomenon in which these compounds are generated (Bax induced cell death, Reekmans et al., 2005).

Although the process of lipid peroxidation is thought not to occur in bacteria, it can be artificially induced by the addition of linolenic acid to the cells. In the case of *E. coli* this results in the induction of the OYE homologue N-ethylmaleimide A (NemA) reductase (Miura et al., 1997a).

Another interesting observation is that the precursor lipids for the jasmonic acid pathway and the prostaglandin pathway are precisely the lipids from which these aldehydes are derived. A pathway for the generation of trans-2-hexenal from linolenic acid in *Phaseolus* leaves, inoculated with the bacterium *Pseudomonas syringae*, is presented in Figure 1.18 (Croft et al., 1993).
Reducing sulfhydryl groups

Next to lipid peroxidation, the oxidation of –SH groups is one of the earliest observable events during oxidant-mediated damage (Wheeler and Grant, 2004). Since these –SH groups often play a key role in the function of proteins; eukaryotic cells, cyanobacteria and purple bacteria (including the proteobacteria) have adapted a specialised molecule to cope with this kind of damage: glutathione (Thomas, 1984; Sherrill and Fagey, 1998). Glutathione (GSH) is a tripeptide; it acts as a thiol ‘redox buffer’ to maintain the redox state of the cell. Mutants of \textit{E. coli} defective in the synthesis of glutathione were shown to be more susceptible to thiol oxidising agents such as diamide, N-ethymaleimide and acrolein (Apontoweil and Berends, 1975; Fuchs and Warner, 1975). GSH functions by forming adducts with the oxidants, which are either pumped out of the cell (Kaluzna and Bartosz, 1997) or decomposed in recycled GSH and reduced oxidant. Alternatively GSH molecules can form glutathione disulfide (GSSG) dimers that need to be reduced by specialised glutathione reductases in order to recycle the GSH and maintain the GSH:GSSG balance. In addition, a whole gamut of proteins exist in cells for the catalysis of various GSH dependent reactions, such as glutathione reductases, thioredoxines, glyoxalases,… (Wheeler and Grant, 2004).

As has been noted in section II.B.3, OYE2 of \textit{S. cerevisiae} is able to reduce the disulfide bridge between actin filaments and possibly of other protein disulfide substrates (Haarer and Amberg, 2004). This suggests the possibility that oxidative damage to sulfhydryl groups is not made undone only by glutathione related mechanisms but also by OYE homologues.
II.C. Biotechnological applications of OYE homologues

In the review article ‘New uses for an old enzyme’ (Williams and Bruce, 2002) some possible biotechnological applications are summarised for the OYE enzymes. It concerns the production of semi-synthetic alkaloids, the remediation of TNT and the production of dual chiral nitroalkanes. Some of these application are in the meantime worked out in detail (alkaloids), others are still under development (TNT, nitroalkanes), and one new application was added (actinol). These processes are discussed in more detail in the following sections.

II.C.1. Production of hydromorphone from morphine in a two-step process

Hydromorphone is a derivative of the alkaloid morphine which can be isolated from the opium poppy. Hydromorphone is an analgesic which is 5 to 7 times more potent than morphine (Moody et al., 1999, Hailes and Bruce, 1993). The chemical synthesis is difficult, since the C-6 hydroxy group has to be specifically oxidised and is sensitive to strong acids and bases (Hailes and Bruce, 1993).

When bacteria able to grow on morphine as the sole source of carbon were discovered, this opened up possibilities for the semisynthetic production of opiate drugs (Hailes and Bruce, 1993). These bacteria were isolated from industrial waste liquors of a pharmaceutics plant and were identified as Pseudomonas putida M10. The first two enzymes in the morphine degrading pathway were shown to be morphine dehydrogenase (morA, Walker et al., 2000) and morphinone reductase (morB), producing morphinone and hydromorphone respectively. Since these compounds are of medical value, and are troublesome to produce in a chemical manner, the reactions catalysed by these enzymes were investigated in detail. Morphine dehydrogenase specifically catalyses the oxidation of the C-6 hydroxyl group of morphine, thereby generating morphinone. Morphinone in turn is reduced at the 7-8 double bond by morphinone reductase, thus generating hydromorphone. The reaction is presented in Figure 1.19. The production process for hydromorphone and hydrocodone was optimised by Boonstra et al. (2000), using an engineered morphine dehydrogenase enzyme and a soluble pyridine nucleotide transhydrogenase for cofactor cycling between the NADP⁺-using morphine dehydrogenase and the NAD⁺-generating morphinone reductase.

Fig.1.19. Production of hydromorphone (hydrocodone) from morphine (codeine) in a two-step process (Hailes and Bruce, 1993). The OYE homologue morphinone reductase is indicated in red.

The Old Yellow Enzyme homologue is morphinone reductase, which reduces the α,β-unsaturated keton morphinone. Morphinone reductase was shown to reduce, beside
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Morphinone, the alkaloids neopinone and codeinone and the small cyclic enone 2-cyclohexene-1-one (French and Bruce, 1994). Codeinone is an important antitussive compound (Hailes and Bruce, 1993); thebaine, its precursor for synthesis, is often in limited supply (Moody et al., 1999) and the above semi-synthetic production pathway is a great improvement compared to the classical synthetic ones.

Morphinone reductase is a dimeric enzyme with a very wide access to the active site, thus allowing the entrance of the bulky alkaloid substrates. The active site accommodates also bulky steroid compounds as cortisone and progesterone; which are inhibitors of the enzyme (Moody et al., 1999, French and Bruce, 1994).

Morphinone reductase differs from the other OYE homologues by the fact that the general acid tyrosine which protonates the reaction products after hydride transfer is replaced by a cysteine (Fig.1.20). Many studies have been undertaken to identify the nature of the proton donor. By mutation studies combined with nuclear magnetic resonance (NMR) and stopped flow kinetics, it has been shown that the proton is not derived from the cysteine, or from the substrate binding histidine186, nor from tyrosine356 and tyrosine72, leaving a water derived proton as the only possibility (Messiha et al., 2005a; Messiha et al., 2005b). Hydride transfer from the FMN has been shown to proceed by quantum H-tunnelling (Basran et al., 2003).

Fig.1.20. The active site of morphinone reductase with the FMN indicated in orange. The amino acids presented in green are Cys191, His186, Asn189, Tyr356 and Tyr72. The tyrosine depicted in blue is the equivalent tyrosine191 of PETN reductase. The picture was generated with Pymol.

II.C.2. The reduction of explosive compounds: nitro-esters and nitro-aromatics

There are two common types of nitro-containing explosives: nitro-aromatic and nitro-ester compounds. Both are true xenobiotics. They exist solely through the production of man-made industries and no compounds alike exist in nature[5].

Nitro-esters are the condensation products of organic alcohols and nitric acid. Their general structure is C-O-NO₂ (Ramos et al., 1996). Most of them are produced for use as vasodilators (delivering NO in the treatment of angina pectoris) and as explosives. The most prevalent compound is glycerol trinitrate (GTN), but other nitro-esters are also in use, such as

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[5] A nitro-ester type of insect sex-pheromone is known, but these pheromones are derived from long chain alcohols and contain only one nitro-substitution (Binks et al., 1996).
pentaerythritol tetranitrate (PETN), isosorbide dinitrate (ISDN), ethylene glycol dinitrate (EGDN) and propylene dinitrate (PDN) (Ramos et al., 1996; Meah et al., 2001). Nitro-aromatic compounds are chemical intermediates in the production of fertilisers, foams, dyes... and of course explosives. TNT (trinitrotoluene) is the best known member of this class and it is the among the most toxic compounds; other compounds are RDX (royal demolition explosive or hexahydro-1,3,5-triazine), TETRYL (trinitrophenylmethylnitramin or N-methyl-N-2,4,6-tetranitroaniline), HMX (high melting point explosive or octahydro-1,3,5,7-tetranitrotetrazocine) (Hooker and Skeen, 1999), …

The contamination of soils and waters with these compounds is of general concern. There are not only huge contaminations at the production sites, but also at storage depots and munitions testing sites, and there is also contamination due to historical reasons. In addition, the explosives can leak from the soil and thus contaminate the ground water. Nitro-aromatics are often toxic to nature, resulting in land devoid of plant life. Some lands are so contaminated (>200 mg/kg soil, Hooker and Skeen, 1999) that explosive potential exists. As a consequence, numerous studies have been performed for the remediation of both nitro-ester and nitro-aromatic compounds. Different bacteria, fungi and cyanobacteria have been noted for use in bioremediation. Among these, several bacteria have been identified in which an OYE homologue is responsible for the ability to reduce both types of nitro-compounds. Although some authors refer to these bacterial homologues as nitro-ester reductases, this naming is quite confusing, regarding the similarity to the type I oxygen insensitive nitroreductases. These enzymes are also NAD(P)H dependent FMN-containing, monomeric or dimeric flavoproteins, but have a different molecular weight of 25 kDa per monomer and a different fold. They catalyse the denitrification of nitrate-esters and the reduction of TNT to hydroxylaminodinitrotoluene (HADNT) and aminodinitrotoluene (ADNT) (Koder et al., 2002).

In the following section the reduction of nitro-esters and nitro-aromatics will be discussed in more detail.

**Nitro ester reduction**

Although poly-substituted nitro-esters are not natural compounds, they are readily used by both bacteria and fungi (e.g. *Phanerochaete*, Esteve-Nunez et al., 2001) as a source of nitrogen and, upon complete dinitrification, as a source of carbon. Most studies focus on the most prevalent compound: glycerol trinitrate (the explosive component of dynamite). It has been shown that it is possible to remove all three nitrates from glycerol trinitrate, every step proceeding with greater difficulty. In fact, many organisms catalyse the dinitrification to dinitrates and mononitrates, but little are known that catalyse the last step to glycerol. Among these few organisms we note *Rhodococcus* strains which have been shown to completely denitrify GTN (Marschall and White, 2001). Besides bacteria isolated from GTN contaminated soils and water, a study testing 13 laboratory strains of *E. coli*, *Pseudomonas* sp., and *Klebsiela* sp. showed that 9 of these bacterial strains, thought to have never been in contact with nitro-esters, readily reduce GTN. The cells can however not tolerate as high concentrations of GTN as the strains isolated from munitions plants (Blehert et al., 1997). This indicates that previous exposure to GTN is no a prerequisite for bacteria to reduce nitro-esters, but that it might be advantageous.

Four OYE homologues were isolated from bacteria selected for growth on GTN as sole source of nitrogen. It concerns PETN reductase of *Enterobacter cloacae* (French et al., 1996), GTN reductase of *Agrobacterium radiobacter* (Snape et al., 1997), XenB reductase (xenobiotic reductase B) of *Pseudomonas putida*, and XenA reductase of *Pseudomonas fluorescence* (Blehert et al., 1999). They catalyse the stereochemical removal of the first (and
second) nitro-group of GTN. A comparison between 5 different members of the OYE family showed that the ability to denitrify nitro-esters is common (Williams et al., 2004). Homologues thought to have never been in contact with nitro-esters can catalyse the denitrification reaction. It is believed that the denitrifying activity is inherent to these flavoproteins and not brought about by adaptation to an environmental need (Williams et al., 2004). This is in analogy with the study demonstrating that lab bacterial strains can denitrify nitro-esters. The denitrification process in the OYE homologues has been examined, and is described in Section II.A.3. Most studies focus on the detoxification of the nitro-ester glycerol trinitrate. But although contamination with this compound is common, many organisms are able to degrade and mineralise it. The nitro-esters EGDN and ISDN are much more recalcitrant and can form a greater environmental concern, and should therefore be included in remediation studies.

Reduction and bioremediation of TNT

Compared to GTN, TNT is a very recalcitrant compound. In addition, it is highly toxic to nature and numerous studies have been performed regarding its breakdown. Fungi and bacteria have been found to catalyse a few steps in the breakdown of TNT. Overall, two possible pathways are recognised: (1) reduction of the nitro groups, with the production of hydroxyaminodinitrotoluene (HADNT) and aminodinitrotoluene (ADNT) intermediates and highly recalcitrant azoxydinitrotoluene dimers, and (2) direct reduction of the aromatic ring, with the production of hydride-Meisenheimer (H-Meisenheimer) and dihydride-Meisenheimer (2H-Meisenheimer) complexes (Figure 1.21, 2-6). Most studies have focussed on the nitro-group reduction pathway, since H-Meisenheimer complexes were thought to be stable end products, which could not be further reduced. Recent studies however indicate that the 2H-Meisenheimer complex can be further metabolised (Williams et al., 2004). Some of the OYE homologues originally isolated for their ability to reduce nitro-esters can reduce TNT as well. Most homologues only catalyse the nitro-group reduction pathway; a few enzymes however catalyse both reactions, namely XenB reductase and PETN reductase (Pak et al., 2000; French et al., 1998). A comparison between 5 different OYE homologues has shown the importance of the HxxH sequence in TNT reduction. OYE homologues using H_{191}xxN_{194}^6 for substrate binding can only catalyse the reduction of the nitro group, whereas enzymes possessing the H_{191}xxH_{194} sequence can catalyse both the nitro group and aromatic ring reduction (Williams et al., 2004). XenB reductase of *Pseudomonas putida* and PETN reductase of *E. cloacae* have been examined in detail regarding TNT reduction. For the first enzyme the pathway and reaction products have been determined, for the latter enzyme, investigations have been focussed on the reaction mechanism. We will discuss the reaction pathway as determined for XenB (Fig.1.21) first, we will then use the pathway determined to discuss the reactions of PETN reductase. There are two pathways, both being enzymatically catalysed. In the horizontal pathway, two NADPH equivalents are consumed to produce HADNT. HADNT can be reduced by further enzymatic conversion to ADNT. HADNT’s can dimerise to form azoxydinitrotoluene dimers. In the vertical pathway, two NADPH molecules are consumed to subsequently reduce the aromatic ring to the H-Meisenheimer and the 2H-Meisenheimer complexes. The products of the two pathways can react non-enzymatically with each other, producing amino-dimethyl-tetranitrobiphenyl isomers and nitrite. Both types of dimers (azoxydinitrotoluene dimers and

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^6 OYE1 numbering
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the dimethyl-tetranitrobiphenyl isomers) are thought to be resistant to further breakdown. The process consumes loads of NADPH and produces little nitrite (7 NADPH molecules for one nitrite molecule). The reaction is thus not advantageous to the cells. In addition, the reaction products of XenB reductase are thought to be toxic. For PETN reductase it has been shown that the first steps in TNT reduction are similar to those for XenB reductase (Williams et al., 2004). Both reaction pathways are present and TNT is both reduced to (H)ADNTs as well as to 2H-Meisenheimer complexes. The subsequent reaction products have not been identified yet, but there are indications that PETN reductase is able to further reduce the H-Meisenheimer complexes and does not form the amino-dimethyl-tetranitrobiphenyl isomers or the azoxydinitrotoluene dimers.

Fig. 1.21. Reaction products as determined for the reduction of TNT by XenB reductase. 1. TNT, 2. H-Meisenheimer complex, 3-6. 2H-Meisenheimer complex, 8. HADNT, 9. ADNT, 11. azoxydinitrotoluene dimer, 12. amino-dimethyl-tetranitrobiphenyl isomer (Pak et al., 2000).
A high resolution structural study of PETN reductase complexed with TNT has shown a key role for Trp102 in TNT reduction (Khan et al., 2004). The tryptophane hinders the binding of TNT and must move aside to let TNT enter the active site. A mutational study of PETN reductase in which Trp102 was mutated to Phe or Tyr shows higher occupancy for TNT in the active site, and the distribution between the reaction products of the horizontal and vertical pathway is shifted towards the production of the H-Meisenheimer complex. If the identification of the breakdown products of the 2H-Meisenheimer complex shows further reduction, and even opening of the ring, PETN reductase is of great promise in TNT bioremediation.

II.C.3. Phytoremediation approaches for the detoxification of TNT

Bioremediation is the process in which biological means are used to clean up the xenobiotic contamination of the environment. The remediatory action can proceed by means of bacteria or plants. Both have advantages and disadvantages. The remediation process by plants is called 'phytoremediation'. It has some advantages over using bacteria. Plants have an extensive root system that can abstract xenobiotics from the water table. Diluted xenobiotics can thus be extracted from the soil/ water by the root system and can be concentrated in the vacuole (the waste dump of plants cells). In addition, many bacteria live in close contact with the roots, in the rhizosphere. There is, however, one problem using plants because some compounds are so toxic that they just kill the plants that need to detoxify them. Bacteria can more easily adapt to different environmental stresses. Their enzymes catalyse many different reactions with great specificity. On the other hand, laboratory bacteria fail to thrive in natural environments where they have to compete with natural bacteria (Hanninck et al., 2001). A transgenic combination, in which bacterial enzymes are expressed in plants holds the best of both worlds and shows great promise.

In relation to the reduction of TNT by OYE, two articles concerning phytoremediation have been published. The first article (French et al., 1999) discusses the transgenic expression of PETN reductase in Tobacco plants. The introduction of PETN reductase in Tobacco is twofold: (1) the authors hope the enzyme will catalyse the starting reactions in the breakdown of TNT, (2) the introduction of this gene in plants reduces the toxicity of TNT to the plant, one of the major bottlenecks in phytoremediation (Hanninck et al., 2001). Whereas the latter goal was reached, no analysis of the breakdown products has been conducted yet. The second article (Mezarri et al., 2005) focuses on the plants themselves. Plants are more susceptible to the toxicity of TNT than bacteria. Therefore, if one wants to use plants in phytoremediation approaches, it is important to understand the detoxification and stress mechanisms in plants. Here, the authors take a closer look at some enzymes that might be involved in the remediation by Arabidopsis thaliana. Relatively little research has been performed in this field. It is believed that there are three fates a xenobiotic can have in a plant cell: conjugation, conversion and compartmentalisation (Mezzari et al., 2005). The compartmentalisation is the transport of the xenobiotic to the vacuole. Conjugation is the addition of, for example, GSH to the reactive sites of a xenobiotic, making it less toxic. Conversion is either oxidation, reduction,… In this light, the enzymes of study were three glutathione S-transferases (GSTs, conjugation) and two OPRs (conversion) (Old Yellow Enzyme homologues). The study indicated that TNT is toxic to the plant and that OPR is upregulated in the presence of TNT, but no conclusion could be drawn as to whether these enzymes are induced by TNT and convert it, or that they are involved in a general stress response. Both articles fail to discuss the real detoxification: the breakdown of the xenobiotics. It is clear there is still a long way to go, before the detoxification and breakdown of TNT in the environment can be realised.
II.C.4. Production of chiral compounds: actinol and nitro-alkanes

In the review article ‘New uses for an old enzyme’ (Williams et al., 2002) it was suggested that OYE might be used to produce chiral nitro-alkane compounds via a nitronate intermediate. Thus far no article has been published describing this kind of reaction. However, the production of another chiral compound, actinol, has been described. It was observed that Candida macedoniensis is able to stereochemically reduce ketoisophorone to levodione, which can subsequently be reduced to actinol. Other known methods (both chemical and bacterial) for the production of actinol yield racemic mixtures. The enzymes responsible for these actions are an OYE homologue and levodione reductase. Both the Candida OYE protein and OYE2 of Saccharomyces are able to catalyse the reduction of ketoisophorone. The reaction was optimised by the addition of a NADH regenerating system (using glucose dehydrogenase) (Wada et al., 2003; Kataoka et al., 2004).

![Diagram of the production of actinol from ketoisophorone in a two-step process](image)

*Fig.1.22. Production of actinol from ketoisophorone in a two-step process (Wada et al., 2003). Old Yellow Enzyme is indicated in red.*

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7 Actinol is an anti-osteoporosis drug, is used in pharmaceutics and is a precursor for carotenoides. Carotenoids are naturally occurring anti-oxidants with anti-cancer and anti-eye disease properties. They are used as colorants and additives for food, drink and cosmetics.
III. The *Shewanella* Yellow Enzymes

### III.A. Identification of four Old Yellow Enzyme homologues in *Shewanella oneidensis*: presence of multiple OYE homologues in bacterial species

The *Shewanella oneidensis* genome has been sequenced by the TIGR institute ([www.tigr.org](http://www.tigr.org)) and the analysis was published in the journal Nature Biotechnology by Heidelberg *et al.* (2002). The genome consists of a circular 4,969,803-base pair chromosome and a plasmid of 161,613 base pairs.

In order to identify enzymes with possible applications in bioremediation in *S. oneidensis*, we performed a BLAST search on the *Shewanella* genome using the amino acid sequence of OYE1 of *S. carlsbergensis* as the probe, since OYE homologues are likely candidates for bioremediation regarding their broad substrate specificity and the ability to reduce many xenobiotics. Four OYE homologues with a homology score higher than 400 were identified. The result of the BLAST search is represented in Figure 1.23.

![Table](image)

**Table 1.23. Results of a BLAST search on the Shewanella oneidensis genome using OYE1 as the probe. The SO number represents the identification number of the gene, followed by the functional annotation. The score gives the likeliness with the probe. A score above 200 is significant. Expect and P are statistical variables.**

We named the four genes *syel* to *sy4* (*Sye* standing for *Shewanella Yellow Enzyme*) in analogy to the *K*lyveromyces (*Miranda et al.*, 1995) and *H*ansenula (*Komduur et al.*, 2002) genes that were named *kye* and *hye* respectively.

*syel* = SO2454  
*sy2* = SO2453  
*sy3* = SO4153  
*sy4* = SO3392

BLAST searches on related bacteria such as *Pseudomonas sp.* and *Vibrio sp.* showed us that the presence of multiple OYE homologues in bacteria is widespread. Figure 1.24 depicts two representative results of BLAST searches performed on the TIGR website.
**III.B. Genomic surroundings of the sye genes**

The identification numbers of the *S. oneidensis* OYE genes indicate that two genes, SO2453 and SO2454, are neighbouring genes. If one takes a look at the genomic organisation for this region one gets the result given in Figure 1.25 below.

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**Fig. 1.24** Results of a BLAST search on the *Pseudomonas putida* KT2440 (left panel) and *Vibrio parahaemolyticus* RIMD 2210633 (right panel) genomes using OYE1 as the probe.

**Fig. 1.25** Genomic organisation of the region surrounding the SO2453 and SO2454 genes. The numbered genes encode for a zinc-containing alcohol dehydrogenase, a putative NEM reductase (OYE homologue), a FMN binding oxidoreductase (OYE homologue), a transcriptional regulator of the LysR family, and two conserved hypothetical proteins.

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The genomic organisation in the region of the SO4153 gene is:
For the SO3392 gene the result is as follows:

When comparing the genomic regions that surround the *Shewanella* Yellow Enzyme genes, there is one similarity to note: every sye gene is adjacent to a transcriptional regulator gene. The sye1, sye2 and sye3 genes are preceded by a LysR type regulator, the sye4 by a TetR type regulator. This is quite remarkable since *Shewanella*, compared to similar organisms, has little transcriptional regulators on its genome (Heidelberg et al., 2002). Analysis of the genomic regions surrounding the above noted *Pseudomonas* and *Vibrio* genes shows that three of the six *Pseudomonas* genes are preceded by an ArsR type regulator and one of the *Vibrio* genes by a LysR type regulator. Browsing in the articles discussing the cloning of the bacterial OYE homologues, we learned that the genes encoding GTN reductase of *Agrobacterium radiobacter* (Snape et al., 1997) and XenA reductase of *Pseudomonas fluorescens* I-C are adjacent to a ArsR type regulator (Blehert et al., 1999) and that the XenB reductase gene is accompanied by another type of regulator, an AcrR type regulator (Blehert et al., 1999). The LysR-type transcriptional regulators comprise the largest family of prokaryotic transcription regulators. They activate the transcription of operons and regulons involved in extremely diverse cellular functions, often requiring a ligand that acts as a coinducer. This is mostly a small molecule, like a sugar, an amino acid, an inorganic anion,… (Zaim and Kierzek, 2003). The TetR family has recently been reviewed by the Spanish group of Ramos et al. (2005). They have developed a general profile for the proteins belonging to the TetR repressor family and have screened genome and protein databases. Their search produced 2353 family members, but, the set of genes they regulate is only known for 85 regulators (3.6%). It concerns the control of multidrug efflux pumps (e.g. tetracycline resistance), pathways for antibiotic biosynthesis, response to osmotic stress and toxic chemicals,…
ArsR and AcrR transcriptional regulators are said to be involved in arsenate detoxification and resistance to acriflavine drugs, respectively (Blehert et al., 1999). The functionality of these regulators regarding regulation of the OYE homologues has never been discussed.

### III.C. Comparing the Shewanella Yellow Enzymes with OYE homologues of different organisms

When we compare the amino acid sequence of the SYE proteins with OYE homologues of different organisms, we see that the identities range from as low as 29% (YqjM) to as high as 79% (Vibrio and Photobacterium proteins) (Table 1.2.).

![Sequence alignment of members of the Old Yellow Enzyme family generated by ClustalW. The same abbreviations as in Table 1 are used (except Vc_OR : Vibrio cholerae Oxidoreductase). Consensus sequence: boldface capital letters, residues conserved in all sequences; capital letters, conserved in all but one sequence; small letters, conserved in all but two sequences; c underlined, conservative substitutions. The accession numbers of the above noted proteins are Pp_oX : YP_133033, Vp_Nem : BAC62116, Pp_Nem : YP_133310, Vc_Nem : NP_233377 and Vc_OR : AAF96894 (see alignment).](http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi)

![Table 1.2. Sequence identities (%) of the four different SYE proteins with OYE homologues of different species. Represented are, starting with Pp_MorB : morphinone reductase of Pseudomonas putida, PETN reductase of Enterobacter cloacae PB2, OPDA reductase of Lycopersicon esculentum (tomato), OYE of S. cerevisiae, YqjM of Bacillus subtilis, a putative oxidoreductase of Photobacterium profundum, a NEM reductase of Vibrio parahaemolyticus, a NEM reductase of Photobacterium profundum, and finally a NEM reductase of Vibrio cholerae. For every SYE the highest and lowest scores are respectively indicated in green or red. The percentages identity were determined with the bl2seq program available on the ncbi website (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi).](http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi)

We can conclude from this table that the SYE enzymes are clearly not isozymes. They share no more sequence identity to each other than enzymes of different species do. The highest identities (indicated in green) of the different SYEs are either with OYEs of Vibrio sp. or of Photobacterium profundum (a bacterium that just as the Vibrio sp. belongs to the family of the Vibrionaceae; [http://www.cme.msu.edu/Bergeys/](http://www.cme.msu.edu/Bergeys/)). This result might have been expected since it was noted in Heidelberg et al. (2002) that about one third of all Shewanella oneidensis genes are highly homologous to Vibrio cholerae genes.

An extended sequence alignment (Fig.1.28) shows that the SYE enzymes have all the amino acids, except one (discussed Section II.A.2), required to make a functional OYE enzyme, including the catalytical residues (green) or the FMN binding residues (yellow). Some amino acids that will be discussed in more detail in Chapter four are depicted in magenta.

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8 The accession numbers of the above noted proteins are Pp_oX : YP_133033, Vp_Nem : BAC62116, Pp_Nem : YP_133310, Vc_Nem : NP_233377 and Vc_OR : AAF96894 (see alignment).
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annotation of the secondary structure is indicated by extra-

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IV. References


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**Williams, R.E., and Bruce, N.C. (2002)** ‘New uses for and old enzyme’- the Old Yellow Enzyme family of flavoenzymes. *Microbiology* 148, 1607-1614


CHAPTER TWO: CLONING, EXPRESSION AND PURIFICATION

I. Cloning and expression

Since we had found four OYE homologous genes to be present on the *Shewanella oneidensis* genome, we decided to characterise the corresponding enzymes, in order to determine possible differences between them. Therefore, large amounts of pure protein are needed. Since it is not easy to purify a protein from its cell of origin, due to low expression yield, modifications, and so on, it is common practise to clone and overexpress genes in a host organism, such as e.g. *E. coli*. Different cloning vectors are available to modulate the rate of expression, to add ‘tags’ to a protein that aid in purification or that help in stabilising a protein, to provide tRNA for a rare codon, etc. The cloning and expression techniques we used are standard (Ausubel *et al.*, 1990; Sambrook *et al.*, 1989) and will not be discussed in this thesis. Overall, primers were designed based on the sye gene sequences available on the TIGR website. The full sequences of the genes were cloned and constructs were confirmed by DNA sequencing before proceeding to protein expression. The identity of the purified proteins was confirmed by mass-spectrometry and corresponded in all cases to the calculated molecular weights.

I.A. SYE1

*Sye1* was the second *Shewanella oneidensis* OYE homologue we cloned, but the first that we could express in soluble form. The *sye1* gene was at first cloned in a pQE70 vector under control of a T5 promoter, but no expression could be shown. The *sye1* gene was subsequently cloned in a pGEX-4T-2 vector which allows for expression as a GST-fusion protein; the tag added to the N-terminus of the protein. This tag was chosen since GST-fusion proteins often show a good expression, are easy to purify and the tag can be used as a solubility enhancer (Derewenda, 2004). This cloning mode resulted in a high amount of soluble protein. The GST-fusion protein could be removed from SYE1 using thrombin, since a linker region containing a thrombin recognition site (LVPRGS) was present. The success for SYE1 prompted us to clone the other homologues in a similar manner. It is to note, however, that both SYE1 and SYE4 contain a sequence alike the recognition site for thrombin: VPR (Chapter one, Fig.1.28, underlined), thus always exhibiting some secondary proteolytic damage. Addition of a protease inhibitor after incubation with thrombin prevents further damage, but the protein fragments cannot be separated from the uncut protein, so the completely pure enzyme cannot be obtained (Fig.2.1).
The secondary recognition sites for SYE1 and SYE4 are located at the capping subdomain. Scission at these sites probably does not interfere with the action of the protein, but does infer additional freedom to the already mobile capping domain, preventing the protein from being crystallised. For crystallisation purposes, another cloning mode was used to express SYE1. The pACYCDuet-1 vector was used, a low-copy plasmid with two T7 promoters.

The cloning modes of sye1 are summarised in Table 2.1.

<table>
<thead>
<tr>
<th>Cloning modes for the sye1 gene</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>pQE70</td>
<td>NO</td>
</tr>
<tr>
<td>Forward primer</td>
<td>AGATCTATGACGCAATCACTGTTT</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>AAGCTTCTAAAGCTTGATAAGTTG</td>
</tr>
<tr>
<td>Modes of expression tested</td>
<td>28 and 37°C</td>
</tr>
<tr>
<td></td>
<td>1 mM IPTG</td>
</tr>
<tr>
<td></td>
<td>LB, SOB and 2 x YT medium</td>
</tr>
<tr>
<td></td>
<td>Sorbitol and betaine</td>
</tr>
<tr>
<td></td>
<td>E. coli MC1061 cells</td>
</tr>
<tr>
<td>pGEX-4T-2</td>
<td>YES</td>
</tr>
<tr>
<td>Forward primer</td>
<td>GAATTCCTATGACGCAATCACTGTTT</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>GCGGCCGCTAAAGCTTGATAAGTTTG</td>
</tr>
<tr>
<td>Modes of expression tested</td>
<td>E. coli BL21 (DE3) cells</td>
</tr>
<tr>
<td></td>
<td>28 and 37°C</td>
</tr>
<tr>
<td></td>
<td>1.0 and 0.5 mM IPTG</td>
</tr>
<tr>
<td>pACYCDuet-1</td>
<td>YES</td>
</tr>
<tr>
<td>Cloning mode</td>
<td>Cloned directly from the pGEX-SYE1 construct</td>
</tr>
<tr>
<td>Modes of expression tested</td>
<td>E. coli BL21 (DE3) cells</td>
</tr>
<tr>
<td>Expression</td>
<td>28°C, 0.5 mM IPTG</td>
</tr>
</tbody>
</table>

Table 2.1. Cloning and expression modes of sye1.
I.B. SYE2

Sye2 was the first *Shewanella oneidensis* OYE homologue cloned, but in the first cloning mode no soluble protein was produced. So we tested different expression vectors, cloning modes and other experimental conditions; despite this we have not been able to obtain the enzyme in soluble form. We will describe here the different cloning modes tested; a summary can be found in Table 2.2.

1. The first cloning mode of the gene was in the pET11a vector. This vector has a T7 based promoter, inducible with isopropyl thiogalactoside (IPTG). Expression of SYE2 was observed, but the protein was almost entirely present in insoluble form. Different conditions of expression were tested in order to optimise expression and improve the solubility. The best expression condition was *E. coli* BL21(DE3) cells, grown at 28°C, in LB medium supplemented with 0.5 M sorbitol and 2.5 mM betaine, and induced with 1 mM IPTG. Sorbitol and betaine can stabilise expressed proteins (Barth et al., 2000; Van Wuytswinkel et al., 1995). But, neither the addition of these compounds, nor lowering of the growing temperature, nor addition of lower concentrations of IPTG could improve solubility. The soluble SYE2 protein, produced in low amounts, resisted all purification attempts and precipitated upon further handling.

2. Considering the success of SYE1, we proceeded to clone the *sye2* gene in the pGEX-4T-2 vector. Large amounts of insoluble GST-SYE2 protein were produced. Some soluble GST-SYE2 could be extracted from the cells, and it could be purified on a Glutathione-Sepharose affinity matrix, but GST-SYE2 contained no FMN (colourless) and could not be reconstituted.

3. The *sye2* gene was subsequently cloned under control of an arabinose promoter in the pBAD-TOPO expression vector, with and without a C-terminal His-tag. Different concentrations of L-arabinose were tested to induce the promoter; different *E. coli* strains and even *S. oneidensis* cells were used as host, but no expression could be shown.

4. Since the *sye2* gene is present in the *S. oneidensis* genome only 8 bases downstream of the *sye1* gene, and heterodimer formation is known for the *Saccharomyces* homologues, we presumed that co-expression along with the *sye1* gene might be needed to stabilise the protein. Therefore, we amplified the total genomic fragment containing *sye1* and *sye2* and cloned it into a pBAD-TOPO vector. This construct did not result in expression of either SYE1 or SYE2.

5. Since for some genes it is noted that co-expression is only possible if both genes have their own promoter, we cloned the two genes separately in the two cloning sites of the pACYCDuet-1 vector. The *sye1* gene was cloned downstream of the His-tag coding sequence, and the *sye2* gene without a tag. However, once again no expression of SYE2 could be shown.

Table 2.2 summarises the cloning modes of *sye2*.

---

| Cloning modes for the *sye2* gene | pET11a | Forward primer CATATGTTCAAGGCAATTCAAGGTT | Reverse primer GGATCCTATGGCTATAGGCAGGATA B | Modes of expression tested *E. coli* BL21 (DE3) and BL21 (DE3)^* cells | Expression at 37°C and 28°C | Addition of 0.5 M sorbitol and 2.5 mM betaine | 0.1-0.5-1.0 mM IPTG | *E. coli* BL21 (DE3) cells, 28°C, 0.5 M sorbitol and 2.5 mM betaine, 1 mM IPTG | Best expression |
|---|---|---|---|---|---|---|---|---|

---

9 Since SYE2 was almost entirely insoluble, the identity of this protein was confirmed by peptide mapping. This technique can be performed on a protein sample extracted from a SDS-PAGE gel.

10 Expression of the pBAD-TOPO constructs was assayed by Western analysis.
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<table>
<thead>
<tr>
<th>Cloning modes for the sye2 gene for coexpression with the sye1 gene</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pBAD-TOPO (without His-tag)</strong></td>
</tr>
<tr>
<td>Forward primer</td>
</tr>
<tr>
<td>Reverse primer</td>
</tr>
<tr>
<td>Modes of expression tested</td>
</tr>
<tr>
<td>Expression</td>
</tr>
<tr>
<td><em>E. coli</em> BL21 (DE3), <em>S. oneidensis</em> cells</td>
</tr>
<tr>
<td>Expression at 28°C and 12°C</td>
</tr>
<tr>
<td>0.5 and 0.25 mM IPTG</td>
</tr>
<tr>
<td>YES</td>
</tr>
<tr>
<td><strong>pBAD-TOPO (with His-tag)</strong></td>
</tr>
<tr>
<td>Forward primer</td>
</tr>
<tr>
<td>Reverse primer</td>
</tr>
<tr>
<td>Modes of expression tested</td>
</tr>
<tr>
<td>Expression</td>
</tr>
<tr>
<td><em>E. coli</em> BL21 (DE3), <em>S. oneidensis</em> cells</td>
</tr>
<tr>
<td>0.2-0.02 % L-arabinose</td>
</tr>
<tr>
<td>NO</td>
</tr>
<tr>
<td><strong>pACYCDuet-1</strong></td>
</tr>
<tr>
<td>Cloning sye1 forward primer</td>
</tr>
<tr>
<td>Reverse primer</td>
</tr>
<tr>
<td>Modes of expression</td>
</tr>
<tr>
<td>Expression</td>
</tr>
<tr>
<td><em>E. coli</em> BL21 (DE3)</td>
</tr>
<tr>
<td>28°C, 0.5 mM IPTG</td>
</tr>
<tr>
<td>Only His-SYE1</td>
</tr>
</tbody>
</table>

Table 2.2. Cloning and expression modes of sye2.

We considered several possible explanations for the extremely low solubility of SYE2:

1. It has been noted that the $H_{191}^{194}$ mutant of OYE (Brown *et al*., 1998) was less soluble than the original $H_{191}^{N_{194}}$ enzyme. Sterical hindrance for FMN binding, in the mutant form of OYE, caused by the histidine causes the enzyme to be predominantly present in the apoenzyme form. SYE2 also has such a HxxH sequence whereas all other SYEs have HxxN. This does not explain, however, why XenB reductase (Blehert *et al*., 1997) and PETN reductase (French *et al*., 1996), enzymes which both have the HxxH sequence, show good solubility.

2. SYE2 has a higher pI than the other SYE proteins (6.46 compared to 5.21, 5.09 and 5.23). The barely soluble OPR1 enzyme purified from *Arabidopsis thaliana* by Schaller and Weiler (1997) has a pI of 7.14.

3. Since both SYE2 and SYE4 were insoluble when produced from pET11a, but only SYE4 was soluble as GST fusion, the low solubility may be caused by sterical hindrance between SYE2 and the GST fusion due to the very short linker region.

4. It has been estimated that about 33-50% of all expressed non-membrane proteins are not soluble (Golovanov *et al*., 2004). So it can be that this protein just belongs to that group of proteins and all solubilisation attempts are futile.

5. Or, and this possibility might be the most probable one, a conserved arginine (amino acid 317) of SYE2 is replaced by a threonine. This arginine is responsible for binding two of the phosphate oxygens of the FMN ribityl tail. It is possible that threonine can not substitute for the lost interactions with the positively charged arginine. When the ribityl tail can not be accommodated in the active site, FMN can no longer bind the protein and the protein is destabilised and precipitates. The Arg→Thr transition can be the result of a G→C point mutation in the DNA sequence of sye2 (Chapter one, Fig.1.28, red amino acid).
I.C. SYE3

Sye3, such as sye1, was first cloned in the pQE70 expression vector. However, also here, no expression of recombinant protein occurred. Regarding the successful cloning of sye1 in the pGEX-4T-2 vector, sye3 was also cloned in this vector, resulting in the production of high amounts of soluble protein.

Table 2.3 summarises the cloning modes of sye3.

<table>
<thead>
<tr>
<th>Cloning modes for the sye3 gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Modes of expression tested</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>pQE70</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward primer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse primer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Modes of expression tested</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expression</td>
<td>GGATCCATGAGTGTATTTACCGCA</td>
<td>AAGCTTATGCTACCTCATC</td>
<td>E. coli MC1061, M15 and XL1Blue cells</td>
<td>NO</td>
</tr>
<tr>
<td>Expression</td>
<td>GAATTCCATGAGTGTATTTACCGCA</td>
<td>TTAAATTAAACTTGCTAACATC</td>
<td>E. coli BL21 (DE3) cells</td>
<td>YES</td>
</tr>
</tbody>
</table>

Table 2.3. Cloning and expression modes of sye3.

I.D. SYE4

Sye4 was at first cloned in the pET11a vector. As was the case for the SYE2 protein, additions of sorbitol and betaine were needed to ensure a good expression. However, SYE4 also turned out to be insoluble. Expressing SYE4 as a GST-fusion protein, once again, was the solution to produce large quantities of pure protein.

Table 2.4 summarises the cloning modes of sye4.

<table>
<thead>
<tr>
<th>Cloning modes for the sye4 gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Modes of expression tested</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET11a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward primer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse primer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Modes of expression tested</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expression</td>
<td>CCATATGACGATAGAAATACCA</td>
<td>GGATCCCTAAATACTGGCTAA</td>
<td>E. coli BL21 (DE3) and BL21 (DE3)*</td>
<td>YES</td>
</tr>
<tr>
<td>Expression</td>
<td>GGATCCATGACGATAGAAATACGTT</td>
<td>GAATTCTAAATATACGTTGAACATC</td>
<td>E. coli BL21 (DE3) cells</td>
<td>YES</td>
</tr>
</tbody>
</table>

Table 2.4. Cloning and expression modes of sye4.
II. Protein production

The different recombinant SYE proteins were expressed, for purification purposes, using the pGEX-SYE1, pGEX-SYE3, pGEX-SYE4 and pACYC-SYE1 constructs, and the following protocol was used.

Cultures of *E. coli*, transformed with the plasmid of interest, were grown in LB medium, supplemented with the appropriate antibiotic, at 28°C and under constant shaking. When an optical density (@600 nm) of 0.4-0.6 was reached, cultures were induced with 0.5 mM of IPTG and allowed to grow for another 6-8 hours, until an optical density of 1.8-2.2 was reached. After this induction period the cells were harvested by centrifugation to separate the cells from the medium. The cell pellets were subsequently solubilised with buffer (30 ml of buffer per litre of culture grown) and frozen till further use.

III. Purification

III.A. Purification of GST-tagged protein (GST-SYE1, GST-SYE3 and GST-SYE4)

Frozen cells were thawed on ice, ruptured by sonication using a Branson Sonifier and centrifuged for 30 min at maximum speed (20800g, 4°C) to pellet down the cellular debris. The supernatant was removed and kept frozen at -20°C until further use. Appropriate volumes of supernatant were loaded on a 10 ml Q-Sepharose FF column equilibrated with 50 mM Tris-HCl, pH 8.0. Using a step gradient of 50 mM Tris-HCl, pH 8.0, 1 M NaCl, the different GST-fusion proteins eluted between 200 and 300 mM NaCl. The fractions containing GST-SYE1, GST-SYE3 or GST-SYE4 were pooled, dialysed against PBS buffer (PBS : Phosphate Buffer Saline; 11.5 g Na_2HPO_4, 2.96 g NaH_2PO_4 and 5.84 g NaCl/l) and loaded onto an 8 ml Glutathione-Sepharose 4 FF column, a GST-affinity matrix. The bound GST-SYE proteins were eluted in 50 mM Tris-HCl, pH 8.0, supplemented with 10 mM reduced glutathione. The eluates (approximately 95 % pure) were concentrated, dialysed against 50 mM Tris, pH 8.0, 100 mM NaCl, and loaded onto a Superdex-HL16/60 prep75 gel filtration column, connected to an Åkta Explorer purification system, to remove the final contaminants. The yield obtained for the different proteins was at least 50 mg/l.

III.B. Removal of the GST-tag

III.B.1. Purification of SYE1 and SYE4

After the two initial purification steps (Q-Sepharose FF and Glutathione-Sepharose 4 FF, see above), the fractions containing GST-SYE1 or GST-SYE4 were dialysed against 50 mM Tris-HCl, pH 8.0, to remove glutathione, after which thrombin was added in a ratio of 1 unit/1 mg fusion protein in order to enzymatically cleave the GST-tag from the different fusion proteins. The concentration of the 95 % pure protein solution was approximately 1 mg/ml. An incubation time of 8 hours at room temperature (22°C) was sufficient to cleave GST-SYE1 and GST-SYE4 completely. The mixtures of the untagged SYE1 and SYE4 proteins with the removed GST were dialysed against PBS and loaded onto an 8 ml Glutathione-Sepharose 4 FF column. SYE1 and SYE4 eluted in the flow-through volume while GST remained bound.
to the matrix. The flow-through fraction was concentrated and applied onto a Superdex-HL16/60 prep75 gel filtration column as a final step.

III.B.2. Purification of SYE3

While 8 hours were sufficient to remove the GST-tag from GST-SYE1 and GST-SYE4, GST-SYE3 was only partially cut, even after an increased incubation time of 24 hours (Fig.2.2). The small fraction (+/-10%) of untagged SYE3 enzyme was enriched using the Glutathione-Sepharose 4 FF affinity matrix, with SYE3 and GST-SYE3 being present in the flow-through and GST-SYE3 and GST bound to the column. The flow-through fraction was then concentrated and loaded onto a Superdex-HL16/60 prep75 gel filtration column in a PBS buffer supplemented with 1 M NaCl, 10% glycerol and 5 mM DTT in order to separate SYE3 and GST-SYE3 and obtain pure SYE3.

Fig.2.2. SDS-PAGE gel analysis of GST-SYE3 cut with thrombin after 8 hours (lane 1) and 24 hours incubation (lane 2). Lane 3: protein marker.

The difficult scission of the GST-tag is to be ascribed to the relatively short N-terminus of SYE3 located before the β-sheet that closes off the bottom of the barrel. This results in a short linker region between the GST protein and the SYE barrel; making it very difficult for the thrombin to bind the cleavage recognition site. SYE4 has a very long N-terminus, allowing scission in 2-4 hours. Due to the different cloning mode SYE1 has an intermediate N-terminus and cuts in 6-8 hours. SYE3 has a very short linker region and thus the GST moiety is hardly cut off at all.

In addition, the long C-terminal tail of SYE3 contains several charged residues, probably allowing SYE3 to form dimers. It has been noted that, because GST itself is expressed as a dimeric protein, an oligomeric protein expressed as a GST-tagged protein may form aggregates (Derewenda, 2004). This might explain why SYE3 and GST-SYE3 could not be separated on the Glutathione-Sepharose 4 FF matrix and an additional gel filtration step using a specific buffer was needed.

III.C. Modified purification protocol for SYE4

As will be discussed in Chapter four, no crystals were obtained for the initial crystallisation attempts of SYE4. A dynamic light scattering experiment was undertaken to analyse the influence of buffer on the behaviour of the SYE4 protein (Chapter 4, section III). This experiment has shown that phosphate buffer directed the protein’s oligomerisation behaviour
towards aggregation. Since it is known that not only the final buffer, but every intermediate buffer has an influence on protein crystallisation, the purification protocol of SYE4 noted in Section III.B.1 was slightly modified. We omitted the first step and proceeded directly to the separation by means of the affinity tag. We replaced the PBS buffer by a 50 mM Tris-HCl, pH 7.4, 100 mM NaCl buffer. This buffer change reduced the affinity of the tag for the glutathione matrix, resulting in a lower yield of GST-SYE4. The GST-tag was removed as described above (Section III.B.1).

Before proceeding to the ‘polishing step’ (gel filtration), we tried to remove some last contaminating bands (Fig. 2.3) by an ion exchange step. A Source30Q column was run in Tris-HCl, pH 7.4, and eluted with a linear salt gradient. The contaminants were not removed and it was attempted to remove these harsh contaminants by a gel filtration step. However, the contaminants co-eluted with SYE4. It was only at this point that we realised that the contaminants were breakdown products of SYE4 generated by thrombin: in solution the fragments could maintain their native form, explaining why they co-migrated with SYE4 on the gel filtration column, but under the denaturing conditions of SDS-PAGE the fragments were separated.

![Fig.2.3. SYE4 and the two degradation products of SYE4 on SDS-PAGE. Lane 1: protein marker, lane 2-3: SYE4, at different amounts. The weak band is in fact composed of two degradation products which are barely separated on the gel because of their similar molecular weight.]

### III.D. Purification of SYE1 for crystallisation

SYE1 was expressed from the pACYCDuet1 vector. The purification was performed in four steps. Protein inhibitor (Complete, Roche) and FMN were added to prevent proteolytic breakdown during purification and to ensure complete saturation of SYE1 with FMN respectively. The first step was performed on a Q-Sepharose FF column equilibrated with 50 mM Tris, pH 8.0. After a washing step of 50 mM NaCl, protein was eluted with 300 mM NaCl. This fraction was dialysed against 50 mM Tris, pH 8.0. The dialysed fraction was loaded onto a Source 30Q column connected to an Äkta Explorer. A linear gradient of 2 mM NaCl/ml was applied. SYE1 eluted between 80-100 mM NaCl. The eluate was dialysed against 50 mM Mes, pH 6.5, and loaded onto a MonoQ column. Again a linear gradient of 2 mM NaCl/ml was applied. SYE1 eluted between 40-60 mM NaCl. The purest fractions were pooled, concentrated and dialysed against 50 mM Tris, pH 8.0, 100 mM NaCl. The concentrated sample was loaded onto a Superdex 200 column in 50 mM Tris, pH 8.0, 100 mM NaCl.
Fig. 2.4. Pure SYE1 protein loaded on a SDS-PAGE gel coloured by silver staining. Lane 1: Protein marker, lane 2-3: SYE1, different elution fractions.

IV. References


CHAPTER THREE:
BIOCHEMICAL CHARACTERISATION OF THE SYE PROTEINS

In this work we wondered whether the four different OYE homologues have different physiological functions. A first indication would be the existence of different biochemical characteristics for these enzymes.

First, we determined some characteristics of the flavin cofactor (Section I):
   I.A. Measurement of the UV-Vis spectrum
   I.B. and I.C. Determination of the identity of the cofactor to be FMN or FAD
   I.D. Photoreduction analysis of FMN
   I.E. Determination of the dissociation constant of FMN

Then, we proceeded to examine a special property of OYE homologues, namely the formation of long wavelength charge-transfer complexes with phenolic ligands (Section II). In connection with this, the dismutase reaction is discussed (Section II.E).

Finally, we proceeded to determining some kinetic features of the SYE proteins (Section III):
   III.A. Preference for NADH or NADPH
   III.B. pH optimum
   III.C. Substrate preferences and kinetic constants

I. The flavin cofactor

I.A. UV-Vis spectrum

I.A.1. Introduction

In 1935, Theorell purified the Old Yellow Enzyme and showed it to be composed of a colourless apoprotein and a yellow dye, the latter being similar to riboflavin (vitamin B\textsubscript{2}).

Twenty years later (Theorell, 1955), he was able to prove that the riboflavin derivative is flavin mononucleotide (FMN). Nowadays we know that two types of riboflavin derivatives can occur as prosthetic group in proteins: flavin mononucleotide and flavin adenine dinucleotide (FAD).

An oxidised flavin molecule has a characteristic UV-Vis spectrum with peak maxima at 360 and 450 nm (Methods in Molecular Biology, Vol. 131, 1999); the spectra of FMN and FAD are indistinguishable.

\footnote{Part of the work in this chapter is incorporated in a paper entitled ‘Comparative characterization and expression analysis of the four Old Yellow Enzyme homologs from \textit{Shewanella oneidensis} indicate differences in physiological function’ to be published by the Biochemical Journal.}
Bound in the active site of a protein, the absorbance properties of the flavin molecule are modulated and the peaks at 360 and 450 nm are red or blue shifted. Although the UV-Vis spectrum in itself has little diagnostic value, it is common practise to measure it and determine the peak maxima. The spectrum can indeed be used when for example a mutated and a wild type enzyme are compared (Brown et al., 1998) since shifts in the maxima of the absorption peaks indicate differences in the respective active sites (Fitzpatrick et al., 2004). Evidently it can also be used to determine the extinction coefficient of a particular OYE homologue.

I.A.2. Measurement of the UV-Vis spectrum and determination of the extinction coefficients

The six different (GST)-SYE proteins were diluted in 0.1 M Tris-HCl, pH 8. Absorption spectra were recorded between 300 and 800 nm using an Uvikon 943 double beam UV-Vis spectrophotometer (Kontron Instruments). The spectra were recorded multiple times; represented are the most typical data. Extinction coefficients were determined by comparing the flavin absorbance of the pure protein with the absorbance of the free flavin released upon denaturation in 2% SDS. The assays were performed in triplicate. The extinction coefficients of (GST)-SYE1, (GST)-SYE3 and (GST)-SYE4 were determined at the respective absorbance maxima of the untagged forms. Represented are the averaged data, rounded to the nearest 100 M$^{-1}$ cm$^{-1}$ value.

I.A.3. Results

![UV-Vis spectra SYEs](image)

**Fig.3.1. UV-Vis spectra of SYE1 (blue), SYE3 (green) and SYE4 (red).**

The spectra of SYE1, SYE3 and SYE4 are shown in Figure 3.1; the peak maxima obtained for all (GST)-SYEs are summarised in Table 3.1.
Table 3.1. Peak maxima of the UV-Vis spectra of the different SYEs and free FMN as reference.

The extinction coefficients of (GST)-SYE1, (GST)-SYE3 and (GST)-SYE4 were determined to be $\varepsilon_{\text{SYE1, 458}} = 11\,500\, \text{M}^{-1}\text{cm}^{-1}$, $\varepsilon_{\text{SYE3, 459}} = 10\,900\, \text{M}^{-1}\text{cm}^{-1}$ and $\varepsilon_{\text{SYE4, 462}} = 11\,800\, \text{M}^{-1}\text{cm}^{-1}$.

I.A.4. Conclusion

We see little difference in the spectra of the GST-bound and GST-free enzymes. This is as expected since the GST is not supposed to affect the flavin environment. All three enzymes are red shifted compared to free FMN. The shifts for the second peak differ little between the different SYEs; the shift for the first peak, however, is 8-10 nm larger for SYE4 than for SYE1 or SYE3 (Arrows, Fig.3.1). This might be caused by a different protein environment in the flavin binding pocket of SYE4, which does not contain the Phe350 and Met25 (SYE1 numbering) of SYE1 and SYE3, but an Ile355 and Leu34 (SYE4 numbering) instead (see Fig.1.28).

The slight red shift of SYE4 compared to SYE1 and SYE3 was visible, describing the colour of SYE1 and SYE3 as intense orange but SYE4 as salmon/orange. A greater divergence can be observed for the extinction coefficients of the different SYEs. SYE4 has the highest extinction coefficient and SYE3 the lowest.

I.B. Identification of the FMN cofactor by thin layer chromatography

I.B.1. Introduction

By measuring the UV-Vis spectrum of the flavin, we are not able to determine the identity of the cofactor as FMN or FAD. Due to the different hydrophobicity, size, of FMN and FAD, the two molecules will behave differently during a chromatographic separation. By comparing the behaviour of synthetic FAD and FMN with a flavin sample obtained from the SYE protein, we conclude which cofactor is present in the molecule. As an easy to perform method of separation, we choose thin layer chromatography (TLC).

I.B.2. Thin layer chromatography

The identity of the flavin prosthetic group was determined as described in Marschall et al. (2004). Purified SYE proteins were boiled in PBS buffer for 5 min and the denatured protein was removed by centrifugation in a microcentrifuge (20800 g, 10 min). Supernatant samples were loaded onto 5 x 10 cm Silica gel 60 F254 plates next to standards of FAD and FMN. The plates were eluted in 2 % [wt/vol] Na2HPO4 in water, air-dried and, if the spots were not visible by eye, examined under UV light for areas of quenched fluorescence. The assays were performed in triplicate and the corresponding Rf (Retention factor) values were determined.
I.B.3. Results

A representative migration profile is presented in Fig.3.2. The picture is taken under UV light.

![Migration Profile](image)

*Fig.3.2. A typical migration profile of the SYE4 cofactor sample compared to FMN and FAD standards.*

The R\textsubscript{f} values for FMN, FAD and the three protein samples are summarised in Table 3.2.

<table>
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<tr>
<th></th>
<th>FAD</th>
<th>FMN</th>
<th>SYE1</th>
<th>SYE3</th>
<th>SYE4</th>
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<tr>
<td>R\textsubscript{f} value</td>
<td>0.64 +/- 0.01</td>
<td>0.47 +/- 0.02</td>
<td>0.41 +/- 0.02</td>
<td>0.43 +/- 0.02</td>
<td>0.41 +/- 0.02</td>
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</table>

*Table 3.2. Averaged R\textsubscript{f} values for FAD, FMN and the cofactor samples of SYE1, SYE3 and SYE4.*

Table 3.3 represents the analogous results for GTN reductase obtained by Marschall *et al.*

<table>
<thead>
<tr>
<th></th>
<th>FAD</th>
<th>FMN</th>
<th>GTN reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td>R\textsubscript{f} value</td>
<td>0.68</td>
<td>0.49</td>
<td>0.43</td>
</tr>
</tbody>
</table>

*Table 3.3. R\textsubscript{f} values according to Marschall *et al.* (2004).*

I.B.4. Conclusion

Comparison of the R\textsubscript{f} values for FAD and FMN in Table 3.3 and Table 3.2 shows that we succeeded well in reproducing the experiment reported by Marschall *et al.* Since the migration coefficient of all three SYE cofactor samples corresponded more to the value obtained for FMN than to the value of FAD, we can conclude the cofactor was FMN in all samples.
I.C. Identification of the FMN cofactor by mass spectrometry

I.C.1. Introduction

A method not often used to determine the cofactor presence in OYE homologues is mass spectrometry. To apply this method successfully the molecular species under investigation needs to be ionised and volatilised. Ionisation, however, requires denaturing methods. In the case of flavoproteins, there is a risk that the flavin cofactor would be completely removed. Nevertheless, by only partially denaturing the sample, we were able to obtain spectra displaying both apo- and holoenzyme.

I.C.2. Measurement of the mass spectrum

Measurement of the molecular weight of the enzyme, both with and without FMN, was conducted on an electrospray Q-TOF mass spectrometer (Micromass; Wythenshawe, United Kingdom). Prior to loading, the enzyme was partially denatured by the addition of 132 nM formic acid.

I.C.3. Results and conclusion

The mass spectrum was only determined in the case of recombinant SYE1. Four peaks were observed (Fig.3.3). Peak one corresponds to the apoenzyme, the measured weight of 40103.0 Da being close to the calculated mass of 40100.5 Da. The second can be accounted for by a phosphate or sulphate substituting group (94.97 Da or 96.07 Da) being bound to the apoenzyme. With respect to the experimentally solved structure of SYE1, sulphate is the most likely candidate (Chapter four, Section VI). The third peak of 40558.5 Da agrees perfectly well with the value of 40556.8 Da calculated for SYE1 + FMN. The fourth peak at 40643.5 Da is not accounted for.

![Fig.3.3. Mass spectrum of partially denatured recombinant SYE1.](image)
I.D. Photoreduction

I.D.1. Introduction

Flavin may have different oxidation states: fully reduced FMN-H$_2$ (hydroquinone), fully oxidised FMN (quinone), and the intermediate semiquinone FMN-H$^\cdot$. The semiquinone can exist in three states: anionic, neutral and cationic (Methods in Molecular Biology, Vol. 131, 1999) (Fig. 3.4).

![Fig. 3.4. The different oxidation states of FMN (Enzymes, Second edition).](image)

In flavoproteins, only the neutral and anionic semiquinones have been observed thus far (Methods in Molecular Biology, Vol. 131, 1999). One can discern the different oxidation states by measuring the UV-Vis spectrum. Oxidised flavin shows the two characteristic peaks at 360 and 450 nm depicted in Fig. 3.5. Reduced flavin is practically colourless, and does not absorb; the neutral or red semiquinone has a characteristic absorbance band between 500 and 650 nm with a maximum around 580 nm, and the anionic or blue semiquinone shows a distinct peak at 380 nm.

The different oxidation states of flavin can be observed when reducing the flavin by light in the absence of oxygen and the presence of EDTA as an electron donor. The electrons are transferred from EDTA to the free flavin molecules that are in equilibrium with the protein solution (Massey et al., 1978) and subsequently to the bound flavin molecules.

Most flavoproteins only stabilise one semiquinone form to a more or lesser extent (Massey et al., 1978). For OYE and OPR, the blue semiquinone was observed (Straţner et al., 1999; Stewart and Massey, 1985) (Fig. 3.5).

If the flavin is directly reduced to the two-electron reduced form, without proceeding through an intermediate semiquinone, an isosbestic point at about 330 nm is expected (Methods in Molecular Biology, Vol. 131, 1999).
I.D.2. Measurement of the photoreduction spectra

Photoreduction spectra were recorded for SYE1, SYE4 and GST-SYE3. The concentrated (15 mg/ml) protein solutions were diluted in 0.5 x PBS buffer supplemented with 5 mM EDTA, made anaerobic by incubation in an anaerobic glove box. The diluted samples were transferred to sealable anaerobic cuvettes and illuminated with a slide projector as a source of white light.

Spectra between 300 and 700 nm were recorded on an Uvikon 943 double beam UV-VIS spectrophotometer (Kontron Instruments) at regular time intervals.

I.D.3. Results and discussion

Similar spectra were observed for the three SYEs. After an incubation period, where no reduction was observed (15 minutes for SYE1 and SYE4 and 30 minutes for GST-SYE3) the flavin was fully reduced in the course of the following hour. No appearance of the blue or the red semiquinone was observed. At 331 nm a single isosbestic point could be discerned, which indicates that only two flavin species (oxidised and reduced) are present in the solution (Fig.3.6). The flavins were slowly reoxidised upon addition of oxygen, without formation of semiquinone. The slow rate by which the flavin reoxidises is consistent with the low diaphorase rate observed for SYE proteins (Section III.A.3).
When comparing the photoreduction spectra of the OYE homologues, one can note a difference between the eukaryotic OYE homologues and the bacterial ones. Whereas OYE (Stewart and Massey, 1985) and OPDA reductase (Straßer et al., 1999) are able to stabilise the blue anionic semiquinone form of FMN, no semiquinone formation has thus far been observed for the bacterial homologues XenA and XenB reductase (Blehert et al., 1997), morphinone reductase (Craig et al., 1998), PETN reductase (Khan et al., 2002), YqjM (Fitzpatrick et al., 2003) and the SYE proteins. Koder et al. (2002) noted that theoretical studies have proven that semiquinone species are planar, and that a butterfly bending of about 15° around the theoretical N₁-N₃ axis would severely destabilise semiquinone formation. This bending has been observed for the type I oxygen insensitive nitroreductases, YqjM (Kitzing et al., 2005) and SYE1 (Chapter four, Section VI), but not for PETN reductase (Barna et al., 2001) and morphinone reductase (Barna et al., 2002). Clearly, this theory might be true for the type I oxygen insensitive nitroreductases, and the flavin bending observed for YqjM and SYE1 might help destabilising semiquinone formation. But an additional or different mechanism is needed to explain the lack of semiquinone formation in the bacterial OYE homologues PETN and morphinone reductase which have a planar flavin ring system.
**I.E. Dissociation constant of FMN**

**I.E.1. Introduction**

It has been noted that the flavin can reversibly be removed from some flavoproteins, including OYE. In order to deflavinate the proteins, different methods are described (Hefti et al., 2003). First, flavin must be released by the protein. This can be done by addition of high concentrations of KBr, $(\text{NH}_4)_2\text{SO}_4$, acidification of the protein solution or partial denaturation of the protein by chaotropic agents (urea or guanidinium hydrochloride) or combinations of some of these methods. Thereafter, the flavin and the apoprotein need to be separated. Methods reported are dialysis, protein precipitation, chromatography, enzymatic breakdown of the flavin,…

After preparation of the apoprotein, free FMN can be titrated with the apoprotein, and the dissociation constant can be determined by spectrophotometric (Valton et al., 2004) or fluorometric means (Koder et al., 2002).

**I.E.2. Results and discussion**

We tried several methods to deflavinate the SYE proteins. Lowering the pH below the pI (<5) did allow release of the flavin, but the proteins were irreversibly denatured. High concentrations of KBr and $(\text{NH}_4)_2\text{SO}_4$ (up to 3 M) had no effect on the proteins. A combination of low pH and salt did not stabilise the deflavinated proteins and again irreversibly denatured protein was observed. As a final method, partial denaturation of the protein by chaotropic agents was tried. At a concentration of 1.5 M guanidinium hydrochloride, partial denaturation of the protein was observed but the flavin was not released. The experiments made us conclude that either the SYE proteins are unstable without FMN, or the methods used were too harsh. In the former case it is not possible to prepare apoprotein that can be reconstituted to determine the $K_d$. 
II. Ligand binding titrations

II.A. Introduction

In the early days of OYE investigations, a green form of OYE was purified (Matthews and Massey, 1969), with a characteristic UV-Vis spectrum showing an additional band between 500-800 nm (Fig.3.5). The green colour could be removed by dialysis, which indicated that ‘something’ was bound to the enzyme. Six years later, the molecule could be identified as p-hydroxybenzaldehyde (Mathews and Massey, 1975), and studies were performed to gain insight in the reaction (Abramovitz and Massey, 1976a; 1976b). Nowadays, it is widely recognised that oxidized members of the OYE family have the ability to form charge-transfer (CT) complexes with a variety of phenolic ligands.

II.B. Experimental measurements

The six different (GST)-SYE proteins were titrated up to saturation with increasing concentrations of a variety of para-substituted phenolic ligands in 0.1 M Tris-HCl, pH 8. Absorption spectra were recorded between 300 and 800 nm using an Uvikon 943 double beam UV-Vis spectrophotometer (Kontron Instruments). The spectral changes at the absorbance maximum of the resulting long wavelength transitions were plotted against the ligand concentration. The dissociation constants for the protein-ligand interaction were determined using the formula $\Delta \text{Abs} = \Delta \text{Abs}_{\text{max}} / (1 + (K_d / [\text{ligand}] ))$. Assays were performed in duplicate; represented are the most typical results. Linear regression analysis was performed on the plots between the Hammett para-constants of the ligands and the wave numbers of the absorbance maxima of the charge-transfer bands.

II.C. Results

All six (GST)-SYE enzymes were titrated with a series of 9 phenolic compounds to compare the characteristics of the anticipated long wavelength transitions. In most cases, an increase in ligand concentration resulted in spectral changes that are typical for the formation of a CT complex, i.e., the appearance of new long wavelength absorption bands with maxima between 550 and 686 nm, accompanied by reciprocal hypochromic changes in the 350 to 520 nm region (Fig.3.7). Bands appearing in the region below 350 nm can be ascribed to perturbation of the absorption spectrum by the phenol.

Figure 3.7 shows a series of spectra obtained for SYE4. By ordering the spectra according to the $pK_a$ of the phenolic ligands (low to high) a first characteristic of the CT complexes can be derived when comparing the spectra. In the spectra obtained for titration with ligands of low $pK_a$, the long wavelength band is little more than a shoulder of the 460 nm peak, having a maximum at about 550 nm. Upon titration with ligands of higher $pK_a$, the CT band is red-shifted and the band is no longer observed as a shoulder but as a distinctive peak. The maximum of the band lies above 600 nm. Similar spectra were obtained for SYE1, SYE3 and the GST-tagged enzymes. We can conclude that the peak maximum of the absorbance band is red shifted (towards higher wavelengths) as the $pK_a$ of the phenolic ligand increases. This indicates that less energy is required for the CT transition when the phenolic compounds have a higher $pK_a$. 
Fig. 3.7. Titration of SYE4 with 0-220 µM pentafluorophenol (a), 0-2570 µM p-hydroxybenzaldehyde (b), 0-3265 µM p-hydroxyacetophenone (c), 0-1870 µM p-chlorophenol (d), 0-4125 µM p-fluorophenol (e) and 0-3760 µM p-cresol (f). The arrow indicates the approximate maximum of the charge-transfer band, which increases with an increasing pKₐ of the ligand.
The dissociation constant of the different ligands was subsequently determined by plotting the \( \Delta \text{absorbance} (@\lambda_{\text{max}}) \) in function of the ligand concentration (Fig. 3.8) and fitting the data to the equation given in Section II.B.

![Fig. 3.8. \( \Delta \text{Absorbance at 604 nm in function of ligand concentration based on the data of SYE4 titrated with p-chlorophenol. Shown in blue are the experimental data, in magenta the theoretical fit.} \)](image)

A comparison of the absorption maxima of the formed CT-complexes and their dissociation constants \( (K_d) \) is shown in Table 3.4. Results as reported for LeOPR and OYE1 were added to the table for comparison.

In general, the \( K_d \) values for the untagged proteins are lower than for the tagged GST-SYE proteins, whereas the long wavelength absorbance maxima are rather similar. This is also the case for the GST-tagged and untagged LeOPR proteins (Straßner et al., 1999). Although the dissociation constants of the SYE proteins are higher than those reported for OYE1 (Abramovitz and Massey, 1976a), they are generally lower than those of LeOPR and YqiM (Fitzpatrick et al., 2003). This difference is most significant for the \( K_d \) values of the (GST)-SYE4 CT-complexes with ligands having a pK\text{a} of 9.34 or more.

A second correlation between the pK\text{a} of the phenol and CT complex can be observed for the dissociation constant and is most apparent in the SYE4 and GST-SYE4 proteins: the higher the pK\text{a} of the ligand, the higher the dissociation constant. When the pK\text{a} of the ligand increases, less molecules will be in the phenolate form necessary to interact with the FMN.

In contrast to (GST)-SYE4, the (GST)-SYE1 and (GST)-SYE3 proteins failed to give long wavelength charge transitions at pH 8.0 when titrated with ligands having a pK\text{a} of the free phenol equal to or larger than 9.34. It is known from the crystal structure of p-hydroxybenzaldehyde bound OYE1 (Fox and Karplus, 1994) that side chains of the active site residues His191 and Asn194 (OYE1 numbering) form stabilizing hydrogen bonds with the p-hydroxyl group, while the hydroxyl group of Tyr375 forms a hydrogen bond with the aldehyde moiety. These stabilizing hydrogen bonds result in a decrease of the pK\text{a} of the bound phenol with several pH units, which is evidenced by the fact that the H191N and H191N/N194H mutant enzymes do not form charge transfer transitions at pH 7.0 when the pKa of the free phenol is 8.0 or greater (Brown et al., 1998). Also, all phenols tested were found to bind much more weakly in both mutants than in wild type OYE1. This means that small changes in the ligand binding environment can have important repercussions on ligand binding properties. In addition, while Tyr375 is conserved in plant and yeast OYEs, it is replaced by a phenylalanine in SYE1 and SYE3, and by the nonaromatic isoleucine in SYE4 (Fig.1.28).
Table 3.4. Dissociation constants (in µM) of the SYE proteins with phenolic ligands. The dissociation constants and maxima of the CT-complexes of OPR and OYE according to Straßner et al., 1999 and Abramovitz and Massey, 1976a were added for comparison.

As was also observed for the LeOPR CT complexes, there is a hypochromic shift of the absorbance maxima of the SYE CT complexes (with the same ligand) compared to those of OYE1. The redox potential of the enzyme-bound FMN is therefore more positive, and/or the redox potential of the phenolic ligand more negative, in the yeast OYE1 CT-complexes compared with their tomato and bacterial counterparts.

For all six SYE proteins, a linear correlation could be demonstrated between the long wavelength transition energies for the ligand-SYE complexes and the Hammett para-constants (σ\(_p\), linear free energy parameter) of the different ligands (Fig.3.9). The reciprocals of the available wavelength maxima (ν\(_{CT}\) or wave number) were plotted against the σ\(_p\) of the different ligands. Shown in Figure 3.9 are the fits of SYE1, SYE3 and SYE4. The fits of the linear regressions all had positive slopes, as expected for a correlation between an acceptor compound and a series of donor compounds. As shown in Fig.3.9.C, the correlation coefficients of the (GST)-SYE4 CT complexes were very similar to those of the OYE1 and LeOPR CT complexes. The slopes of the SYE1 and SYE3 linear regressions were almost zero. This is caused by the limited range of data points obtained for these enzymes and the fact that the uttermost left data point (p-hydroxybenzaldehyde) is a statistical outlier which is ‘normally’ corrected for by another outlier : the p-chlorophenol data point. The corresponding data points for OPR1 are indicated by arrows in Fig.3.9 (a); similar points can be observed for OYE and (GST)-SYE4.

---

<table>
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<tr>
<th>Ligands</th>
<th>SYE1</th>
<th>GST-SYE1</th>
<th>SYE3</th>
<th>GST-SYE3</th>
<th>SYE4</th>
<th>GST-SYE4</th>
<th>OPR</th>
<th>OYE1</th>
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<td>Pentafluorophenol</td>
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<td>+/- 0.5</td>
<td>+/- 28.7</td>
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<td>(550)</td>
<td>(555)</td>
<td>(550)</td>
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<td>5</td>
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<td>p-hydroxybenzaldehyde</td>
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<td>+/- 6.8</td>
<td>11.6</td>
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<td>+/- 7.7</td>
<td>4.2</td>
<td>2.3</td>
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<td>(pK_a = 7.62)</td>
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<td>(570)</td>
<td>(570)</td>
<td>(570)</td>
<td>(555)</td>
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<td>(550)</td>
<td>12</td>
</tr>
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<td>4.4</td>
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<td>+/- 5.7</td>
<td>3.6</td>
<td>45.0</td>
<td>+/- 18.5</td>
<td>6.7</td>
<td>10.1</td>
</tr>
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<td>(578)</td>
<td>(560)</td>
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<tr>
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<td>4.0</td>
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<td>+/- 8.7</td>
<td>7.1</td>
<td>28.2</td>
<td>+/- 6.5</td>
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<td>(pK_a = 8.05)</td>
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<td>21</td>
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<tr>
<td>p-chlorophenol</td>
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<td>17.2</td>
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<td>-</td>
<td>(604)</td>
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<td>51.1</td>
<td>+/- 24.6</td>
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<td>-</td>
<td>-</td>
<td>(642)</td>
<td>(645)</td>
<td>1280</td>
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\(^a\) Phenolic p\(K_a\) according to Abramovitz and Massey (1976 a)
Fig 3.9. Linear correlation between the Hammett para-constant ($\sigma_p$) of para-phenols and the long-wavelength transition energies ($\nu_{CT}$) of the (GST)-SYE, LeOPR and OYE1 CT complexes. The Hammett para-constant values of the following compounds were as reported by Jaffe (1953): p-methoxyphenol (-0.268), p-cresol (-0.170), p-fluorophenol (0.062), p-hydroxybenzaldehyde (0.216), p-chlorophenol (0.227), p-hydroxycetophenone (0.516), p-cyanophenol (0.665) and p-nitrophenol (0.778). The wave numbers of the absorbance maxima of the CT-complexes are represented as magenta (LeOPR), yellow (OYE1), blue straight line (SYE), and blue dashed line (GST-SYE). Panel (a) represents the data of (GST)-SYE1, (b) of (GST)-SYE3 and (c) (GST)-SYE4. The arrows in (a) indicate the p-hydroxybenzaldehyde (top) and p-chlorophenol (bottom) data points of OPR1.
II.D. Discussion

It was quite remarkable that both SYE1 and SYE3 failed to form long wavelength charge transfer complexes with phenolic compounds of high pK$_a$. Such an observation has thus far only been made for OYE (Abramovitz and Massey, 1976a), which failed to form CT complexes with salicylic acid (pK$_a > 13$). We suspect, however, that not the pK$_a$ may be the cause of the failure, but the different binding mode of phenolic compounds in the active site of SYE1 (Chapter four, Section VI). The ‘normal’ binding mode for p-hydroxybenzaldehyde in OYE consists of two hydrogen bonds of the phenolate oxygen to His191 and Asn194, and of a hydrogen bond between Tyr375 and the carbonyl oxygen. In SYE1 the two hydrogen bonds to His181 and Asn184 are conserved; the hydrogen bond to Tyr375, however, is replaced by a bond to Trp274 (Chapter four, Section VI).

II.E. Dismutase reaction

As noted in Chapter one, OYE1 is able to perform a dismutase reaction with, for example, cyclohexenone (Vaz et al., 1995). Cyclohexenone reduces the flavin and the flavin subsequently reduces a second molecule of cyclohexenone.

\[
\begin{align*}
OYE_{\text{ox}} + \text{cyclohexenone} & \rightarrow OYE_{\text{red}} + \text{phenol} \\
OYE_{\text{red}} + \text{cyclohexenone} & \rightarrow OYE_{\text{ox}} + \text{cyclohexanone} \\
OYE_{\text{ox}} + \text{phenol} & \rightarrow \text{CT complex}
\end{align*}
\]

This reaction was recognised by the fact that the phenol formed was able to form a CT complex with the flavin.

Regarding the limited range of phenolic compounds that can form CT complexes with SYE1 and SYE3, we first checked whether a CT-complex can form between phenol and the SYEs. SYE1, SYE3 and SYE4 were titrated with increasing concentrations of phenol (pK$_a$ 9.95), but only SYE4 was shown to form a CT-complex. Then SYE4 was incubated with cyclohexenone in 0.5 x PBS buffer and a spectrum between 300 and 800 nm was taken to assay the appearance of a CT band. Although initial results showed promising, the experiment lacked reproducibility. As a consequence, no conclusions can be drawn about the ability of SYE4 to perform the dismutase reaction.

---

12 It is to note that upon addition of high concentrations of p-chlorophenol (10 mM) to SYE1 a very small perturbation of the spectrum could be observed, indicating an unusually high K$_d$ of SYE1 for p-chlorophenol.

13 2-cyclohexen-1-one is in the text mostly referred to as cyclohexenone for short.
III. Enzyme kinetics

III.A. Preference for NADH or NADPH

III.A.1. Introduction

The OYE reaction mechanism proceeds by a two step ‘Ping-Pong’ mechanism. In the first step oxidised OYE is reduced by either NADH or NADPH and in the second step substrate is reduced, thereby oxidising OYE. NADH and NADPH can not be used indiscriminately; OYE homologues either prefer NADPH or NADH. No specific nucleotide binding motif for the OYE family has been determined yet, so cofactor preference needs to be determined prior to kinetic experiments.

The experiment used to determine this preference is based on the fact that OYEs convert oxygen to hydrogen peroxide in the presence of NAD(P)H; this is called ‘diaphorase activity’. Since the amount of oxygen in the air is constant, one simply compares the rates of NADPH or NADH oxidation by the enzymes.

III.A.2. Measurement

The six GST-SYEs were diluted in phosphate buffer, pH 7.3, and were incubated with either NADH or NADPH. The rate of oxidation of NAD(P)H was monitored by measuring the absorbance at 340 nm in a Uvikon 943 double beam UV-VIS spectrophotometer (Kontron Instruments). Results were cross-checked by the addition of 5 mM NEM to the reaction. Assays were performed in triplicate; represented are the averaged data.

III.A.3. Results and discussion

The diaphorase rates for the six different (GST)-SYE’s are presented in Table 3.5 (values reported in mol NADPH oxidised per min and per mol of enzyme bound flavin). Compared to the rates reported for OYE 1, OYE2 and YqjM which range from 33 to 51 mol NADPH oxidised per min and per mol of enzyme bound flavin (Stott et al., 1993; Fitzpatrick et al., 2003), the values obtained for the SYE enzymes are very low (Table 3.5).

<table>
<thead>
<tr>
<th></th>
<th>SYE1</th>
<th>GST-SYE1</th>
<th>SYE3</th>
<th>GST-SYE3</th>
<th>SYE4</th>
<th>GST-SYE4</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH + O2</td>
<td>6.3</td>
<td>4.4</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>2.0</td>
<td>4.1</td>
</tr>
<tr>
<td>NADPH + O2</td>
<td>&lt;1</td>
<td>1.7</td>
<td>1.6</td>
<td>3.0</td>
<td>7.4</td>
<td>11.1</td>
</tr>
</tbody>
</table>

Table 3.5. Diaphorase rates for the SYEs in mol NAD(P) oxidised per min and per mol flavin reduced.

So far, only GTN reductase (Snape et al., 1997) and morphinone reductase (French and Bruce, 1994) showed preference for NADH; therefore, we cross checked the preferences by the addition of 5 mM NEM. The values obtained are presented in Table 3.6. We can see that SYE1 has a strong preference for NADH, and can not accept NADPH as an alternative electron donor. SYE3, which appears to prefer NADPH based on the rate by which oxygen was reduced, showed an 80 times higher rate of NEM reduction with NADH compared to NADPH, establishing the cofactor preference for this enzyme to be NADH. SYE4 showed
Debbie van den Hemel

reactivity for both NADH and NADPH, preferring the latter. This is a phenomenon that is quite often observed. The OYE homologues enzymes which prefer NADH can not accept NADPH, but the ones that prefer NADPH do accept NADH. This indicates that the mechanism that confers preference for NADH/NADPH to the enzymes needs to be sought in a mechanism that excludes NADPH from the enzymes and not in a mechanism that discriminates between NADH and NADPH.

<table>
<thead>
<tr>
<th></th>
<th>SYE1</th>
<th>GST-SYE1</th>
<th>SYE3</th>
<th>GST-SYE3</th>
<th>SYE4</th>
<th>GST-SYE4</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH + 5 mM NEM</td>
<td>1241.4</td>
<td>324.6</td>
<td>1296.3</td>
<td>2796.1</td>
<td>32.1</td>
<td>50.8</td>
</tr>
<tr>
<td>NADPH + 5 mM NEM</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>16.4</td>
<td>33.4</td>
<td>301.2</td>
<td>748.8</td>
</tr>
</tbody>
</table>

Table 3.6. Reaction rates for the SYEs with 5 mM NEM, in mol NAD(P) oxidised per min and per mol flavin reduced.

III.B. pH optimum

III.B.1. Introduction

The pH of an enzyme solution can affect the overall stability and catalytic activity in a number of ways. Like all proteins, enzymes have a native tertiary structure that is sensitive to pH, and denaturation generally occurs at extremely low or high pH values. The pH range over which the native state of an enzyme will be stable varies from one protein to the next. While most proteins are stable at physiological pH (7.4), some display maximum activity at much lower or higher pH values. The appropriate pH range for a specific enzyme must therefore be determined empirically. Typically, one finds that protein conformation is maintained over a relatively broad pH range, say 4-5 pH units. Within this range, however, the velocity of the enzymatic reaction varies with pH (Enzymes, 2nd Edition).

In addition to the effect on the stability of an enzyme, pH may have effect on certain acid-base groups involved in catalysis, substrate binding, etc. A wrong protonation state can thus influence the activity of the enzyme.

III.B.2. Measurements

The pH optimum was determined in the presence of 100 µM NAD(P)H, 50 nM enzyme and 3 mM NEM, by assaying a series of phosphate buffers ranging from pH 4.5 to 11. We measured the rate of NAD(P)H oxidation at 340 nm using an Uvikon 943 double beam UV-VIS spectrophotometer (Kontron Instruments). Assays were performed in triplicate, represented are the most typical data.
III.B.3. Results

The pH optima of SYE1 and SYE4 could be determined unequivocally to be at 7.75 and 7.25 respectively. SYE3, on the contrary, appeared to have multiple pH optima. A broad range of high reaction rates could be discerned between pH 7.5 and 9.5.

![pH optimum of SYE1](image)

*Fig. 3.10. pH optimum as determined for SYE1.*

III.C. Steady state kinetics

III.C.1. Introduction

Depending on the aspects of an enzymatic reaction that needs to be explored, one can perform different experiments, ranging from simple assays, to confirm the activity of the enzyme, to stopped flow enzyme kinetics, that decompose a single reaction in multiple steps. For our purpose, the determination of the substrate specificity and the comparison of the different enzymes, we choose to perform steady state kinetic analysis.

The theory was developed in the early 1900’s by Henri and later improved by Michaelis and Menten (Enzymes, 2nd edition).

The steady state equation

For every enzymatic reaction the following equation can be written:

$$E + S \xrightleftharpoons[k_s]{k_i} ES \xrightarrow{k_2} E + P$$

We can see that the velocity of product formation will be directly proportional to the concentration of the Enzyme-Substrate complex (ES):

$$v = k_2 [ES]$$
Early in a reaction, $[S] >> [E]$ and, as a consequence, the rates of composition and decay of the ES complex will be equal, or $[ES] = ct$. This early phase is called the steady state of the reaction.

Using the assumption that $[S] >> [E]$ and $d[ES]/dt = 0$ (the derivative of a constant is zero), one can deduce the so-called (Henri)-Michaelis-Menten equation for the velocity:

$$V = V_{\text{max}} [S] / (K_m + [S])$$

The kinetic constants are defined as:

$V_{\text{max}}$ = the maximum velocity obtained if the substrate concentration reaches infinity

$K_m = (k_{-1} + k_2) / k_1$

= the substrate concentration that provides a reaction velocity that is half of the maximal velocity obtained under saturating condition ($V_{\text{max}}$)

A third constant can be derived:

$k_{\text{cat}} = V_{\text{max}} / [E]$

= the turnover number = how many turnover events (molecules of substrate converted) occur per unit time and per enzyme molecule

If we like to compare the efficiency of different enzymes for a particular substrate, we use the parameter $k_{\text{cat}}/K_m$ (catalytic efficiency). The higher $k_{\text{cat}}/K_m$, the more efficient the enzyme is in catalysing the reaction.

**Substrates for OYE homologues**

Searching the literature of OYE, one can find a whole series of possible substrates for OYE. We can divide these substances in different classes (Fig.3.11).

1. Simple aldehydes and ketones : comprising cyclohexenone, N-ethyimaleimide, trans-2-hexanal,…
2. Complex aldehydes and ketones : comprising steroids, alkaloids,…
3. Nitro-olefins : comprising for example the nitro-aromatic compounds TNT, picric acid and RDX and the non-aromatic compound nitrocyclohexene.
4. Nitro-esters : comprising glycerol trinitrate, PETN, IPDN, …
5. In one rare case the acid maleic acid has been reported as substrate (Straßer et al., 1999).

**III.C.2. Measurements**

All kinetic parameters were determined under strict anaerobic conditions and all assays were performed in triplicate. Represented are the averaged data. Reaction mixtures consisting of 200 mM sodium phosphate buffer, pH 7.3, 60-100 µM NAD(P)H and varying concentrations of substrate were prepared within an anaerobic glove box and transferred to sealable quartz cuvettes (Hellma). The concentrations of NADH and NADPH were determined spectrophotometrically using a molar extinction coefficient of 6220 M$^{-1}$ cm$^{-1}$ at 340 nm.
Enzyme solutions were prepared in sealed flasks and made anaerobic by bubbling with N₂-gas. Reactions were initiated by the addition of enzyme to the reaction mixtures using a Hamilton needle. Enzyme concentrations ranged from 34 to 135 nM depending on the observed reaction rate with each substrate. SYE activity was assayed by following the oxidation of NADH or NADPH at 340 nm using an Uvikon 943 spectrophotometer. Apparent steady state kinetic constants were determined by least squares fitting procedures to the standard Michaelis-Menten equation using the software GraphPad prism4.

Fig. 3.11. Different substrates for OYE homologues. Presented are from left to right: (1) 3-penten-2-one, 3-methyl-2-butanal, 2-cyclohexene-1-one, trans-2-dodecenal and duroquinone, (2) 1,4-androstadiene-3,17-dione and morphinone, (3) picric acid, (4) glycerol trinitrate and isosorbide dinitrate, and (5) maleic acid.
III.C.3. Results

Comparison of the activity of the SYE enzymes with different substrates

We choose different representatives of the above noted five substrate classes for assaying activity with the SYE enzymes.

1. Simple aldehydes and ketones: N-ethylmaleimide, 2-cyclohexenone and acrolein
2. Complex aldehydes and ketones: 1,4-androstadiene-3,17-dione and progesterone
3. Nitro-olefins: picric acid
4. Nitro-esters: glycerol trinitrate
5. Acids: fumaric and maleic acid

No activity was observed for fumaric acid and maleic acid. This was no surprise since only one enzyme has thus far been reported to reduce maleic acid (Sträßer et al., 1999).

In addition, progesterone and 1,4-androstadiene-3,17-dione showed no reactivity under the conditions used. Both substrates were sparsely soluble, and the possibility exists that under different assay conditions reduction can occur.

Also no activity was observed for picric acid. However this experiment was repeated under different assay conditions (p70).

For the other substrates, kinetic constants were determined by plotting the velocity by which the NAD(P)H is oxidised in function of the substrate concentration and then fitting the data to the Michaelis-Menten equation. Figure 3.12 gives an example of a typical curve.

![Figure 3.12: Velocity in function of ligand concentration for the reduction of NEM by SYE1.](image)

The kinetic constants obtained are summarised in Table 3.7.
Table 3.7. Kinetic constants of the different SYE enzymes. NR : No Reaction.

(1) We can see that the GST-tagged enzymes generally have a lower catalytic activity compared to the untagged ones. A comparable difference in catalytic activity was also seen between the GST-tagged and untagged form of YqJ from *B. subtilis* (Fitzpatrick et al., 2004). The lower catalytic efficiency of GST-YqJ is mainly reflected in the $k_{cat}$ values which are, depending on the substrate tested, 4-10 times lower than for YqJ. This is in contrast with the SYE proteins, where the difference, for the major part, is displayed in $K_m$ values being generally 1.6-10 times higher in the tagged forms than in the untagged forms.

(2) The catalytic properties of the SYE4 protein were found to differ from those of SYE1 and SYE3. Not only is the activity with the tested substrates higher for SYE4 than for SYE1 and SYE3, in addition, only SYE4 is able to use the more bulky nitroglycerine as a substrate. SYE4 thus appears to be the only SYE protein with nitrate ester reductase activity.

(3) The apparent kinetic constants for NEM and cyclohexenone are of the same order of magnitude as those determined for *LeOPR* (Straßner et al., 1999) and YqJ (Fitzpatrick et al., 2003).

(4) We have shown for the first time that bacterial OYE family members can reduce acrolein, a lipid peroxidation product that is formed endogenously in eukaryotic cells. Among all α,β-unsaturated aldehydes it is by far the strongest electrophile and therefore highly toxic to the cell.
Additional experiments for SYE1

For SYE1, some additional substances were tested for reactivity. They were chosen based on the experimental structure determined for SYE1. It concerns t-2-hexenal, t-2-decenal, and t-2-dodecenal. These results are discussed in Chapter four, Section VI.

Reactivity with picric acid

It has been noted for PETN reductase that the reduction of picric acid could not be measured during the time course of a kinetic experiment. However a change of colour was observed when PETN reductase was incubated for 15 hours with the picric acid solution, indicating the formation of the H-Meisenheimer complex (Khan et al., 2002). It has been shown that the production of a H-Meisenheimer complex with TNT is restricted to enzymes possessing the HxxH sequence (Williams et al., 2004). The possibility that only enzymes capable of aromatic ring reduction of TNT are able to reduce picric acid to the H-Meisenheimer complex has been proposed by Khan et al. (2002), but Nigel Scrutton (personal communications) noted that the reaction with picric acid is such that it is not possible to unequivocally conclude that there is no reaction or that the reaction is extremely slow.

When incubating the three SYE enzymes with picric acid and the appropriate reductant, a different colouration was observed for SYE4 (Fig. 3.13), indicating the formation of a H-Meisenheimer complex. This is the first report of an enzyme, different from PETN reductase, able to reduce picric acid. If indeed the production of a H-Meisenheimer complex of picric acid is restricted to enzymes possessing the HxxH sequence, then this is the first report of the production of a H-Meisenheimer complex by an enzyme possessing the HxxN sequence. It is however absolutely necessary to assay the reaction with TNT to draw a conclusion.

![Image of solutions with different colourations](Fig.3.13. Solutions of picric acid incubated overnight (16 hours) with NAD(P)H and SYE enzyme. From left to right: no enzyme, SYE1, SYE3, SYE4.)

IV. Conclusion

Based on these initial characterisations, we can divide the SYE enzymes in two clearly distinct groups: SYE1 and SYE3 on the one hand compared to SYE4 on the other. Not only are there clear differences based on the sequence alignment, but also differences based on the ligand binding and the kinetic characterisations.

1. The sequence alignment revealed that SYE4 compared to the other SYE proteins has a shorter L3 loop, which comprises the capping subdomain, and lacks a part of the conserved C-terminus. The protein is, however, fully functional. In addition, SYE1 and SYE2 have an
identical C-terminus ‘YQA’. In SYE3, 14 amino acids, comprising several charged residues (R and D), are present. This results in a very long C-terminus for SYE3.

(2) The inability of SYE1 and SYE3 to form charge transfer complexes with phenolic ligands of high pK_a may be linked to the special binding mode of these ligands in the active site and is discussed in more detail in Chapter four, section VI and Chapter six, Section I.

(3) Where both SYE1 and SYE3 accept only NADH as a reductant, SYE4 accepts both, with a preference for NADPH. Comparing the co-enzyme preferences of OYE homologues led to the assumption that the enzymes preferring NADPH can not discriminate between NADH and NADPH, and that the enzymes preferring NADH must have some mechanism for excluding NADPH.

(4) SYE1 and SYE3 are only able to reduce the ‘small’ substrates NEM, acrolein and cyclohexenone. SYE4 accepts the more bulky nitroglycerine as an additional substrate. Two possible explanations exist for this observation: (i) the substrate can not enter the active site due to sterical hindrance, or (ii) the enzymes do not have the reducing power required to denitrify nitroglycerine.

(5) Only SYE4 is able to reduce picric acid to the hydride-Meisenheimer complex, this being the first report of an enzyme other than PETN reductase being capable of catalysing this reduction.

The division of the SYE proteins in two groups based on their biochemical properties indicates that two different physiological functions may exist for these enzymes.
V. References


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CHAPTER FOUR: 
Comparative modelling, dynamic light scattering, protein crystallisation and structure determination

After biochemical characterisation of the three soluble SYE enzymes we aimed to gain insight in their tertiary structure. We especially wanted this for the SYE1 or SYE3 enzymes because of the special features that were recognised in the binding of phenolic ligands. The elucidation of an experimental structure complexed with phenolic ligand might explain why not all ligands could bind to SYE1 (SYE3). In addition, we wanted to solve the structure of SYE4, which lacks a large part of the conserved C-terminal domain. Some of the amino acids of this domain are known to be involved in active site interactions. It would be interesting to know what effects the truncation has on the overall structure and the active site structure in particular. In order to experimentally solve a structure by X-ray crystallography protein crystals of good quality are needed. These were not easy to obtain. Therefore we first calculated structural models of the SYE’s.

The topics that will be discussed in this chapter are:
I. Calculation of structural models of the SYE enzymes
II-V. Protein crystallisation aided by dynamic light scattering
VI. The experimental structure of SYE1
VII. Comparison of the experimental structure of SYE1 with the theoretical models.

VI. Comparative modelling of the SYE enzymes

I.A. Introduction

To date, more than 32000 protein structures are deposited to the Protein Data Bank (http://www.rcsb.org/pdb/). The number of protein and DNA sequences available in databases worldwide, however, exceeds this number by at least a factor of 2. No structural information is thus available for the majority of proteins. Although progress is made in the field of nuclear magnetic resonance (NMR) and crystallography it is still a long and troublesome way to solve a protein structure. To bridge the gap between the sequences known and the structures solved, many theoretical approaches have been made. Comparative modelling is one of the best techniques; it generates a 3D structure for a given sequence based on known structures of homologous proteins. The theoretical model can provide valuable insights into the molecular basis of the protein, allowing an effective design of experiments, such as site directed mutagenesis, studies on disease related mutations or structure-based design of specific inhibitors (Schwede et al., 2003).

However, the SYE proteins were modelled to see whether the biochemical differences observed are reflected in structural differences.
I.B. Modelling

The SYE models were calculated using the Swiss-Model programme (Schwede et al., 2003) and verified by comparison to models obtained from the CPH-Models server (Lund et al., 2002), the ESyPred3D server (Lambert et al., 2002) and 3Djigsaw (Bates et al., 2001). Structure quality was evaluated using the ProCheck program (Laskowski et al., 1993). The models were visualised and superimposed for comparison using Deep View Swiss-PdbViewer (Guex and Peitsch, 1997).

I.C. Results

When the four original models were calculated (W. Carpentier, pers. communic.), four structures of OYE homologues were known: PETN reductase (Barna et al., 2001), morphinone reductase (Barna et al., 2002), OPDA reductase (Breithaupt et al., 2001) and OYE1 (Fox and Karplus, 1994). Therefore, modelling was performed using these structures. OYE homologues are composed of an eight stranded α,β-barrel. The loops on the N-terminal end of the barrel are short and the barrel is closed at the bottom by an antiparallel β-sheet. The loops on top of the barrel are long and contribute to make the substrate binding site. These loops adapt different conformations in the different structures, the most apparent being the loop between β-strand 3 and α-helix 3, which makes up a ‘capping’ subdomain. This capping subdomain, in OYE1, is composed of 2 α-helices and 2 β-strands, in OPDA reductase of two times two β-strands making up a β-sheet, and in the bacterial homologues PETN reductase and morphinone reductase of a very short α-helix followed by a β-sheet composed of two β-strands (Fig.4.1). Loop 4 and Loop 8 fold into an α-helix said to be involved in substrate recognition or FMN binding respectively.

Fig.4.1. The different capping domains: OYE1 (blue), OPDA reductase (yellow), morphinone reductase (green) and PETN reductase (red). The β-strands of OYE1 and OPDA reductase were not correctly recognised by Pymol and arrows are added to the picture to indicate their location. 14

14 All structural models presented in this chapter were generated with Pymol (DeLano Scientific, San Carlos, CA, USA).
The SYE models impose particularly well upon the other bacterial structures (morphinone reductase and PETN reductase), showing they are more related to these structures than to the eukaryotic ones. More precisely, the barrels impose almost perfectly, and the loops adopt conformations very similar to either morphinone reductase, PETN reductase or an intermediate formation (Fig. 4.2).

*Fig. 4.2. Superposition of the theoretical models of the SYEs with morphinone reductase. green : SYE1, magenta : SYE4, red : morphinone reductase, blue : SYE3 and yellow : SYE2.*

The only structural elements that do not seem to fit the picture were the capping subdomains of SYE1 and SYE4. In SYE1 the short α-helix which lies before the β-sheet is disordered; in SYE4, Loop 3 is very short and not folded into a β-sheet (Fig. 4.3).

Based on the sequence alignment of the four SYE proteins it was already noted that Loop 3 of SYE4 would be shorter than the corresponding loops in SYE1, SYE2 and SYE3. This structural difference may account for the differences in the biochemical characteristics.
VII. Crystallisation attempts for the GST-tagged and ‘untagged’ SYE1 and SYE4 proteins

II.A. Introduction

To reduce the free energy of a solution system, molecules can assume an ordered lattice, called a crystal (McPherson, 1990). A well known crystal is rock salt. For a long time people thought crystallisation was restricted to small molecules. Proteins, however, were later found to be able to also form regular lattices. Their crystals are necessary to determine the structure of proteins. However, protein crystals are not easy to obtain. As a first requirement one needs very pure protein, in large quantities (in the order of mg). Even the smallest contaminations in the protein sample or even a difference in the purification procedure can prevent crystallisation (alternatively called ‘crystallogenesis’). The protein solution must be concentrated (e.g. 10 mg/ml) and crystallisation trials set up. Different methods can be used to obtain the protein crystals, although they are all based on a common theory.

When the objective is to grow crystals of any compound, a solution of the molecule must be brought into the supersaturated state (Fig.4.4). This can be achieved, for example, by a saturated solution from which solvent is gradually withdrawn by evaporation. The supersaturated state can be divided in two states: the metastable and the labile region. In the labile region, stable nuclei of a size such that the rate of recruitment exceeds the rate of loss of molecules, can form and grow. In the metastable region the nuclei are dissolved as rapidly as they form; nuclei initiated in the labile region can continue to grow in the metastable region until saturation is reached.

Fig.4.3. The differences in the capping subdomains of SYE4 (red), SYE1 (blue) compared to morphinone reductase (yellow).
The method we used to obtain such saturation conditions is the ‘hanging drop vapour diffusion’ method. A small droplet of protein, diluted twofold in crystallisation solution, is hung over a reservoir filled with crystallisation solution (or precipitant, Fig.4.5). The droplet evaporates to equilibrate with the reservoir solution and so (hopefully) reaches the condition of supersaturation. Many conditions of e.g. buffer, pH, precipitant and additives must be screened in order to obtain crystals. Commercial screens of different crystallisation solutions are available to aid in crystallogenesis.

II.B. Crystallisation

Crystallisation trials were set up for SYE1, SYE4 and SYE4-GST. Many buffer conditions were tested, but no crystals were observed. Some ‘promising’ conditions were examined in more detail but failed to give reproducible results. Table 4.1 mentions the crystallisation screens tested for the different SYE enzymes.

To overcome the problem of crystallisation, dynamic light scattering (DLS) experiments were conducted.
**Enzyme** | **Screen** |
---|---|
SYE1 | Molecular Dimensions Structure Screen 1  
pH/ammonium sulphate screen  
pH/PEG1000 screen  
pH/PEG6000 screen  
pH/PEG400 screen  
pH/NaCl screen |
GST-SYE4 | Molecular Dimensions Structure Screen 1  
Molecular Dimensions Structure Screen 2  
Jena Bioscience Screen  
PEG/ion Screen (Hampton research) |
SYE4 | Molecular Dimensions Structure Screen 1 (22°C and 4°C)  
Molecular Dimensions Structure Screen 2  
pH/ammonium sulphate/NaCl screen based on condition 14 of MD SSC2  
pH/PEG20000 screen based on condition 22 of MD SSC2  
pH/PEG/ammonium sulphate screen based on condition 26 of MD SSC2 |

Table 4.1. Different crystallisation conditions tested with the SYE1, GST-SYE4 and SYE4 enzymes. The pH/xx screens were home-made to test various buffers in a range from pH4-9 in function of the concentration of the additive.

### VIII. Dynamic light scattering

#### III.A. Introduction

Dynamic light scattering is a technique that allows measuring the translational diffusion coefficient ($D_T$) of a macromolecule undergoing Brownian motion in solution. Monochromatic light scattered by moving particles will display intensity fluctuations corresponding to particulate motion, and a decay analysis of the autocorrelation function (a measure of the time-dependence of intensity fluctuations) of the light scattering signal can yield quantitative information about the behaviour in solution of the dissolved particles. Molecular weight can be estimated from the measured value of $D_T$, using a calibration curve obtained from assorted globular particles of known mass (Ferré-D’Amaré and Burley, 1994). Three cases commonly occur for proteins in solution: (I) the protein is in its pure monomeric form (or in whatever its natural single-sized state may be), (II) the protein monomer is accompanied by lower order oligomers (dimers, trimers,…), or (III) the protein monomer is present with higher order protein aggregates. Cases (II) and (III) are described as solutions that are ‘polydisperse’ (Wilson, 2003). Empirical observations suggest that macromolecules that are monodisperse (i.e. are all the same size) under ‘normal’ solvent conditions crystallise readily, whereas random aggregating or polydisperse systems rarely, if ever, yield crystals. DLS can thus be used as an aid in protein crystallography by determining the solution states in which the protein is monodisperse.
III.B. Measurements

Dynamic light scattering experiments were performed for SYE4 on the DLS apparatus at the Laboratory of Ultrastructure at the University of Brussels (VUB). Protein was diluted in different buffers of different pH to a final concentration of 1 mg/ml and centrifuged for half an hour at maximum speed to pellet down small contaminating particles that could disturb the measurements. The protein samples were transferred to DLS cuvettes and illuminated with green light. The diffraction pattern was measured and interpreted by an appropriate software program.

III.C. Results and discussion

Two different graphic representations of the measurements are generated by the program, the most informative being the radius histogram. Figure 4.6 and 4.7 show typical outputs for SYE4. The radius of the particles in solution is represented on the horizontal axis. In addition, the radius is correlated to the molecular weight of a theoretical, spherical particle.

The first round of measurements consists of a pH/buffer screen. This screen showed us that phosphate buffer was among the worst buffers we could have used for crystallisation. Almost all protein was present as higher order oligomers or aggregates (case III, as described on previous page). The best buffers were Tris ((hydroxymethyl)-aminomethane) and MES (4-morpholinemethanesulfonic acid), in which the protein was mostly present in the monomeric state accompanied with lower order oligomers (case II). The histograms indicate that the sample was not yet monodisperse and that an additive was probably needed.

![Radius histogram]

Fig.4.6. Radius distribution of SYE4 proteins in phosphate buffer, pH 5, suggesting the presence of different high molecular weight oligomers.
To assay the effect of additives, we decided to start from the Tris, pH 8.0 buffer solution, for which the best radius histogram was obtained. As a first additive, salt was assayed at various concentrations: 25 mM, 50 mM and 100 mM. The simple addition of 50 mM NaCl to the buffer was sufficient to obtain a monodisperse sample (Fig. 4.8) and no further additives needed to be tested. The radius obtained correlates to a protein of approximately 40 kDa, which is in agreement with the molecular weight of a SYE protein. A similar correlation between protein aggregation and salt concentration was observed for PETN reductase. It has been suggested that this protein forms dimers under low salt conditions (French et al., 1996), a phenomenon which could also occur for SYE4.
IX. Crystallisation trials for SYE4 purified without phosphate buffer

From the DLS experiments (Section III) we concluded that phosphate was probably the worst buffer we could have used for protein purification, so we decided to make a batch of SYE4 purified without phosphate. For this purpose, we had to adapt our purification protocol. The new protocol is described in Chapter 2, Section III.C.

Applying this purification, it was observed however, that SYE4 was subject to proteolytic cleavage by thrombin. We noted the presence of two faint bands upon SDS-PAGE (Fig.2.2) that could not be separated from SYE4. This made us conclude that the species responsible for these two bands might interact with each other and are breakdown products of SYE4, certainly because their combined molecular weight corresponded to the molecular weight of SYE4 itself. Inspection of the sequence of SYE4 showed a thrombin-like recognition site. The normal recognition site is LVPRGS, SYE4 possesses a VPR site, which is apparently sufficient for being recognised by thrombin. Inspection of SYE1 also showed a VPR site, whereas SYE2 and SYE3 do not contain such a site. Secondary cleavage by thrombin is commonly observed for proteins (Derewenda, 2004). By using gel filtration as the final step, we were able to remove the nicked SYE4 enzyme, at the price of loosing approximately 95% of the protein sample loaded onto the gel filtration column. A single crystallisation screen was set up (Molecular Dimensions Structure Screen 1), but we did not observe any promising crystallisation conditions. The fact that the proteolytic damage is not always apparent during the purification procedure, explains why it was not recognised earlier. Traces of thrombin can remain in the protein sample and damage the supposedly pure protein sample during the crystallisation trial.

X. Crystallisation of SYE1 derived from the pACYCDuet-1 vector

As said above, it was observed for SYE4 that secondary proteolytic damage occurred during purification due to thrombin. In addition, the DLS measurements performed showed that phosphate buffer had to be excluded from the purification process. As for SYE4, initially no crystals of SYE1 were observed. In addition, the SYE1 protein possessed an internal thrombin recognition site, just as reported for SYE4. It was thus likely that similar problems were to be expected for SYE1. To avoid them, a novel cloning and expression mode was assayed for SYE1. The protein was cloned in the pACYCDuet-1 vector. Compared to the expression yield of GST-SYE1, very little SYE1 was produced. However, the enzyme could be purified to homogeneity in a 4-step process (Chapter two, Section II.D).

The protein was concentrated to 10 mg/ml in a 100 mM Tris-HCl, pH 8.0, and 50 mM NaCl buffer. A first crystallisation trial was set up with Molecular Dimensions Structure Screen 1. After merely two days, crystals shaped as needles were observed in condition 30. This condition consisted of 2 M ammonium sulphate, 100 mM Hepes (4-(2-hydroxyethyl)-1-piperazinethane sulfonic acid), pH 7.5, and 2% PEG 400 (polyethylene glycol 400). Novel crystallisation trials were set up to reproduce the condition and to assay the effects of pH, ammonium sulphate and PEG400 concentration on crystal formation. These trials showed (1) that PEG400 was an absolute necessity for crystal formation and that the initial concentration of 2% was optimal, (2) that the pH could be varied between pH 6 and 9 without affecting
crystal formation, and (3) that by lowering the concentration of ammonium sulphate to 1.9 M higher quality needles could be obtained. These observations also suggested that an additive was needed to proceed from the formation of needles to the formation of higher quality crystals. We took the ‘best’ condition (100 mM Tris-HCl pH 8.0, 1.9 M (NH₄)₂SO₄, 2% PEG400) and added 0.2 µl of additive (Additive Screen, Hampton Research) to every 2 µl droplet. Most conditions produced needles, some droplets remained clear, but in condition 11 (0.5 % β-octyl glucoside) protein crystals grew overnight. We optimised the crystal growth by screening once again the effects of buffer/pH, ammonium sulphate concentration and the concentration of β-octyl glucoside. The use of Hepes buffer, pH 7.3, clearly propagated nuclei formation. Many small crystals were observed in this buffer. Contrarily, in Tris buffer, pH 8.0-8.5, few large crystals were obtained. Nuclei formation was also dependent on the concentration of ammonium sulphate. By lowering the concentration to 1.75 M, the crystal formation was slowed down; lower concentrations failed to support crystal growth, possibly because the supersaturated state was not reached. Clearly, whereas the number of crystals depended on the buffer and precipitant concentration, the quality of the crystals depended on the additive concentrations. β-Octyl glucoside concentrations could not be lowered below 0.25 % without effecting crystal quality. So the crystals used were grown in 100 mM Tris-HCl buffer, pH 8.0, 1.75 M (NH₄)₂SO₄, supplemented with 0.25 % β-octyl glucoside (Fig.4.9). Two crystal forms could be observed in the droplets. These were measured at the synchrotron source in Hamburg. The hexagonal crystals were shown to diffract to 2.3 Å, and the more strangely shaped pyramidal crystals to 1.4 Å. The latter crystals were used for diffraction analysis and shown to contain one molecule in the asymmetric unit, having a Vₘ value of 2.24 Å³/Da.

Fig.4.9. Crystals of SYE1. Note the two different crystal forms.
VI. The crystal structure of the *Shewanella oneidensis* OYE homologue SYE1 reveals a novel binding mode for phenolic ligands in the active site\(^{15}\)

VI.A. Abstract

The crystal structure of the *Shewanella oneidensis* Old Yellow Enzyme homologue SYE1 has been determined at 1.4 Å resolution. In addition to the oxidised form, which binds a molecule of PEG400 in its active site, the structures of the NADH reduced and the p-hydroxybenzaldehyde (pHBA) and p-hydroxyacetophenone (pACE) complexed forms were solved at 1.7 Å resolution. The overall structure reveals a monomeric enzyme comprising the typical eight stranded α,β-barrel, known for all OYE homologues, with one molecule of FMN bound on top of the barrel. The C-terminal loops of the barrel, with L6 being the most prevalent, are all orientated towards the centre of the barrel, making the active site tunnel quite narrow. As recently noted for YqjM of *Bacillus subtilis*, the FMN group in SYE1 is strongly butterfly bended in both the oxidised and the reduced state. A comparison of the SYE1 and YqjM structures indicates a role for Met25 in butterfly bending. Both phenolic ligands, when bound to the enzyme, are stacked in a similar manner above the flavin. The hydroxyl group is hydrogen bonded to His181 and Asn184 and the active site acid Tyr186 is located 3.5 Å above the ring of pHBA and pACE. This novel binding mode might explain why SYE1 is only able to form charge transfer complexes with a limited range of phenolic ligands. In addition, in the pHBA complexed form, L3 adopts a different conformation compared to the other solved structures. We can describe these conformations as a ‘closed’ and an ‘open’ form. In the ‘open’ form, a hydrophobic cleft opens up besides the active site entrance and accommodates a second pHBA molecule. This conformational shift and special binding mode might resemble how NADH is bound in the active site of SYE1.\(^{16}\)

VI.B. Introduction

The OYE family has been studied since the 1930’s when Warburg and Christian isolated a yellow enzyme from brewers’ bottom yeast (*S. carlsbergensis*). Theorell purified the enzyme in 1935 and showed that it was composed of colourless apoprotein and a yellow dye. The dye turned out to be a small vitamin B\(_2\) derived molecule (FMN) and to be absolutely necessary for catalysis. When a second yellow enzyme was isolated from yeast, the first enzyme was named ‘Old Yellow Enzyme’ or OYE, a name that has persisted till this day. OYE became a model for flavoprotein study and early studies demonstrated several important biochemical concepts (Williams and Bruce, 2002). Although the enzyme has been studied for over seven decades and the catalytic mechanism has been examined in detail (Brown *et al*., 1998; Kohli and Massey, 1998; Xu *et al*., 1999), the physiological role is only recently being unravelled (Lee *et al*., 1999; Haarer and Amberg, 2004; Reekmans *et al*., 2005).  

\(^{15}\) This section is the adapted version of a manuscript to be submitted to the Journal of Molecular Biology.  
\(^{16}\) (Loops are numbered after the β-strand they follow, thus L6 lies between β-strand 6 and α-helix 6.)
With the advent of modern cloning and sequencing techniques, many enzymes were added to the OYE family. These include the bacterial nitro-ester reductases PETN reductase (French et al., 1996), GTN reductase (Snape et al., 1997) and XenA/B reductase (Blehert et al., 1999), the bacterial morphinone reductase (French an Bruce, 1994), the plant OPDA reductases (Straβner et al., 1999; Schaller and Weiler, 1997), the different yeast OYE’s (Miranda et al., 1995; Komduur et al., 2002; Stott et al., 1993; Niino et al., 1995), and an enzyme involved in prostaglandin synthesis in Trypanosoma cruzi (Kubata et al., 2002). Although these enzymes originate from different organisms and catalyse different reactions, many similarities were found. All enzymes are able to reduce simple and complex unsaturated aldehydes and ketones (Vaz et al., 1995, French and Bruce, 1994), nitro-esters (French et al., 1996; Snape et al., 1997; Blehert et al., 1999; Meah and Massey, 2000) and nitro-aromatic substrates (Pak et al., 2000; French et al., 1998; Williams et al., 2004), yet distinct preferences are observed. OYEs can form long wavelength charge transfer interactions with phenolic compounds (Abramovitz and Massey, 1976), which are oriented in the active site by hydrogen bonding of the phenolate oxygen to a strictly conserved histidine and asparagine/histidine residue (Fox and Karplus, 1994). The reaction mechanism proceeds by a Ping-Pong mechanism, separated in an oxidative and reductive half reaction. The first crystal structures reported were very similar, with the greatest difference being the fold of the capping subdomain (Fox and Karplus, 1994; Breithaupt et al., 2001; Barna et al., 2001). Although the overall characteristics of the OYE enzymes are very similar, minor and major differences emerge with every new study: morphinone reductase lacks the general acid (tyrosine) that protonates the substrate (Messiha et al., 2005), enzymes possessing two histidines (Williams et al., 2004) for substrate binding can catalyse the reduction of TNT to the H-Meisenheimer complex, whereas the enzymes with only one histidine cannot and YqjM has a flavin that is bended in both the reduced and the oxidised state (Kitzing et al., 2005). While the initial studies focussed on the similarities between these enzymes, the focus nowadays lies in the differences.

Here we report the structure of SYE1, an Old Yellow enzyme homologue of Shewanella oneidensis. S. oneidensis is a gram-negative γ-proteobacterium with a very diverse metabolism (Heidelberg et al., 2002). During a BLAST search on the genome (www.tigr.org) using yeast OYE1 as a probe we identified 4 Old Yellow Enzyme homologues (SO2453, SO2454, SO3392, and SO4153) which we named SYE1-4 for Shewanella Yellow Enzyme. SYE1 (SO2454) is the Shewanella OYE homologue with the greatest sequence homology to OYE1. Based on the sequence alignment, we expected the structure of SYE1 to be quite similar to the bacterial homologues PETN reductase and morphinone reductase. Although the SYE1 structure does show good overall similarity to these enzymes, some important differences are found. SYE1 shows a strong butterfly bending of the flavin ring as noted for YqjM of Bacillus subtilis, a very narrow active site tunnel, a different binding mode of phenolic ligands in the active site and two distinct conformations for the capping subdomain.

VI.C. Materials and methods

VI.C.1. Materials

The Q-Sepharose FF and Source 30Q matrices and the MonoQ and Superdex 200 columns were purchased from Amersham Pharmacia, the pACYCDuet-1 vector from Novagen. Structure screen 1 was from Molecular dimensions LTD and the additive screen from Hampton Research. Trizma base, ammonium sulphate, FMN, MES, Na2HPO4 and NaH2PO4 were purchased from Sigma, HEPES and PEG400 from Fluka, NaCl and NADH from Merck,
t-hexenal, t-decenal, and t-dodecenal from Acros and IPTG (isopropylthiogalactoside) from Duchefa.

VI.C.2. Cloning, purification and crystallisation of SYE1

The cloning and purification SYE1 is discussed in Chapter two, Section I.A and III.D. The crystallisation is discussed in Section V.

VI.C.3. Data collection, structure solution and refinement

X-ray diffraction data were collected by members of the structural biology group of the Laboratory for Protein Biochemistry at the EMBL beamlines X13 and BW7A in DESY, Hamburg. All diffraction data were processed using the programs Denzo and Scalepack (http://www.hkl-xray.com/). Data statistics are presented in Table 4.2. A random 5% set of the possible reduced data was selected and used to flag reflections for the calculation of $R_{\text{free}}$ values. The same set of reflections was used for the calculation of $R_{\text{free}}$ for all datasets. The SYE1-PEG400 structure was solved by molecular replacement using the molecular replacement–phaser program of CCP4 (http://www.ccp4.ac.uk/main.html). The search model was generated from the coordinates of morphinone reductase, replacing non conserved residues by alanine (or glycine) and deleting all insertions and the FMN. The sequence identity between the two enzymes is 50%. After initial rigid body minimisation, refinement was performed by alternating model building using the program “O” (http://www.bioxray.dk/~mok/o-files.html) and crystallographic refinement using CNS 1.1 (http://cns.csb.yale.edu/v1.1/). FMN and the sulphate ion were added in the first rounds of refinement, PEG400 and β-octyl glucoside in the last. Ligands were added using atomic parameters extracted from the HICUP server (http://xray.bmc.uu.se/hicup/). p-Hydroxyacetophenone was composed by combining two molecules (phenol and an acetyl group). For all three soaking experiments difference density maps were calculated using coefficients $F_{\text{soak}} - F_{\text{obs,nat}}$ and calculated phases from the final refinement of the native structure. These structures were further refined after insertion of the proper ligand. The rmsd (root mean square distance) for the different models were calculated by the program ‘Sequoia’ (http://bruns.homeip.net/~bruns/sequoia.html) and figures were drawn in ‘Pymol’ (http://pymol.sourceforge.net/). The Ramachandran plot was generated by the program ‘Procheck’ (http://www.biochem.ucl.ac.uk/~roman/procheck/procheck.html).

VI.C.4. Soaking experiments

Crystals were washed two times with 100 mM Tris, pH 8.0, 2M ammonium sulphate and incubated in 100 mM Tris, pH 8.0, 2M ammonium sulphate, supplemented with 50 mM of the wanted ligand. Complex formation in the crystals was monitored via the rapid change of colour from yellow to lime-green, in the case of the phenolic ligands, and from yellow to colourless in the case of NADH. After incubation, the crystals were flash frozen in liquid nitrogen.

VI.C.5. Kinetic studies

All kinetic parameters were determined under strict anaerobic conditions and all assays were performed in triplicate. Reaction mixtures consisting of 0.5 x PBS buffer, pH 7.3 (5.75 g Na₂HPO₄, 1.49 g NaH₂PO₄ and 2.92 g NaCl/l), 60-120 µM NADH, and varying
concentrations of substrate were prepared within a glove box (Coy Laboratories, Grass Lake, Michigan) and transferred to sealable quartz cuvettes (Hellma). The concentration of NADH was determined spectrophotometrically using a molar extinction coefficient of 6220 M$^{-1}$ cm$^{-1}$ at 340 nm. Enzyme solutions were prepared in sealed flasks and made anaerobic by bubbling with N$_2$-gas. Reactions were initiated by the addition of enzyme to the reaction mixtures using a Hamilton needle. Enzyme concentration was kept constant at 100 nM. SYE activity was assayed by following the oxidation of NADH using a double beam Uvikon 943 spectrophotometer. Apparent steady state kinetic constants were determined by least squares fitting procedures to the standard Michaelis Menten equation.
Table 4.2. X-ray data-collection and refinement statistics of SYE1, p-hydroxybenzaldehyde (pHBA) and p-hydroxyacetophenone (pACE) complexed SYE1 and NADH reduced SYE1.

<table>
<thead>
<tr>
<th>Data collection</th>
<th>Native-SYE1</th>
<th>pHBA-SYE1</th>
<th>pACE-SYE1</th>
<th>NADH-SYE1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source, wavelength</td>
<td>DESY/EMBL-X13, 0.8034 Å</td>
<td>DESY/EMBL-X13, 0.8034 Å</td>
<td>DESY/EMBL-X13, 0.8067 Å</td>
<td>DESY/EMBL-X13, 0.8067 Å</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>1.4</td>
<td>1.7</td>
<td>1.7</td>
<td>1.7</td>
</tr>
<tr>
<td>Space group</td>
<td>P2₁,2₂,1</td>
<td>P2₁,2₂,1</td>
<td>P2₁,2₂,1</td>
<td>P2₁,2₂,1</td>
</tr>
<tr>
<td>Unit cell parameters (Å, °)</td>
<td>a = 48.45, b = 83.60, c = 87.53, α = β = γ = 90</td>
<td>a = 47.99, b = 83.91, c = 88.36, α = β = γ = 90</td>
<td>a = 48.56, c = 83.68, c = 87.48, α = β = γ = 90</td>
<td>a = 48.20, c = 83.74, c = 88.10, α = β = γ = 90</td>
</tr>
<tr>
<td>Vₐₐₐ (Å³ Da⁻¹)</td>
<td>2.239</td>
<td>2.247</td>
<td>2.245</td>
<td>2.245</td>
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<tr>
<td>Solvent content (%)</td>
<td>45.06%</td>
<td>45.25%</td>
<td>45.18%</td>
<td>45.21%</td>
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<tr>
<td>Unique Reflections</td>
<td>69169</td>
<td>38033</td>
<td>39223</td>
<td>38791</td>
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<tr>
<td>Redundancy</td>
<td>6.8 (6.1)²⁷</td>
<td>6.9 (6.4)</td>
<td>6.5 (5.9)</td>
<td>7.0 (6.2)</td>
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<tr>
<td>Completeness (%)</td>
<td>99.9 (99.3)</td>
<td>99.5 (97.4)</td>
<td>99.7 (98.4)</td>
<td>99.9 (99.6)</td>
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<tr>
<td>Rₚₛₚ (%):&lt;sup&gt;²&lt;/sup&gt;</td>
<td>0.031 (0.232)</td>
<td>0.056 (0.450)</td>
<td>0.033 (0.090)</td>
<td>0.056 (0.248)</td>
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<td>Average 1/σ(I)</td>
<td>42.9 (7.6)</td>
<td>32.9 (4.0)</td>
<td>49.0 (18.2)</td>
<td>32.7 (7.3)</td>
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Refinement

| Resolution (Å)                   | 30-1.4      | 30-1.7     | 20-1.7    | 20-1.7    |
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VI.D. Overall structure

The structure of oxidised SYE1 was determined and refined at 1.4 Å resolution, to a final \(R_{\text{cryst}}/R_{\text{free}}\) value of 0.158/0.174, by molecular replacement, using morphinone reductase as the search model. All residues were found in the most favoured, additional or generously allowed regions of the Ramachandran plot except for the aromatic amino acid Trp274 which lines the active site tunnel.

SYE1, like OPR1 and PETN reductase (Breithaupt et al., 2001; Barna et al., 2001), consists of a single 361 residue domain (monomer) that folds into a typical eight stranded parallel \(\alpha,\beta\)-barrel, containing one non-covalently bound FMN molecule as the prosthetic group (Fig. 4.10).

The flavin, which is located towards the centre and C-terminal end of the barrel, is surrounded by the central \(\beta\)-strands. Residues in and directly following these \(\beta\)-strands make up most of the contacts with the flavin.

Like morphinone reductase and PETN reductase (Barna et al., 2001; Barna et al., 2002), SYE1 has four extra-barrel elements (Fig. 4.10), the N-terminal beta-hairpin which closes of the bottom of the barrel, the big excursing between \(\beta\)-strand 3 and \(\alpha\)-helix 3 which makes up the capping subdomain of the barrel, the \(\alpha\)-helix between \(\beta\)-strand 4 and \(\alpha\)-helix 4 which in OPR1 (Breithaupt et al., 2001) is thought to aid in substrate recognition, and the \(\alpha\)-helix between \(\beta\)-strand 8 and \(\alpha\)-helix 8 which is necessary for the binding of FMN. The capping subdomain of SYE1 is composed of a small helix (half a turn), followed by a pair of anti-parallel \(\beta\)-strands connected by a hairpin loop, as is the case for morphinone reductase and PETN reductase (Barna et al., 2001; Barna et al., 2002).

![Fig. 4.10. Topology diagram of SYE1. The arrows represent the \(\beta\)-strands, whereas the cylinders represent \(\alpha\)-helices, the barrel elements are numbered 1-8, whereas the extra-barrel elements are indicated by letters. The numbers (>8) refer to the amino acids.](image)

The capping subdomain of SYE1 is very mobile and the amino acids are not always well discerned. Additionally, the C-and N-terminus and some of the charged amino acids on the surface are disordered.
The overall structure of SYE1 closely resembles that of morphinone reductase, and 344 residues can be superimposed with an r.m.s.d. of 1.2 Å. The largest differences can be seen in the capping subdomain and the loop between β-strand 6 and α-helix 6. The β-hairpin that connects the two β-strands of the capping subdomain is, in SYE1, orientated towards the inside of the barrel. In addition, in SYE1, Loop 6 protrudes into the active site tunnel, making it also very narrow compared to morphinone reductase.

SYE1 can be compared with the other known structures of OYE homologues and imposes with PETN reductase with a r.m.s.d. of 1.3 Å over 347 residues, followed by OPR1 (1.4 Å over 317 residues), OYE (1.6 Å over 293 residues) and YqjM (1.7 Å over 217 residues). During refinement, the Fo-Fc map of SYE1 showed clear additional density for three non-protein elements: a PEG400 molecule, a sulphate ion and a β-octyl glucoside molecule (Fig.4.11).

PEG400 was absolutely necessary for crystallisation, and could be traced in the active site of SYE1. A similar observation has been made for OPR1 (Breithaupt et al., 2001), but whereas the PEG400 molecule is bended double in the active site of OPR1 thus resembling the substrate 12-oxophytodienoic acid (OPDA), the PEG400 molecule in SYE1 is fully extended. In addition, only the first 6 atoms, corresponding to two ethylene glycol units, can enter the active site tunnel; the rest of the molecule bends around the hydrophobic active site entrance and sticks out like a pig’s tail. Interactions between the PEG molecule and SYE1 consist of van der Waal’s interactions with the hydrophobic lining of the active site. Although the PEG400 molecule could be completely traced, the molecule has considerable mobility both in the active site and outside the enzyme.

The sulphate ion and the β-octyl glucoside molecule participate in intermolecular contacts between two adjacent SYE1 molecules in the crystal lattice. The β-octyl glucoside molecule lies on one side parallel to His39 and is coordinated on the other side by Lys*261 (* indicates amino acid belonging to symmetry related molecule). The tail of the β-octyl glucoside molecule, in contrast to the ring is not well defined and possibly mobile.

The sulphate ion is coordinated by His337 and Arg336, and on the other side by His*344, Arg*301 and Arg*323. The latter two amino acids are also involved in the coordination of the flavin phosphate oxygens.
VI.E. The active site structure

The active site is composed of a shallow and narrow tunnel about 10-15 Å long, and is lined by aromatic and hydrophobic residues. At the end, the tunnel opens up towards the flavin. Loop 6, folds towards the centre of the barrel and makes up an active part of the lining of the tunnel. The flavin in SYE1 is strongly butterfly bended around the hypothetical N5-N10 axis in both the reduced and oxidised state (Fig.4.12). Reduction of the enzyme does not result in a further bending of the flavin. A similar feature is reported for the flavin group in *Bacillus* YqiM (Kitzing *et al.*, 2005).

![Fig.4.12. Superposition of SYE1 (blue) and PETN reductase (green) centred at the FMN prosthetic group. Note the bended flavin of SYE1 (orange) compared to the planar flavin of PETN reductase (magenta).](image)

The bending must be brought about by the apoprotein environment of the flavin, but without the availability of structures to compare with, Kitzing *et al.* did not make any assumptions as to which amino acids might be the determinants. Comparison of the flavin region in both SYE1 and YqiM, however, shows one similarity for these two enzymes: Leu25 is replaced by Met25. Sequence alignments of different OYE homologues (Fig.1.28) show that only Leu and Met occupy this position. This residue lies directly under the dimethylbenzene ring of FMN (Fig.4.12). The functionality of a butterfly bended FMN group is not yet known, and no abnormalities caused by this bending are observed thus far.

FMN is tightly bound in the protein, exhibiting low B-factors for all models (Fig.4.13). The amino acids that contact the riboflavin moiety of FMN are conserved among the different known OYE structures (Fox and Karplus, 1994; Barna *et al.*, 2001; Barna *et al.*, 2002; Breithaupt *et al.*, 2001; Kitzing *et al.*, 2005): the flavin N(5) interacts with the main chain amide of Thr26, O(4) is hydrogen bonded to the amino acid side chain of Thr26 and the main chain amide of Gly58, and N(3) and O(2) interact with the side chain of Gln100. O(2) has an additional hydrogen bond with the amide of His181 and the guanidine group of Arg233.
Fig. 4.13. Binding mode of FMN in the active site of SYE1. Some of the hydrogen bonds are represented with dashed lines. Dotted spheres represent water molecules.

Differences can be seen in the amino acids binding the ribityl hydroxyl groups and the phosphate oxygen atoms. The first two hydroxyl groups are each hydrogen bonded to the guanidino group of Arg233, and to the main chain carbonyl oxygen of Pro24 and a water molecule, respectively. The third OH group and the phosphate oxygens are involved in an extensive hydrogen bonding network comprising the side chains of Arg323 and Arg301, the main chain amides of these two arginines and of Gly300, and three water molecules.

In contrast to the si-face of the flavin, the ribityl side chain and the dimethylbenzene ring, which are all solvent accessible, the re-face of the flavin is buried in the structure and is surrounded by amino acids of Loop 1.

VI.F. Enzyme kinetics

Thus far, activity of SYE1 has only been shown with relatively small molecules: N-ethylnmaleimide, acrolein and 2-cyclohexenone (Chapter three, Section III.C.3). The bulkier molecules glycerol trinitrate and androstanediene/progesterone show no reaction with SYE1. It is likely that these molecules cannot reach the narrow active site tunnel of SYE1. The presence of a PEG400 molecule in the active site tunnel of SYE1 prompted us to explore whether SYE1 can accept long chain unbranched α,β-unsaturated aldehydes as a substrate. Therefore steady state kinetic analysis was performed with trans-2-hexenal, trans-2-decenal and trans-2-dodecenal, as described in Materials and Methods. Trans-2-hexenal, t-2-decenal and t-2-dodecenal are naturally occurring volatile components of plant oils, with bactericidal properties shown against Salmonella (Croft et al., 1993; Kubo et al., 2004). Their corresponding alkanals are less toxic.

SYE1 shows reactivity to all three substrates; the results are summarised in Table 4.3.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (µM/s$^{-1}$)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$ µM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>t-2-hexanal</td>
<td>2320</td>
<td>0.67</td>
<td>6.72</td>
<td>0.00289</td>
</tr>
<tr>
<td>t-2-decenal</td>
<td>2350</td>
<td>0.29</td>
<td>2.92</td>
<td>0.00124</td>
</tr>
<tr>
<td>t-2-dodecenal</td>
<td>2090</td>
<td>0.31</td>
<td>3.10</td>
<td>0.00148</td>
</tr>
</tbody>
</table>

Table 4.3. Apparent kinetic constants of SYE1 with t-2-hexenal, t-2-decenal and t-2-dodecenal.

The $k_{cat}/K_m$ values of SYE1 for these substrates are comparable to each other, differing only by a factor two. This is in contrast to OPR of tomato where the activities of t-2-hexenal and t-2-dodecenal differ by a factor 20 (Straβner et al., 1999). This difference is probably due to the different binding mode of these substrates into the two enzymes. Whereas OPR can accommodate for the full length chain in its active site (Breithaupt et al., 2001), SYE1 only binds the first 6 atoms. The rest of the molecule is situated outside the tunnel entrance. A preference can be seen for the shorter chain of t-2-hexenal. A similar observation was made for LeOPR, which has the highest catalytic efficiency with the small molecule NEM.

VI.G. Complexes of SYE1 with phenolic ligands and NADH

We have performed three experiments in order to obtain complexes of SYE1 with the cofactor reductant (NADH) and phenolic ligands. During incubation with NADH, the yellow colour of the crystals faded, indicating reduction of the flavin. However the occupancy of NADH in the active site was too low to allow modelling of this molecule in the active site. This appears to be a recurring theme in this family of enzymes as attempts to obtain such complexes in morphinone reductase (Barna et al., 2002), OPR1 (Breithaupt et al., 2001) and OYE (Fox and Karplus, 1994) also failed. The ligands were p-hydroxybenzaldehyde (pHBA) and p-hydroacetophenone (pACE). The choice of ligands for the soak was limited by the fact that previous experiments had shown that SYE1 is not capable of binding phenolic ligands with high pK$_a$ (Chapter three, Section II.C).

Upon soaking with the phenolic compounds, the crystals turned from yellow to green. Analysis of the crystal structures showed full occupancy for these ligands in the active site. The reduced and the complexed structures were solved by difference Fourier analysis to a resolution limit of 1.7 Å and final $R_{cryst}/R_{free}$ values of 0.188/0.222, 0.161/0.191 and 0.168/0.195 respectively.

VI.G.1. NADH reduced structure

The crystals soaked with NADH turned colourless, indicating the reduction of SYE1. NADH, however, could not be discerned in the active site. It has been noted for other homologues that reduced flavin harbours ordered water molecules above the ring which replace previously bound ions (Cl$^-$) (Fox and Karplus, 1994; Barna et al., 2001, Breithaupt et al., 2001). Although no ions are present in SYE1 due to the presence of PEG400, upon reduction, three ordered water molecules could be detected in the active site.
VI.G.2. pHBA and pACE complexed structures

The two phenolic compounds were well defined in the difference density maps and the orientation of the molecules could easily be determined in the active site. pHBA and pACE are orientated in the same fashion (Fig. 4.14). The phenolate oxygen is placed above the C2 atom of the flavin, forming a hydrogen bond with His181 and Asn184. While the aldehyde carbonyl of p-hydroxybenzaldehyde in OYE is hydrogen bonded to Tyr375 (Fox and Karplus, 1994), no bond of this kind is possible in SYE1 since Tyr375 is replaced by Phe350; instead the carbonyl oxygen is hydrogen bonded to Trp274, explaining the special position of Trp274 in the Ramachandran plot. The active site amino acid Tyr186 is located above the phenolic ring (Fig. 4.14).

![Fig. 4.14. FMN (orange), pHBA (blue) and pACE (magenta) bound in the active site of SYE1. Hydrogen bonds are represented by dotted lines.](image)

It has been shown for morphinone reductase that a Tyr356Phe mutation (equivalent of Tyr375) elevates the $K_m$ for NADH with a factor of 10. For SYE1 the $K_m$ for NADH binding is 9.3 µM. A comparison with different OYE homologues (Table 4.4) shows that this is a ‘normal’ value. Possibly, Trp274 not only substitutes for the lost interaction of Tyr375 with the phenolic ligand, but also for NADH binding.


<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m^{NAD(P)H}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYE1 (this study)</td>
<td>9.3</td>
</tr>
<tr>
<td>Morphinone reductase$^{18}$</td>
<td>8.9</td>
</tr>
<tr>
<td>Morphinone reductase Y356F</td>
<td>61.2</td>
</tr>
<tr>
<td>OYE1</td>
<td>7.5</td>
</tr>
<tr>
<td>XenB reductase</td>
<td>5</td>
</tr>
<tr>
<td>XenA reductase</td>
<td>28</td>
</tr>
<tr>
<td>GTN reductase</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 4.4. Values of the $K_m$ constants for NAD(P)H for different OYE homologues.

VI.G.3. The ‘open’ conformation of Loop 3

The NADH reduced and the pACE complexed structures differ little from the PEG bound SYE1 structure, the most notable feature is the disappearance of the β-octyl glucoside molecule in the NADH reduced structure, which brings about a 90° flipping of His39 around the hinge. The pHBA structure, on the contrary, was not straightforward to interpret. The soaking increased the internal disorder of the crystals, resulting in the disappearance of the electron density of many side chains. As in the NADH-reduced structure, the β-octyl glucoside molecule was washed away in the soak and, in addition, the sulphate ion became highly disordered.

The active site pHBA molecule could be readily placed. But, quite unexpectedly, a second pHBA molecule could be discerned, being sandwiched between two Phe residues (Phe132 and Phe350). To accommodate for this molecule, Phe132 was flipped 90° around the hinge (Fig.4.15) and the hairpin loop of the capping subdomain was twisted about 20° around amino acid Val133 and 80° around Gly141 towards the outside of the barrel (Fig.4.15), thus creating a hydrophobic cleft besides the active site entrance hole in which pHBA could fit (Fig.4.16). High temperature factors made it impossible to see the entire capping subdomain, thus losing information for the four amino acids that make the ‘tip’ of the loop.

In addition to Phe132 and Phe350, the pHBA molecule was surrounded by the aromatic amino acids Phe142 and Tyr68. Of these amino acids, only Tyr68 is conserved in the OYE family. The placement of Tyr68 is such that it might replace Phe137, creating the possibility of a similar interaction in other OYE homologues. This interaction, however, would not bring about a similar shift in the capping subdomain.

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$^{5}$ Km values of morphinone reductase (wild type and mutant enzyme) were taken from Messiha et al., 2005; the other values from Marschall et al., 2004.
Fig. 4.15. Zoomed-in view of Loop 3 of the SYE1 uncomplexed (blue) and pHBA-complexed (green) structures. Represented in red is the pHBA molecule sandwiched between Phe132 and Phe350, and in orange the FMN. Note the different conformation of Loop3 and of Phe132 (arrow), and the sterical hindrance between Phe132 (blue) and pHBA.

Fig. 4.16. Top view of the pHBA-complexed structure (space fill presentation) showing the two molecules of pHBA (red), one in the active site tunnel of SYE1 and one in the cleft besides the tunnel. Green patches indicate the presence of hydrophobic residues and red and blue patches represent charged residues.
It is tempting to envisage the inner pHBA molecule representing the nicotinamide moiety and the outer pHBA molecule the adenine moiety of NADH. The second pHBA molecule, however, can not be replaced by adenine, but can correspond to the second ribose, the adenine sticking out of the molecule and possibly aligning with Phe142 in the hydrophobic cleft. This model is consistent with the theory proposed by Barna et al. (2001), who proposed that the distal part of the NADH moiety interacts with Loop 3 and argued that the preference for NADPH in PETN reductase is brought about by the presence of 2 arginines (Arg142 and Arg130) in this loop. In morphinone reductase (prefers NADH) these arginines are absent, and a glutamic acid is present (Glu135). If one extends this theory to SYE1, one sees the presence of both a lysine (Lys130) and a glutamic acid (Glu139). Since SYE1 has a preference for NADH, this theory can not be extended to all OYE homologues. Just as one can model NADH to fit the two pHBA molecules, one can model an aliphatic chain in the active site tunnel, combined with a ring system in the hydrophobic cleft, thus generating a retinal-like type of substrate.

VI.H. Discussion

At first sight, the SYE1 structure appeared to be little different from morphinone reductase, but upon refinement and after analysis of the complex with pHBA, some interesting special features were discovered. The flavin molecule is butterfly-bended as in YqjM. Only 10% of all flavoproteins are thought to have a bended FMN group (Haynes et al., 2002). The cause and the functionality of this bending are not yet known. Comparison of the YqjM and SYE1 structures prompt us to propose that Met25 may be a major determinant in the bending of the flavin. Koder et al. (2002) argue that a bended flavin destabilises the formation of semiquinones and thus of one-electron reactions of the flavin. In this respect, it is interesting to note that semiquinone species were observed during photoreduction of LeOPR (Straßer et al., 1999) and OYE (Stewart and Massey, 1985), but not for YqjM (Fitzpatrick et al., 2003) and SYE1. The behaviour of YqjM and SYE1 thus resemble that of other bacterial OYE homologues such as PETN reductase (Khan et al., 2002), morphinone reductase (Craig et al., 2001), XenA reductase and XenB reductase (Blehert et al., 1997). Determination of the X-ray structure of the two former enzymes has shown their flavins to be planar (Barna et al., 2001; Barna et al., 2002). Clearly, a mechanism other than the bending of the flavin is responsible for the lack of semiquinone formation in these enzymes. The active site of SYE1 is very narrow due to Loop 6 protruding deeply towards the inside of the barrel. This was quite remarkable, since, based on the high similarities with the bacterial homologues PETN reductase and morphinone reductase, we had expected an ‘open’ active site. In addition, Trp274, a residue on Loop 6, contributes to the active site by forming a hydrogen bond to the carbonyl oxygen of the phenolic ligands. It replaces the Tyr375 (OYE1 numbering, Fox and Karplus, 1994) interaction that is lost by a mutation to Phe in SYE1. Morphinone reductase has a Trp279 corresponding to Trp274; this residue, however, is located more towards the outside of the barrel. Two scenarios could explain the emerging role of Loop 6. The conformation of Loop 6 in SYE1 arose when the enzyme had to substitute for the lost Tyr375 interaction. Alternatively, Loop 6 appeared first in the active site and since Trp279 could substitute for the Tyr375 interaction, a Tyr→Phe mutation was not harmful for catalysis. A BLAST search on the nr database of the NCBI website using SYE1 as the probe, however, gave us a different picture. We found numerous proteins with high homology to SYE1 possessing the equivalents of Met25 as well as those of Trp274 and Phe350. These proteins are only present in bacteria of two different classes: the β-proteobacteria (Burkholderia,
Ralstonia,…) and the γ-proteobacteria (Shewanella, Vibrio, Photobacterium,…). Previous studies have shown that SYE1 (and SYE3) is not capable of forming CT complexes with phenolic ligands with a pKₐ > 9. A preliminary study of a Vibrio homologue has shown that this enzyme is also not capable of forming a CT complex with p-cresol (Chapter six). These observations indicate that the special binding mode of the phenolic ligands in the active site of SYE1 might not be restricted to this enzyme, and that SYE1 might represent a subclass of the OYE homologues in β- and γ-proteobacteria.

In addition, the shallow and narrow landscape of the active site of SYE1 puts some spatial restraints on possible substrates. Regarding the fact that PEG400 is absolutely necessary for protein crystallisation and is found bound the active site, one can argue for a t-2-alkenal type of biological substrate. Trans-2-alkenals are noted as breakdown products in lipid peroxidation reactions (Croft et al., 1993), produced from poly-unsaturated lipid acids. Substances as t-2-hexenal and t-2-dodecenal are volatile compounds of plant oils, produced upon bacterial injury to the plant. Shewanella oneidensis, however, is not known as a plant pathogen, and poly-unsaturated lipid acids are only present in psychrophilic bacteria (Bowmann et al., 1997; Abboud et al., 2005). Thus an involvement of SYE1 in detoxification of these compounds is unlikely. In addition, the presence of a second pHBA molecule in the pHBA complex, and the hydrophobic nature of the active site entrance indicate that the physiological substrate of SYE1 may be a molecule combining a bulky (possibly aromatic) head connected to a thin tail. The ‘tail’ of the bulky substrate could enter the narrow active site, and the bulky head could be located outside the tunnel, in the cleft besides the active site tunnel.
VII. Comparison of the theoretical SYE models with the experimental SYE1

When the experimental structure of SYE1 was solved (Section VI), we wondered whether its structure resembled the modelled one, described in Section I, the issue being that a comparison of the theoretical and the experimental model would show the power of the programs used, to calculate the theoretical model.

![Comparison of the loops in the theoretical model of SYE1 (blue) and the experimental model (red). Shown are Q32 of Loop 1, N112 and S137 of Loop 3 and W274 of Loop 6 to illustrate the differences.](image)

We found that there are important differences between the theoretical and the experimental model. As expected, the barrels imposed well, but the conformation of the loops was different (Fig.4.17). The part of Loop 3 that was disordered in the theoretical model folded neatly into an α-helix, just as noted for morphinone reductase and PETN reductase. The hairpin loop connecting the two β-strands of Loop 3 was oriented towards the centre of the barrel and not to the outside. In addition, Loop 1 and Loop 6 were orientated towards the centre of the barrel (Fig.4.17).

Since the orientation of the loops was quite different for the experimental and the theoretical model, we wondered whether the structures of the other SYEs would be different when we included the experimental structure of SYE1 in the calculation of the models.

As a consequence, new models were calculated using the Swiss-model server. The sequences were modelled based on the coordinates of SYE1, morphinone reductase, OYE1 and OPDA reductase. These models were not cross-checked by the different programs noted in Section I.B. The new theoretical structures were slightly different from the previous ones, the loops folding into a conformation resembling SYE1 more than morphinone reductase.
The question that instantly arises is: ‘What is the quality of the models if a different input generates a different outcome?’ To answer this question one has to check the C values. Experimental coordinates of protein structures have B-values or temperature factors. These B-factors are an indication of how mobile an amino acid side chain/atom is. In Swiss-Model, a similar factor is added to the coordinates, the C-value. It indicates how dependable the coordinates are. An amino acid that is modelled with good certainty has a value of 10; one of little certainty can have values as high as 99. Pymol gives an easy graphical presentation of the B/C-factors. In Figure 4.18, concerning SYE1, the rigid amino acids are coloured blue and the mobile ones range from green to red. Analysis of the models showed the highest uncertainty to be located in the top loops.

Fig. 4.18. Theoretical model of SYE1, coloured regarding increasing C-score from blue → red.

VII.C.3. Conclusion

With the calculation of different models of OYE homologues based on different input coordinates, we here observe a characteristic that is often noted for OYE homologues: the barrel domain is rigid and well conserved, whereas the loops on top of the barrel adopt different conformations. The exact conformations have to be determined experimentally. Possibly the elucidation of more experimental structures with more similar sequences in the loops will improve the possibility of correct structure prediction in these parts. For now, care must be taken to draw conclusions from these models, since the top loops are exactly those that define the shape of the active site entrance and tunnel. We can thus conclude from the models which amino acids make up the body of the enzyme, how long the loops are, and what amino acids surround the flavin, but the really crucial information, i.e. the shape of the active site, can not be inferred from these models.
VIII. Conclusion

It is interesting to determine the structure of novel OYE homologues, since, although the core structure is very rigid, the loops that make up the active site entrance and determine the substrate specificity are orientated in different ways. These little differences can have serious impact on the enzymatic activity of the protein, in other words, on its structure-function relationship.

The crystallisation of the different SYE proteins was quite troublesome. SYE3 had to be excluded from the trials, since incomplete removal of the GST occurred. SYE1 and SYE4 failed to crystallise when expressed from the pGEX-4T-2-vector and purified as a GST-tagged enzyme, not only due to a bad choice of buffer (phosphate), which is a general standard for use in purifying GST-tagged proteins, but also due to the presence of impurities in the protein sample. Both SYE1 and SYE4 possessed an internal thrombin recognition site. Trace amounts of thrombin in the protein sample, may have resulted in proteolytic breakdown. Since the nick was at the already mobile capping subdomain of Loop 3, it is likely that some additional mobility of this loop prevented crystallisation.

The importance of dynamic light scattering as a tool in protein crystallography was shown in the case of SYE4. This protein was only present in a monodisperse state when a Tris-buffer supplemented with 50 mM NaCl was used.

The use of a different vector for protein expression and a different purification protocol was very successful in the case of SYE1. Due to lack of time, this different cloning mode has not yet been applied to SYE3 and SYE4.

We think, however, that all attempts in recloning, purification and crystallisation should in first instance be directed towards SYE4. This enzyme shows characteristics clearly different from SYE1 and SYE3 and it lacks a large part of the conserved C-terminal domain. It would be interesting to know if SYE4 would adapt a fold similar to YqiM, which also lacks the C-terminal domain, or that it adapts a fold thus far unencountered.
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(additive screen)
XI. Introduction

The physiological role of *Saccharomyces*’ OYE has been a mystery for over 70 years. The ability of the enzyme to reduce a wide range of different substrates prevented the assignment of the ‘true’ physiological substrate, and the lack of a true phenotype for knock-out mutants severely hindered the search for function (Stott *et al*., 1993). Due to novel techniques, indications for a function are found by recent experiments, and the OYE mystery is beginning to be unravelled, even though the picture is as yet far from complete.

The first physiological function was recognised for the plant OYE homologue OPR3. It has a key role in the production of jasmonic acid, catalysing the reduction of OPDA to OPC8:0 (Turner *et al*., 2002). But, as the numbering of the enzyme indicates, there are multiple OYE homologues present in plants. The role of these enzymes is not yet recognised (Schaller *et al*., 1998). For the *S. cerevisiae* homologues OYE2 and OYE3, a function in the protection of the actin cytoskeleton against oxidative stress (Haarer and Amberg, 2004) and a role in Bax-mediated programmed cell death (Reekmans *et al*., 2005) have been suggested respectively. The role of the former was recognised by a yeast-two-hybrid screen whereby OYE2 interacts with actin, and the latter by differential micro-array transcriptional profiling. Possibly other yeast homologues fulfil a similar function.

Besides the physiological function in yeast and plants, the functions of these enzymes in bacteria are beginning to be explored. The onset has been made for the Gram-positive bacterium *Bacillus subtilis*, where induction studies showed the enzyme to be involved in oxidative stress responses (Fitzpatrick *et al*., 2003). By identifying a possible partner for YqjM, these authors hope to gain a better picture of the role of this enzyme in *Bacillus*.

In analogy to the experiments reported for *Bacillus subtilis*, we engaged in exploring SYE protein function. Biochemical and structural characterisation had already shown that the SYE proteins have different characteristics, and could be divided in two subgroups comprising SYE1 and SYE3 on the one hand and SYE4 on the other. This indicated that differences in function might exist (Chapter three and four). Possibly these results could be confirmed by some additional experiments.

A first approach was made by performing induction studies (Section III). Antibodies were generated in rabbit against recombinant SYE1 and used for expression analysis of SYE proteins in *Shewanella oneidensis* cultures grown under different conditions.

In a second series of experiments we directed our focus towards the search for a physiological partner. Different approaches were used for partner search: ProteinChip Technology (SELDI) and pull-down assays. In both techniques SYE1 or SYE4 is used as ‘bait’ isolate out the ‘target’ from a protein solution (Section IV).

Before discussing the results obtained, we will first describe the techniques used for identification of the protein partner (Section II).
XII. Techniques

II.A. ProteinChip technology as a tool in partner search

II.A.1. Introduction

The ProteinChip system of Ciphergen is a quite novel and not widely used technique to study molecular interactions. A different name, often used for this technique is SELDI : Surface Enhanced Laser Desorption/Ionisation. It can be used for different purposes : to study protein-protein interactions, DNA-RNA interactions, to optimise a purification protocol of a specific protein, for immuno-assays,… Because of its novelty we will discuss the technique in some more detail.

The ProteinChip system is comprised of a ProteinChip Reader (or a time-of-flight mass spectrometer) to analyse proteins captured on ProteinChip Arrays. The ProteinChip System detects and accurately calculates the mass of compounds ranging from small molecules and peptides of less than 1000 Da up to proteins of 500 kDa, or more, based on the measured time-of-flight (ProteinChip Users guide, Ciphergen).

We will explain some things in more detail.

‘ProteinChips’ arrays are small chips, which comprise 8 to 12 spots (Fig.5.1). These spots are coated with a specific chemical surface. This can be ‘classic’ chromatographic surfaces, such as hydrophobic surfaces, cationic surfaces, IMAC surfaces, etc. Or it can be a biochemical surface like a protein, a DNA fragment, an antibody,… The naming ProteinChip can be somewhat confusing, since non-protein molecules as DNA, drugs,… can be bound to the Chips as well.

---

**Chemical Surfaces**

- Hydrophobic
- Hydrophilic
- Ionic
- IMAC

**Biochemical Surfaces**

- Antibody
- DNA
- Enzyme
- Receptor
- Drug

---

Fig.5.1. Picture of a ProteinChip (bottom, blue) and graphical presentation of the different coatings (top). Presented are chemical (hydrophobic, hydrophilic, ionic and IMAC) surfaces and biochemical surfaces (antibody, DNA, enzyme, receptor and drug).
The chemical surfaces can be purchased as they are presented. The biochemical surfaces must be made by the user, by covalently attaching a molecule of interest to specialised, preactivated chips: ‘PS Chips’ (Fig. 5.2).

Fig. 5.2. Different biomolecules that can bind the surface of a preactivated ProteinChip.

The spots on these PS chips are coated with, for example, reactive epoxy groups (Fig. 5.3, right panel).

ProteinChip Arrays - Current Derivatized Surfaces

<table>
<thead>
<tr>
<th>Chromatography</th>
<th>Coupling Chemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Hydrophobic [C16], WCX [carboxylate], SAX [quaternary ammonium], IMAC [nitrilotriacetic acid], Normal [silica oxide])</td>
<td>(PS-I [Carbonyldiimazole, amine], PS-II [Epoxy, amine or thiol])</td>
</tr>
</tbody>
</table>

Fig. 5.3. Two different kinds of ProteinChip. Specialised chips with a chromatographic coating (left). General purpose chips with a reactive coating for the covalent coupling of biomolecules (right).

The choice of the chip depends on the application. For designing protocols for protein purification, ionic, hydrophobic and IMAC surfaces can be used. Biochemical surfaces are used for immuno-assays, to analyse protein-protein interactions, to analyse DNA-RNA interactions,…

For example, in this thesis, we wanted to study protein-protein interactions. Therefore the protein of interest (the bait) was coupled to a specialised PS chip (Fig. 5.3, right panel) to generate a biochemical surface exposing the protein of interest (Fig. 5.2. biochemical surfaces, antibody and enzyme).
II.A.2. Study of protein-protein interactions using SELDI-technology

Preparing a ProteinChip with a biochemical surface

In order to study protein-protein interactions, one must first, as noted above, attach the protein of interest (the bait) covalently to a specialised PS Chip (Fig.5.4, (1) and (2)). To prevent molecules to bind non-specifically to the chip, the remaining reactive groups are blocked (Fig.5.4, (3)).

![Diagram of ProteinChip preparation](image)

*Fig.5.4. A biomolecule (bait) is bound on a preactivated surface and the unused sites are blocked. The chip is incubated with a sample and interactions occur between the bait and the molecules of the sample. Unspecific and non-interaction molecules are washed away and the prey remains bound on the bait. The energy absorbing molecule is added and the molecular weight of the prey is determined.*

Protein-protein interaction

Then we proceed to the interaction between the protein bound (the bait) and the target protein. The Chip is incubated with a protein solution (e.g. a cell lysate). Interactions are formed between the bait molecule and possible target molecules in the solution (Fig.5.4, (4)). To remove non-specifically binding proteins, the Chip is washed. Only proteins that display a strong interaction with the bait remain captured on the surface of the chip (Fig.5.4, (5)). The result is a Chip to which a protein of interest is bound: the target.

To every spot EAM (Energy Absorbing Molecule) is added (Fig.5.4, (6)). The application of this EAM in organic solvent causes the protein to dissolve into solution with the EAM. When this solution dries on the Chips surface, a very crude crystal is formed that includes both the protein and a large molar excess of EAM molecules. This EAM is needed for the subsequent analysis of the chip.
Analysis of the Chip

In order to determine the identity of the target protein, the Chips needs to be analysed. This occurs in the ProteinChip reader. This reader is in fact a small time-of-flight mass spectrometer. The Chip is inserted in the reader (imagine a large box with a slit, in which the ProteinChip fits), and every spot is illuminated with a laser beam (Fig.5.4., (7)). This illumination starts the process of ionisation and desorption. The ionisation of the protein results from a poorly understood interplay between the laser energy, the EAM and the protein. Thus the protein becomes charged and is transformed into the gaseous phase. It can therefore be caused to fly upon application of a voltage differential. The voltage differential applies the same energy to all the proteins in the sample, thus resulting in a time-of-flight that is proportional to the mass. The ProteinChip Reader records the time-of flight and from this measurement a highly accurate and precise mass is derived (Fig.5.8).

Identification

The final result of the analysis of a ProteinChip is the molecular mass of the interacting molecule; however, given the complexity of the proteome of an organism, the molecular weight alone is not enough to identify the target protein. As a method for the identification of the captured protein, ‘on chip’ tryptic digests combined with the subsequent analysis of peptide fragments has been proposed by Caputo et al. (2003). Alternatively, one could determine the C-terminus of the captured peptide by the use of carboxypeptidase Y to digest the protein (Cool and Hardiman, 2004). The principle is depicted in the flow chart of Figure 5.5.

![Flow chart](image-url)

*Fig. 5.5. The principle of identification of a bound protein sample by ’on chip digest’.*
A requirement for identification is that the protein is detected as a single peak by the ProteinChip Reader; this implies that the protein needs to be sufficiently pure. Therefore one can purify the protein of interest by standard chromatographic procedures to near homogeneity before analysis. The ProteinChip technology is then used after every subsequent purification step to determine the fraction(s) where the protein of interest is present. If sufficient purity is reached, the aimed protein can then be analysed by the above mentioned technique, or it can separated from the remaining contaminants by SDS-PAGE. The enriched protein can for example be identified by:

1) N-terminal protein sequencing: proteins are separated on SDS-PAGE and blotted on PVDF membrane, the protein with the right molecular weight is cut from the blot and submitted to N-terminal sequence analysis. The obtained sequence can then be identified through a BLAST analysis (such as performed on the *Shewanella oneidensis* genome).

2) Peptide mapping: a tryptic digest is performed on the spot of the chip or on a protein eluted from a SDS-PA gel fragment, the peptides are analysed by mass-spectrometry and compared to a database (MASCOT).

The principle of this method is presented in Figure 5.6.

**II.B. Pull-down assays**

The pull-down assay is an *in vitro* method used to determine physical interactions between two or more proteins. Pull-down assays are useful in both confirming the existence of a protein-protein interaction predicted by other techniques and as an initial screening assay for identifying previously unknown protein:protein interactions. The minimal requirements for a
pull-down assay are the availability of a purified (tagged) protein (the bait) which will be used to capture and ‘pull-down’ a protein-binding partner (the prey).

In a pull-down assay, a target bait protein can either be captured on an immobilised affinity ligand specific for the tag or on a pre-activated medium, such as CNBr-Sepharose, that binds proteins (just as the PS20 Chips) by the means of their free primary amines, thereby generating a ‘secondary affinity support’ for purifying other proteins that interact with the bait protein. The secondary affinity support of immobilised bait can be incubated with a variety of other protein sources that contain putative prey proteins.

We used the pull-down assays to confirm and complete the results obtained with the ProteinChip Protocol. Two different pull-down methods were used: GST-pull down and pull-down by the use of CNBr-activated Sepharose.

**XIII. Induction studies of Shewanella oneidensis**

This section describes the induction studies performed to explore when the SYE proteins are expressed in *S. oneidensis*. First, in order to detect the proteins, specific antibodies against recombinant SYE1 were generated in rabbit (III.A). These antibodies were cross-checked for reactivity with the four SYE proteins (III.A) and it was determined whether SYE proteins expressed constitutively in *S. oneidensis* (III.B). Then expression was induced by growing the cells under different culture conditions. These conditions will be discussed first (III.C) before we draw a general conclusion (III.D).

**III.A. The generation of SYE specific antibodies**

Antibodies were generated in rabbit (Eurogentec) against purified recombinant denatured SYE1. The specificity of the antibodies was checked in two independent ways.

(1) The antibodies were checked for cross-reactivity with all four SYEs by Western analysis. The SYE proteins were denatured.

Pure samples of all four SYE proteins (including insoluble GST-SYE2 fractions) were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes. The membranes were scanned with 25 µl of the rabbit anti-SYE1 serum. Strong, clear bands where interaction had occurred were observed (Fig.5.7) for all four SYEs, indicating that the antibodies could be used for the detection of all four proteins.
(2) The antibodies were checked for specific binding of SYE1 and SYE4 on ProteinChip arrays. The proteins were present in their native form.

Polyclonal rabbit antibodies were purified from the rabbit serum by means of a ProteinG antibody specific affinity matrix. The purified antibody solution was bound on a PS20 chip, which is a general purpose chip that is coated with a surface of free epoxide groups that can form covalent interactions with the free primary amino groups of a protein or other biomolecule. Then the antibody coated chip was incubated with pure samples of SYE1 and SYE4. In addition, the chip was incubated with cell lysate spiked with SYE1 and SYE4 protein. The following protocol was used:

**Standard protocol (PS20-chip):**

- The spots are humified with 3 µl of PBS buffer and the chip is incubated for 5 minutes in a humid chamber at room temperature
- Excess buffer is removed and 1 µl (10mg/ml in PBS) of 0.2-0.5 mg/ml of purified polyclonal rabbit antibody solution is added
- the chip is incubated for one hour in a humid chamber at room temperature
- Excess antibody solution is removed and 3 µl of 500 mM Tris, pH 8.0 is applied to block the remaining active places on the spot
- the chip is incubated for half an hour in a humid chamber at room temperature
- The chip is washed twice with 10 ml PBS + 0,5% Triton-X100 during 15 min at room temperature
- The chip is washed three times with 10 ml PBS, during 5 min at room temperature
- Excess buffer is removed
- The protein samples (either pure SYE1, pure SYE4 or cell lysate spiked with SYE1 or SYE4) are applied to the spots (10 µg sample/spot, in a volume of 5 µl) and the chip is incubated for four hours in the humid chamber
- Every spot is washed three times with PBS + 0,1% Triton X-100 + 0,1 M NaCl
- The spots are cleaned with PBS and water
- The chip is air dried
- The EAM molecule SPA is applied by adding 2 times 0,5 µl
- The chip is analysed by the ProteinChip Reader

**(SPA:** 5 mg SPA (sinapinic acid), 75 µl ACN and 75 µl 1% TFA)
The meaning of this experiment was four-fold: (1) to determine the molecular mass of SYE1 and SYE4 when measured with the SELDI, (2) to confirm the specific binding of SYE1 and SYE4 on the antibodies, (3) to check whether the antibodies are able to isolate the wanted proteins from a protein solution, and (4) to analyse whether antibodies generated against denatured protein are able to interact with native protein.

The results of the experiments where pure SYE1 and SYE4 were bound to the antibodies are shown in Figure 5.8.

As the antibodies (AB) are covalently linked to the chip, only the SYE proteins are released from the chip and measured by the ProteinChip Reader. We can conclude from the figure that the antibodies, although generated against SYE1, also specifically bind SYE4. In addition the antibodies are able to isolate SYE1 and SYE4 out of a mixed protein solution and recognise native protein.

### III.B. Constitutive or inducible expression?

#### III.B.1. Introduction

It is generally believed that when a protein is expressed constitutively, that protein is probably essential for normal cellular function, and little can be learned about its physiological function without the means to construct and examine knock-out mutants. So, the first experiment when determining protein function by induction studies should be to determine if the protein is expressed in normal growth conditions.

#### III.B.2. The experiment

A first experiment was performed in order to evaluate the possibility of constitutive expression. The YqiM protein of *Bacillus subtilis* (Fitzpatrick *et al.*, 2003) was shown to be induced when cells enter the stationary phase. Accordingly, we analysed if the *S. oneidensis’* SYE proteins were induced under similar conditions.
Shewanella oneidensis cultures were grown both aerobically and anaerobically in LB and minimal medium. The compositions of the media are given in Table 5.1. 20 mM of fumaric acid was added to the media used for anaerobic growth.

<table>
<thead>
<tr>
<th>Media and solutions</th>
<th>Composition</th>
</tr>
</thead>
</table>
| **Luria Bertani medium (LB, 1 litre)**  | 10 g trypton  
|                                         | 5 g yeast extract  
|                                         | 5 g NaCl |
| **Minimal Medium (Shewanella) (1 litre)** | 0.95 g NH₄Cl  
|                                         | 100 ml salt solution (a)  
|                                         | 875 ml H₂O (distilled)  
|                                         | 0.1 ml metal supplement (c)  
|                                         | 0.2 ml 0.115 M Na selenate  
|                                         | pH 7.4 – heat sterilise  
|                                         | add 10 ml 0.2 M Na bicarbonate  
|                                         | 15 ml phosphate solution (b)  
|                                         | 20 mM lactate  
|                                         | 0.2 g/l casaminoacids |
| **(a) Salt solution (1 litre)**         | 2 g MgSO₄·7H₂O  
|                                         | 0.57 g CaCl₂·2H₂O  
|                                         | 0.2 g EDTA disodium  
|                                         | 0.012 g FeSO₄·7H₂O  
|                                         | 10 ml trace elements solution (d) |
| **(b) Phosphate solution (1 litre)**    | 30 g KH₂PO₄  
|                                         | 66.1 g K₂HPO₄  
|                                         | pH 7.4 |
| **(c) Metal supplement (100 ml)**       | 1.41 g CoSO₄·7H₂O  
|                                         | 1.98 g Ni(NH₄)₂(SO₄)₆·6H₂O  
|                                         | 0.58 g NaCl |
| **(d) Trace elements (1 litre)**        | 2.8 g H₃BO₃  
|                                         | 0.24 g ZnSO₄·7H₂O  
|                                         | 0.75 g Na₂MoO₄·2H₂O  
|                                         | 0.042 g CuSO₄·5H₂O  
|                                         | 0.17 g MnSO₄·H₂O |

Table 5.1. Composition of LB medium and Shewanella minimal medium.

The aerobic cultures were inoculated by diluting a full grown culture in the appropriate medium and grown at 28°C under constant shaking. The media for anaerobic growth were made anaerobic by incubation in an anaerobic glove box. The anaerobic cultures were inoculated by diluting a full grown culture in the appropriate anaerobic medium and were grown at room temperature in the anaerobic glove box and without shaking. Samples were taken at regular time intervals, until the stationary growth phase was reached. The samples taken were loaded onto SDS-PAGE gels alongside with samples of pure SYE1 or SYE4 to serve as an internal standard. The gels were electroblotted onto nitrocellulose membranes and analysed by Western analysis using SYE-specific antibodies. First the membranes were scanned with SYE-specific antibodies generated in rabbit (the primary antibody), then the membranes were washed and incubated with a secondary goat-antirabbit antibody to which a phophatase was attached. After incubation with the secondary antibody the membranes were washed and sprinkled with luminol, a chemoluminescent substrate of
Debbie van den Hemel

phosphatase. A light sensitive film was laid over the membranes and incubated for 15 minutes in the dark. The film was developed with photographic solutions and the pattern interpreted.

III.B.3. Results

No SYE protein could be detected, although a 60-70 kDa band appeared in the late exponential-early stationary phase. Partial purification of the band, followed by N-terminal sequencing, confirmed the identity of this band to be flavocytochrome (Section III.C). This enzyme consists of 2 domains: a type I α,β-barrel oxidoreductase- the probable cause of the aspecific binding of the antibodies- and a cytochrome (Scrutton, 1993). Since it has a much higher molecular weight than the SYEs, no danger of misinterpretation exists.

III.C. Induction of SYE protein under stress conditions

As already noted above, during our experiments, an article concerning the characterisation of YqiM of Bacillus subtilis was published (Fitzpatrick et al., 2003). In this article different conditions were evaluated that induced YqiM expression. As a first experiment the authors could induce the expression by the addition of the substrates TNT and glycerol trinitrate. Since both substrates are able to exert oxidative stress on the cells by the production of superoxide/hydrogen peroxide and NO radicals respectively; it was evaluated if YqiM induction could be induced directly by addition of peroxides (hydrogen peroxide and t-butyl hydroperoxide) and paraquat (generates superoxide). In addition they assayed if heat shock induced the expression of YqiM. Fitzpatrick et al. were able to obtain induction of YqiM for all conditions mentioned, indicating a function for YqiM in oxidative stress responses.

Our first experiment (Section III.B) indicated that no SYE protein was expressed under ‘normal’ growth conditions. This was in contrast to the results reported for Bacillus subtilis. Therefore we assayed if SYE protein could be expressed under the other conditions mentioned for YqiM: oxidative stress and heat shock. In addition, we performed some additional tests: S. oneidensis cultures were grown in the presence of metals and induced by the substrates NEM, acrolein and cyclohexenone.

In summary, S. oneidensis was grown in the presence of:

1. metals
2. oxidative stress
3. heat shock
4. substrates

III.C.1. Protocols

Metals

Full grown cultures of Shewanella oneidensis were diluted 100 fold in LB medium containing 1g/l (11.5 mM) manganese oxide, 5-10 mM iron chloride, 5-10 mM iron citrate, 0.1, 0.5 or 1.0 mM chromium sulphate. The cultures were grown aerobically at 28°C under constant shaking. Samples were taken at regular time intervals (every hour).
Oxidative stress and substrates

Since we here wanted to reproduce the results obtained for *B. subtilis*, we compared the growth conditions. The *Bacillus* cells were induced in early exponential growth phase (0.25 OD) by the addition of 0.2 mM of the respective substance. *Bacillus* has however a shorter doubling time compared to *S. oneidensis* and growth curves were compared to determine the sampling intervals (Fig.5.9.). The hour scale of the two curves is different to indicate the difference in growth rate. The growth rate between the two organisms differed by a factor 2.

![Growth curve of Bacillus subtilis](image1)

![Growth curve of S. oneidensis](image2)

*Fig.5.9. Comparison of growth curves of S. oneidensis and B. subtilis. The arrow indicates where sampling had stopped for the experiments of B. subtilis. Note the difference in time scale between the two organisms. The optical density was measured at 600 nm.*

We grew *S. oneidensis* cultures accordingly to early exponential phase at 28°C, added different concentrations of the substances and took samples after 15, 30, 60, 120, 180 minutes of induction.
Heat shock

*S. oneidensis* cultures were grown under constant shaking at 28°C until an optical density @ 600 of approximately 0.4 was reached. At that point the cultures were transferred from 28°C to 42°C. Samples were taken at regular time intervals.

**Analysis of the samples**

Samples were taken at regular time intervals (1) to determine the optical density @ 600 nm and (2) to analyse the protein content of the samples. Growth curves were drawn to determine the effect of the different conditions on the viability of the cells, and the expression pattern of SYE protein was assayed by Western analysis as described in III.B.2.

**III.C.2. Growth curves**

For every substance different concentrations were tested and growth curves (Fig. 5.10) were drawn to assay the toxicity of the substances on the *S. oneidensis* cultures and to determine the concentration to be used in the induction experiments (Table 5.2). Due to the insolubility or colouration of some of the metals, however, which changed in time due to reduction by the bacteria; it was not possible to derive a growth curve. Some conclusion could however be made:

1. Manganese and the lower (0.1 and 0.5 mM) concentrations of chromium sulphate were invariant to the growth rate of the cells
2. 1 mM of chromium sulphate reduced the growth rate
3. 5 mM of iron chloride or iron citrate improved the growth rate
Fig. 5.10. Growth curves of S. oneidensis determined under different growth conditions:

**Cumene hydroperoxide**: dark blue: 0 mM, pink: 0.05 mM, yellow: 0.0625 mM and light blue: 0.075 mM.

**Paraquat**: dark blue: 0 mM, pink: 1 mM, yellow: 2.5 mM and light blue: 5 mM.

**t-Butyl hydroperoxide**: dark blue: 0 mM, pink: 0.44 mM and yellow: 0.55 mM.

**Hydrogen peroxide**: dark blue: 0 mM, pink: 0.15 mM and yellow: 0.2 mM.

**Picric acid**: blanc blue: 0 µM, pink: 100 µM, yellow: 200 µM and light blue: 400 µM.

**Glycerol trinitrate**: dark blue: 0 µM, pink: 200 µM, yellow: 400 µM and light blue: 800 µM.

**Heat shock**: dark blue: 28°C and pink: 42°C.

**N-ethylmaleimide**: dark blue: 0 µM, pink: 10 µM, yellow: 25 µM, light blue: 100 µM, brown: 50 µM.

**Acrolein**: dark blue: 0 µM, pink: 34 µM, yellow: 85 µM and light blue: 170 µM.

**Cyclohexenone**: dark blue: 0 mM, pink: 10 mM, yellow: 25 mM, light blue: 100 mM.

The final concentration of the substances (or the growth conditions) used for the induction studies are presented in Table 5.2. The concentrations chosen were the minimum concentration required to unequivocally induce SYE production, as assayed by Western analysis.
<table>
<thead>
<tr>
<th>Substance/Condition</th>
<th>Concentration used in B. subtilis</th>
<th>Concentrations used in S. oneidensis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrogen peroxide</td>
<td>0.2 mM</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>t-Butyl hydroperoxide</td>
<td>0.2 mM</td>
<td>0.55 mM</td>
</tr>
<tr>
<td>Cumene hydroperoxide</td>
<td>-</td>
<td>0.05 mM</td>
</tr>
<tr>
<td>Paraquat</td>
<td>0.2 mM</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>Picric acid</td>
<td>-</td>
<td>0.4 mM</td>
</tr>
<tr>
<td>Glycerol trinitrate</td>
<td>0.2 mM</td>
<td>0.8 mM</td>
</tr>
<tr>
<td>NEM</td>
<td>-</td>
<td>25 µM</td>
</tr>
<tr>
<td>Acrolein</td>
<td>-</td>
<td>85 µM</td>
</tr>
<tr>
<td>Cyclohexenone</td>
<td>-</td>
<td>10 mM</td>
</tr>
<tr>
<td>Iron chloride</td>
<td>-</td>
<td>5 mM</td>
</tr>
<tr>
<td></td>
<td>-</td>
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</tr>
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<td>-</td>
<td>10 mM</td>
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<td>Manganese oxide</td>
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</tr>
<tr>
<td></td>
<td>-</td>
<td>1.0 mM</td>
</tr>
<tr>
<td>Heat shock</td>
<td>48°C</td>
<td>42°C</td>
</tr>
</tbody>
</table>

Table 5.2. The concentrations of the additives added to the S. oneidensis cultures (and the growth temperature used for heat shock) used for final induction studies. Some concentration used for B. subtilis (Fitzpatrick et al., 2003) are mentioned as a reference.
III.C.3. Results of the Western analysis

The final results of the Western analysis are given in Figure 5.11.

In most cases a signal was observed at about 37 kDa, which corresponds approximately to the calculated mass of the SYE proteins: SYE4 (39 kDa), SYE1 (39.6 kDa), SYE2 (40 kDa) and SYE3 (41 kDa).

In addition, in some cases a band was observed at 60-70 kDa. The identity of this protein band was determined by N-terminal sequencing (after partial purification of the corresponding protein as described in (3) below) to be a flavocytochrome (III.B.3).

The identity of the band(s) at about 37 kDa was determined to be SYE4 based on the following observations:

(1) the band co-migrated with recombinant SYE4 (Fig.5.11, iron chloride induction patterns)
(2) SYE4 has the lowest molecular weight of the four SYE proteins: approximately 39 kDa for SYE1, SYE2 and SYE3 compared to approximately 37 kDa for SYE4
(3) the two bands present in, for example, the acrolein induction pattern, were partially purified and their identity determined as follows:

The proteins corresponding to the two bands were partially purified using a three step protocol. Western analysis was performed between each step to determine in which fractions the proteins were present.

(1) Ion exchange: Q-Sepharose FF run in Tris buffer, pH 8.0, and eluted with a stepwise gradient of NaCl.
(2) Ion exchange: Source30Q run in Tris buffer, pH 6.5, and eluted with a linear gradient of NaCl.
(3) Gel filtration: Superdex-HL16/60 prep200 run in a PBS buffer.

When sufficient purity was achieved, the identity of the proteins was determined in two independent ways. First, a polyacrylamide (SDS-PA) gel was electroblotted to PVDF membrane and the bands shown to interact with the polyclonal anti-SYE antibodies were cut out of the membrane and used for N-terminal sequencing. The identity of the lowest band at 40 kDa was confirmed as SYE4 and the intermediate band at 40 kDa was too impure to determine the sequence. Therefore, a second method of identification was used: both the high and low band at 40 kDa were excised directly from a SDS-PA gel and analysed by peptide mapping. Both bands were confirmed to be SYE4. Since a similar double band pattern was sometimes observed on coomassie-stained SDS-PA gels during purification of recombinant SYE, we assume that the upper band represents FMN-bound protein and the lower band FMN-free apoprotein. Although the FMN group is bound noncovalently to proteins of the OYE family, experimental evidence suggests it is very tightly bound.

This result is in agreement with a recent DNA microarray study by Gao et al. (2004) in which the genomic response of S. oneidensis to a temperature upshift from 30° to 42°C was evaluated over a period of 25 minutes: only the sye4 gene was upregulated, whereas the other sye genes were not.
Fig. 5.11. Induction profiles for the SYE proteins as determined by Western analysis. *: indicates the height of recombinant SYE4 on the gel. CHP: cumene hydroperoxide, t-BOOH: t-butyl hydroperoxide and NEM: N-ethylmaleimide.
We can describe the individual induction patterns as follows:

1. No induction of SYE protein was observed for the cultures grown with chromium sulphate and glycerol trinitrate, as a consequence, these are not presented in Figure 5.11.
2. The induction pattern was similar using 2.5 mM paraquat (induction of superoxide production), 0.2 mM hydrogen peroxide (H₂O₂), 0.05 mM cumene hydroperoxide (CHP) and 0.55 mM tert-butylhydroperoxide (t-BOOH): although the expression level was quite low, induction occurred already within the 15 minute (paraquat, H₂O₂ and CHP) or 30 minute (t-BOOH) time interval.
3. Induction with 0.4 mM picric acid, a TNT analog that enhances superoxide and hydrogen peroxide production (Kong et al., 1989), occurred only after 2 hours. At this point, the colour of the culture immediately shifted from yellow to orange. As seen in Fig.5.11, the induction signal had a double band pattern.
4. SYE protein is induced rapidly (15 minutes) when S. oneidensis cultures are transferred from 28°C to 42°C, the induction bands are about two times stronger than those reported for the peroxides (Fig.5.11). A strong induction level is observed even though cells stopped growing (Fig.5.10).
5. Since the SYE proteins are able to use acrolein, NEM and 2-cyclohexen-1-one as substrate, expression was also assayed in the presence of these compounds (85 μM, 25 μM and 10 mM, respectively). All three substrates resulted in a very rapid and strong induction of SYE protein (Fig. 5.11). A similar double band pattern was observed as seen with picric acid. This direct substrate induction yielded expression levels that were at least 2- to 3-fold higher than in the oxidative stress generating conditions mentioned above, even with NEM concentrations as low as 25 μM.
6. The cultures grown with manganese (11.5 mM), iron chloride or iron citrate (5 mM) showed some induction of SYE protein but the results could not be interpreted unequivocally. It was therefore assayed to see whether the induction rate would improve if the cells were grown in the presence of a higher concentration of metals. Induction rates of cultures grown in the presence of 5 mM, 7.5 mM or 10 mM iron chloride or iron citrate were compared. It was shown that the induction rate was correlated to the concentration of the metal. Adding higher concentrations of iron improved the expression level (Fig.5.11).

In conclusion: No basal expression of any of the SYE proteins was observed under normal growth conditions, nor in cultures entering the stationary phase. As it was shown that YqjM from B. subtilis is rapidly induced in conditions of oxidative stress (Fitzpatrick et al., 2003), we analyzed whether the SYE proteins are expressed under similar conditions, as was the case. A labelled band near the 37 kDa marker protein appeared rapidly in the presence of compounds generating oxidative stress conditions (Fig. 5.11). In addition, we were able to prove that SYE proteins can be expressed by when the cells are exposed to heat shock or when iron or manganese is supplied in the growth medium of S. oneidensis cells.

In contrast to B. subtilis YqjM, SYE expression was not induced with 1.5 mM GTN, a compound also shown to increase the production of reactive oxygen species (ROS) (Sydow et al., 2004). As generally higher concentrations of peroxides were needed for the upregulation of SYE4 compared to YqjM, it is possible that higher concentrations of NG are also required. Only SYE4 was detected in vivo, i.e. under conditions of oxidative stress. This may indicate an involvement in oxidative stress response for SYE4, but not for the other three SYE proteins.
III.D. Discussion

The YqjM protein from B. subtilis was found to be induced in conditions of oxidative stress. The expression of YqjM was also upregulated in the presence of nitro compounds (2,4,6-trinitrotoluene and GTN), although the induction may be indirect as these compounds result in the generation of reactive oxygen (ROS) species. We showed that SYE4 can be induced under similar conditions, yet the expression of SYE4 is substantially increased in the presence of the substrates acrolein, NEM and 2-cyclohexen-1-one. Despite the fact that SYE1 and SYE3 show catalytic activity with these compounds, they are not induced under the conditions tested. This implies that the SYE proteins, indeed, have a differential function, which is supported by the finding that the sye genes can be differentially expressed. While sye4 is preceded by a tetR repressor gene, sye1, sye2 and sye3 are preceded by a lysR regulator sequence. This has important implications in the search for the true physiological role of OYE family members, as our results suggest that there may not be a specific conserved functional role for these enzymes. In fact, the function may even be different within the same organism. This hypothesis is supported by the fact that the two OYE isozymes in S. cerevisiae were found to have quite different functions. OYE2 protects the actin cytoskeleton against oxidative damage by reducing a cysteine bridge formed between filaments (Haarer and Amberg, 2004) and OYE3 is induced under conditions of oxidative stress (Lee et al., 1999) and is shown to have a role in Bax-mediated programmed cell death (Reekmans et al., 2005).

The fact that the SYE proteins can catalyze the reduction of acrolein to propionaldehyde and that SYE4 is induced very rapidly and strongly in its presence agrees with the hypothesis that OYE is involved in the detoxification of α,β-unsaturated aldehydes formed during lipid peroxidation cascades (Kohli and Massey, 1998). Acrolein represents a typical example of such physiological relevant substrates as it is an endogenous lipid-derived product that is likely to be ubiquitously formed in eukaryotic cells. This would also explain why the OYE enzymes are upregulated in oxidative stress conditions, taking into account that lipid peroxidation is one of the important events in free radical-induced cellular damage. Lipid peroxidation is a continual process in living aerobic cells, yet the process can be accelerated by physical or chemical means, such as the exposure to t-BOOH (Yoon et al., 2002). Additionally, it has also been shown that acrolein is generated in the early stages of the exposure to ROS and to reactive nitrogen species (RNS) (Takabe et al., 2001). The process of lipid peroxidation is mainly restricted to eukaryotes, as their cell membranes, in contrast to prokaryotes, contain polyunsaturated fatty acids which are the targets for free-radical attack. Yet, some prokaryotes have been shown to increase fatty acid desaturation in cellular response to environmental stress (Guerzoni et al., 2001). Also, bacterial strains from low-temperature and high-pressure marine environments appear to synthesize polyunsaturated fatty acids, such as linoleic acid (Allen and Bartlett, 2002; Yano et al., 1994; Gentile et al., 2003). This is most interesting, as one of these strains was identified as Photobacterium profundum which contains two OYE homologues with a sequence identity of 71% and 78% to SYE2 and SYE4, respectively. Also, Vibrio T3615 and Shewanella sp. GA-22 were among the other strains identified. OYE homologues have been found in close relatives of both strains. Lipid peroxidation, initiated in E. coli by the addition of linoleic acid and oxidising agents, has been shown to result in the induction of the OYE homolog NEM reductase (Miura et al., 1997). This means that, even though the most important physiological function of bacterial OYE members is not likely to be the detoxification of lipid peroxidation products, OYE expression can be triggered by a common factor in bacteria and yeasts.
The finding that yeast OYE can protect the actin cytoskeleton from oxidative stress by **reducing disulfide bonds** in oxidised actin (Haarer and Amberg, 2004) has important implications for explaining the results of our expression analysis, thereby providing insight into the true role of OYE members in bacteria. In oxidative stress conditions, excess ROS can damage a cell’s lipids, proteins or DNA, resulting in a loss of their normal function. For instance, ROS will react with the sulfhydryl groups of cysteines to initiate disulfide bond formation. The exposure to acrolein, NEM or 2-cyclohexen-1-one is not linked to the production of ROS, but they are highly electrophilic compounds that severely damage biological nucleophiles, such as the sulfhydryl groups of proteins. They can cause oxidative stress in two different ways, which may explain why the induction of SYE4 was found to be much stronger in their presence: either directly by the induction of disulfide bonds within and between proteins, or directly by depletion of reduced glutathione (GSH). Because these compounds are readily scavenged by GSH (Uchida, 1999), the formation of such GSH-adducts will result in a reduction of the intracellular level of GSH. This will make the cell more susceptible to oxidative damage since GSH is a key molecule in antioxidant defence and in maintaining the cytosol in a reduced state. Given that (1) yeast OYE can repair disulfide bonds in oxidized actin and (2) all conditions in which SYE4 and YqjM were upregulated have the same outcome, i.e. protein damage by disulfide bond formation, it is possible that the bacterial OYE homologues have a similar function in protecting proteins from oxidative damage. Oxidative damage to cytosolic proteins, in normal oxidative metabolism, is repaired by redox-active oxidoreductases such as glutaredoxin and thioredoxin. This is consistent with our finding that SYE4 is not expressed in normal aerobic growth conditions. However, in conditions of extreme oxidative stress, i.e. when the concentration of ROS has increased to a level that exceeds the defence capacity of the cell, bacterial OYEs might be recruited as an **additional cellular defence mechanism** against oxidative injury. This can be exemplified by SYE4, which is rapidly and strongly induced under such conditions. SYE4 might then have a similar function as glutaredoxin for the repair of unwanted disulfide bridges occurring in either a specific protein, as is the case for yeast OYE2, or in a broader range of proteins. The latter possibility is likely because the activity of glutaredoxin, which is GSH-dependent, may be impaired under conditions of severe oxidative stress. Another possibility is that SYE4 can act as a glutathione reductase, which is the enzyme that catalyzes the reduction of GSSG to GSH, the electron donor for glutaredoxin. This reduction requires NADPH as cofactor, as is the case for SYE4 and most other OYE family members. (Initial results however indicated that SYE4 is not able to reduce GSSG.) A further argument for such a function is that several OYE encoding genes in *Vibrio* species are located in the same operon as the genes encoding glutaredoxin 2 or lactoylglutathione lyase (Chapter six). The latter enzyme catalyzes the GSH-dependent detoxification of glyoxal and methylglyoxal, which are produced during lipid peroxidation and glycolysis, respectively.

In the light of the above discussion, one might look at the induction of SYE protein by **metals** through means of the **Fenton reaction**. In this reaction, hydroxyl radicals are generated by the interaction of Fe$^{2+}$ and hydrogen peroxide, thereby imposing oxidative stress on the cells. The expression of SYE4 is apparently controlled by the oxidative stress regulon. Because GSH is a key molecule in oxidative stress metabolism, depletion of reduced GSH will result in an imbalance of the GSH/GSSG ratio. Exposure to a more oxidized GSH pool will cause a situation of ‘disulfide stress’ in which unwanted disulfide bonds are generated. As a consequence, the redox state of the cytosol becomes altered and the resulting genetic response for restoring the redox homeostasis of the cytosol may then include the upregulation of OYE family members.
In conclusion, at least one possible function can be assigned to bacterial OYE family members, i.e. as a second line of defence in conditions of extreme oxidative stress. Moreover, our study strongly supports the statement that multiple physiological roles may exist for the different OYE homologues within a single organism.

IV. Partner search

Elucidating gene function involves the determination of the function of each gene’s encoded protein product. In the cell, proteins participate in extensive networks of protein-protein interactions. These interactions take the form of dynamic ‘protein machines’, which assemble and disassemble in concert with an ever-changing influx of intra-, inter- and extra-cellular cues. A preliminary step in understanding protein structure and function is to determine which proteins interact with each other, thereby identifying the relevant biological pathways. The ‘classical’ method of identifying interaction partners is a yeast-two-hybrid screen. A similar screen does not exist for prokaryotes, thus some other means of identifying a partner is needed.

A common method to gain insight in a proteins’ function is to examine the genes in the direct vicinity of the gene of interest. Genes are often organised in functional transcription ‘blocks’ called operons. When comparing the genomic neighbours of the different SYE1 homologues genes, we found no regular partners. Some lie in the vicinity of glyoxalases, glutaredoxines, ABC transporters, other in the middle of a sugar regulon, next to a dehydrogenase,… A better approach is to find a possible partner for the enzyme, which can provide a clue for function. We made use of two different techniques: ProteinChip arrays and pull-down assays.

As protein of study we used both the SYE1 and SYE4 enzymes. It has been noted in Chapter four that the special binding mode of phenolic ligands in SYE1 might have significance in β- and γ-proteobacteria. It is, however, unclear what the function of these enzymes would be. The reason why SYE4 was chosen is because of its special properties, clearly distinct from SYE1 and SYE3, and because SYE4 is the only SYE protein shown to be induced, in vivo, when S. oneidensis cultures are grown in the presence of e.g. acrolein.

IV.A. SELDI protocols

When the ProteinChip Reader is used for the first time or has been used for a different type of experiment, the mass spectrometer needs to be calibrated with standards of the appropriate molecular weight. A calibration solution comprised of 5 different proteins ranging in molecular weight from 12 kDa to 116 kDa is supplied by the manufacturer for this purpose.

The Chips used for the experiments in this work were ‘PS20 ProteinChip Arrays’. These are coated with a surface of free epoxide groups that can form covalent interactions with the free primary amino groups of a protein.
Standard protocol (PS20-chip):

- The spots are humified with 3 µl of PBS buffer and the chip is incubated for 5 minutes in a humid chamber at room temperature
- Excess buffer is removed and 1 µl (in PBS) of pure bait protein is applied (10 mg/ml of pure recombinant SYE1/SYE4)
- The chip is incubated for one hour in a humid chamber at room temperature
- Excess protein solution is removed and 3 µl of 500 mM Tris, pH 8.0, is applied to block the remaining active places on the spot
- The chip is incubated for half an hour in a humid chamber at room temperature
- The chip is washed twice with 10 ml PBS + 0.5 % Triton-X 100 during 15 min at room temperature
- The chip is washed three times with 10 ml PBS, during 5 min at room temperature
- Excess buffer is removed
- The protein samples (S. oneidensis cell lysates) are applied to the spots (10 µg sample/spot, in a volume of 5 µl) and the chip is incubated for four hours in the humid chamber
- Every spot is washed three times with PBS + 0.1% Triton X-100 + 0.1 M NaCl
- The spots are cleaned with PBS and water
- The chip is air dried
- The EAM molecule SPA is applied by adding 2 times 0.5 µl
- The chip is analysed by the ProteinChip Reader

(SPA: 5 mg SPA (sinapinic acid), 75 µl ACN and 75 µl 1% TFA)

On chip tryptic digest protocol:

- The chip is prepared as described above, with the exception of the composition of the final wash buffers (Triton X-100 and NaCl are excluded) and analysis steps: the spots are not allowed to air dry and no EAM is added
- 25 ng trypsin (in 5 µl 50 mM ammonium bicarbonate) is applied per spot
- The chip is incubated for 4 hours at 37°C
- The digestion samples are pooled and dried in a Speedvac centrifuge before analysis on a MALDI mass spectrometer

IV.B. A partner for SYE1 and SYE4?

Although it is recognised that many proteins function in co-operation with other proteins; it is possible that a protein functions on its own. When a protein does interact with other proteins, it is possible that very short, transient interactions exist between the proteins, which can not easily be recognised. So as a first test in partner search, pure recombinant SYE1 and SYE4 was bound on a PS20 chip and incubated with S. oneidensis lysates of cultures grown under ‘normal’ conditions and cultures induced with 85 µM of acrolein. The experiment was performed in duplicate and some representative results are presented in Figure 5.12. In both cases, a peak of 43 kDa can be detected for the SYE1 protein, but not for SYE4.
Debbie van den Hemel

Fig. 5.12. SELDI spectrum of SYE4 incubated with lysate of S. oneidensis induced with acrolein (above) and of SYE1 incubated with lysate of S. oneidensis grown under normal growth conditions (below). Note the peak of 43 kDa present for SYE1. The other peaks represent background that is always present in the spectrum.

IV.C. Identification of the SYE1 partner

IV.C.1. A first trial

It has been noted in Section II of this chapter that a protein bound on a chip can be identified with an ‘on chip’ tryptic digest. Before proceeding with this ‘on chip’ digest, we needed to enrich the protein by partial purification. In addition, we choose to grow the cells in the presence of acrolein.

The cell lysate was submitted to a first purification step. The sample was dialysed against 20 mM Tris, pH 8.0, and loaded on a Q-Sepharose 4FF matrix. A stepwise gradient of 100 mM NaCl/step was applied and fractions corresponding to every step were collected. The fractions were concentrated by ammonium sulphate precipitation and spotted on a PS20 chip preactivated with SYE1 (Fig. 5.13).

Fig. 5.13. Mass distribution profile detected by the ProteinChip reader. The position of the 43kDa protein is indicated by an arrow.
An enrichment of the 43 kDa protein could be observed in the 200 mM NaCl fraction. The signal to noise ratio of the starting 43 kDa peak in the cell lysate was 35 (Fig.5.12) whereas this peak had a signal to noise ratio of 133, indicating a 3.8 times enrichment of the protein. The 200 mM fraction was subsequently dialysed against 50 mM Tris, pH 8.0, 100 mM NaCl, and loaded onto a Superdex-HL16/60 prep75 column. Several fractions were spotted on a novel chip pre-activated with SYE1 and analysed. Since the goal was to perform a tryptic digest and analyse the peptide fragments on a MALDI spectrometer, Triton-X 100 and NaCl were excluded from the washing steps. The disadvantage of removing these additives from the washing steps was that a higher amount of background was observed (Fig.5.14).

![Mass distribution profile detected by the ProteinChip reader. Indicated by the arrow is the position of the 43kDa protein on the mass distribution profile.](image)

The protein was predominantly present in the fraction corresponding to a molecular weight > 67 kDa, indicating an oligomeric behaviour of the protein on a gel filtration column. Although the stringency of the washing conditions was lower, resulting in high background, the signal to noise ratio of the protein was still 235, corresponding to a 6.7 times enrichment compared to the crude lysate. A visual representation of the enrichment of the partner from the step of crude cell lysate to that of gel filtration is presented in Figure 5.15.

![A visual representation of the enrichment of the partner. The 43 kDa protein is hardly visible in the crude cell lysate, moderately in the ion exchange sample and visible as a strong band in the gel filtration sample.](image)
A tryptic digest was performed on the chip and the peptide profile determined on a MALDI spectrometer. However, due to the high amount of background proteins, the profile was too complex and it was impossible to assign the peptides to a 43 kDa protein. It was decided not to optimise this tryptic digest, but to purify the protein to a greater homogeneity and then determine the identity of the protein by different means.

IV.C.2. Optimisation of the purification protocol

The first step on the Q-Sepharose FF was not modified.

As a second step, the proteins eluting at 200 mM NaCl (the 200 mM fraction) were dialysed against 0.2 x PBS buffer and loaded onto a Source30Q column (ion exchange). This time a linear salt gradient was applied and 1 ml fractions were collected.

![Chromatogram of the 200 mM fraction run on a Source 30Q.](source)

Fig. 5.16. Chromatogram of the 200 mM fraction run on a Source 30Q. The blue line is the absorbance at 280 nm, red at 220 nm, pink at 460 nm and the light blue line is the conductivity (%). The collected samples are numbered 1-20.

The samples were analysed on a ProteinChip preactivated with SYE1; the results of fraction 3, 5 and 6 are shown in Fig. 5.17.
The protein was predominantly present in fraction 5. This fraction corresponds to a conductivity of 11-14.5 %, or a elution concentration of NaCl of approximately 110-145 mM.

Since we knew the molecular weight of the protein and a strong enrichment was observed, we decided to submit the sample to a gel filtration step. The sample was run in PBS buffer on a Superdex-HL16/60 prep75 column. Fractions of different peaks (Fig.5.18) were concentrated and loaded on a ProteinChip preactivated with SYE1 for analysis (Fig.5.19).

Based on the molecular weight of the protein and the calibration of the gel filtration column we expected the protein to be present in peak 3.
IV.C.3. Conclusion

We were able to identify the molecular mass of a potential partner of SYE1 and enrich this protein in two subsequent purification steps. Identification of the protein by a tryptic digest failed due to impurity of the sample. Therefore, the decision was made to optimise the purification protocol of the possible partner.

Some assumptions about the identity of the protein can already be made from these results: its molecular mass is about 43 kDa, it is no flavoprotein nor cytochrome (based on the specific absorbencies of the protein, no absorbance at 460 nor 400 respectively) and its pI < 7.5.

A further optimisation of the purification protocol, possibly combined with an improvement of the protocol for the tryptic digest, is necessary to identify a partner of SYE1.

IV.D. Pull-down assay for SYE1

SYE1 (the bait) was specifically bound to a CNBr-matrix (Section II.B) using the protocol described by the manufacturer (AmershamPharmacia). The matrix had a yellow colour after application of SYE1 indicating successful binding of the protein to the matrix.

Lysate of a *S. oneidensis* culture induced with acrolein was applied to the matrix in PBS buffer and incubated for two hours to allow for specific binding. After incubation, the column was washed with PBS supplemented with 0.1 M NaCl to remove contaminants and non-
specifically binding proteins. The possible partner was eluted by the addition of PBS supplemented with 1 M NaCl.

SDS-PAGE analysis of the eluate, however, showed that no protein of 43 kDa had specifically bound SYE1.

Compared to the SELDI technology, the sensitivity of a pull-down assay is very low. It is therefore possible that the partner is present in the *S. oneidensis* cell lysate in a concentration far too low for identification by a pull-down assay.

**IV.E. A partner for SYE4**

In the initial test (Fig.5.12) for a partner we had used both SYE1 and SYE4 as bait to scan the *S. oneidensis* lysate. Where the interaction between SYE4 and the 43 kDa protein was not stringent enough to isolate it from a crude protein sample, the 43 kDa protein did react with SYE4 when the ‘200 mM fraction’ obtained from the chromatographic separation of the *Shewanella* lysate on a Q-Sepharose column was applied (Results not shown). We wanted to confirm these results obtained with the SELDI technology by a pull-down assay.

GST-SYE4 was bound to a Glutathione-Sepharose matrix in PBS buffer. Lysate of a *S. oneidensis* culture, induced for one hour with 25 µM NEM, was applied to the column. The matrix was incubated for two hours with the lysate to allow for binding. After incubation, the column was washed once with PBS and once with PBS, supplemented with 0.1 M NaCl to remove contaminants and non-specifically binding proteins. GST-SYE4 and the possible partner were eluted by the addition of 10 mM glutathione.

In parallel with the GST-SYE4 pull-down experiment a control experiment was performed using GST as the bait.

The eluates of both experiments were loaded onto several parallel SDS-PA gels. One gel was electroblotted and (1) analysed by Western analysis, the other gels were used for the identification of the protein bands visible on the gel by either (2) peptide mapping or (3) N-terminal sequencing.

In both cases (GST-SYE4 and GST as the bait), some protein bands could be observed when the eluate was analysed on a SDS-PA gel, but little differences were observed for the GST-SYE4 and the GST-control reactions, indicating that the reactions observed were non-specific for GST. Some protein bands were analysed, but the results were not reproducible.
V. References


IV. Characterisation of OYE homologues of *Vibrio* species

When the induction studies were performed on *S. oneidensis* with N-ethylmaleimide or acrolein as the inducer (Chapter five, Section III.C) strong induction of the *sye* genes was observed, resulting in a high amount of SYE protein expressed. NEM and acrolein are noted to undergo reactions with thiol groups, thereby not only modifying proteins, but also depleting the GSH pool. In addition, the yeast homologue OYE2 has been noted in the reduction of the cysteine disulfide bridge of actin filaments. These observations raised the question whether SYE enzymes interact in some way with detoxifying enzymes which function through the use of glutathione. Since enzymes of similar function are often clustered on the genome, we checked the genomic surroundings of the *sye* genes. However, no genes encoding proteins which function through the use of glutathione could be found in the vicinity of the four *sye* genes.

After elucidation of the *Shewanella oneidensis* genome, it was noted that one third of its genes are most related to *Vibrio cholerae* genes, and, as already mentioned in Chapter five, Section III.D, it was noted that several OYE encoding genes in *Vibrio* species are located in the same operon as the genes encoding glutaredoxin 2 or lactoylglutathione lyase (glyoxalase). Therefore we aimed (1) to express these *Vibrio* Yellow Enzyme (VYE) proteins and characterise them, (2) express and characterise the glutaredoxin 2 and glyoxalase enzymes and (3) investigate possible interactions between these OYE homologues and the glutaredoxin 2 and the glyoxalase enzymes.

*V. vulnificus* and *V. parahaemoliticus* strains were available at our laboratory. The *Vibrio vulnificus* genome encodes for an OYE homologue with identification number Vv21077. The Vv21077 gene is preceded by a glutaredoxin 2 and a transcriptional regulator gene (Fig. 6.1). Glutaredoxin 2 (glutathione disulfide oxidoreductase) is an enzyme thought to catalyse the reduction of mixed disulfides generated by reactive oxygen species (Enoksson *et al.*, 2005).

![Fig. 6.1. Genomic organisation of the region surrounding the Vv21077 gene. The genes encode for (from left to right) a transcriptional regulator, a dehydrogenase, glutaredoxin 2, an OYE homologue, a predicted Zn-dependent hydrolase of the β-lactamase fold, and a transcriptional regulator of sugar metabolism.](image-url)
The *Vibrio parahaemoliticus* homologue is encoded by the *VPa0773* gene and lies adjacent to a lactoyl glutathione lyase (glyoxalase) gene (Fig. 6.2). Glyoxalases catalyse the GSH-dependent detoxification of glyoxal and methylglyoxal, which are produced during lipid peroxidation and glycolysis, respectively.

**Fig. 6.2. Genomic organisation of the region surrounding the VPa0773 gene.** The genes encode for (from left to right) a putative lipopolysaccharide modification acyltransferase, a hypothetical protein, an OYE homologue, a lactoyl glutathione lyase, a hypothetical protein, a maz-G related protein, and a hypothetical protein.

In addition, the enzymes identified showed high similarity to both *sye1* and *sye3*. They possessed all of the ‘special’ amino acids noted for SYE1, namely Met25, Phe132, Trp274 and Phe350 (Chapter four, Section VI). By characterising the *Vibrio* OYE homologues, additional information about the significance of these substitutions can be gained.

An alignment of the VvYE (*Vibrio vulnificus* Yellow enzyme) and the VpYE (*Vibrio parahaemoliticus* Yellow Enzyme) with the *Shewanella* SYE1 and SYE3 enzymes shows the high sequence homology between these four enzymes (Fig. 6.3). Both Vibrio genes have the equivalents of Met25, Trp274 and Phe350 (magenta), indicating an active site similar to SYE1. The most notable difference is the presence of the HxxH sequence instead of HxxN (green).

As a first step we wanted to express and characterise the different *Vibrio* OYE homologues. This characterisation would serve both purposes mentioned above.
I.A. Cloning, expression and purification of VvYE and VpYE

The two vye genes were cloned in the pGEX-4T-2 vector using the restriction sites BamHI/NotI. Both genes were expressed as GST-fusion proteins in E. coli MC1061 cells at 28°C upon induction with 0.5 mM IPTG. The cells were ruptured by sonication and the cell lysate was separated from the cellular debris by centrifugation. The lysate was loaded onto a Glutathione-Sepharose 4 FF affinity matrix, which retains the GST-tagged proteins. In both cases, high amounts of 95% pure yellow protein were obtained. The proteins were subsequently cut with thrombin. About 10% and 2% of the total amount of GST-VpYE and GST-VvYE was cut respectively. The amount of thrombin was raised by a factor 10 and we were able to cut a sufficient amount of VpYE, but were not able to obtain enough VvYE for purification. The resistance to scission with the thrombin can be explained by the relative short N-terminal tail (the amino acids present before the actual barrel) of the proteins. VpYE was purified in 3 subsequent steps: affinity purification on a Glutathione-Sepharose matrix to separate VpYE from GST and GST-VpYE, followed by anion-exchange chromatography, and gel filtration to remove the last contaminants.

I.B. Initial characterisation

I.B.1. Ligand binding titrations

In a preliminary screen VpYE was titrated up to saturation with p-hydroxybenzaldehyde (pK_a = 7.62), p-chlorophenol (pK_a = 9.34) and p-cresol (pK_a = 10.30). Charge transfer interactions could be observed with the first two compounds (Fig.6.4), but even elevated concentrations of 10 mM p-cresol could not induce CT formation. By extending the titration experiment to the phenols p-fluorophenol (pK_a = 9.43) and p-methoxyphenol (pK_a = 10.20), it should in principle be possible to determine the cut-off pK_a where the formation of CT complexes is no longer possible. The maxima of the charge transfer bands lie at 633 nm for p-chlorophenol and 575 nm for p-hydroxybenzaldehyde. The K_d of p-chlorophenol was estimated at about 622 µM, the K_d for p-hydroxybenzaldehyde could not be derived from these preliminary results.

I.B.2. Cofactor dependence

The cofactor dependence of VpYE was determined as described in Chapter three, Section III.A.2. This initial screen showed VpYE to have a relatively low rate of diaphorase activity and a preference for NADH.
These initial screens indicate a similar behaviour for SYE1 and VpYE. VpYE was not able to form a CT interaction with p-cresol but did so with p-chlorophenol. This difference could be caused by the presence of the HxxH motif in VpYE instead of the HxxN motif of SYE1. It is to note that an insignificant CT formation can be observed between SYE1 and p-chlorophenol when a very elevated concentration (20 mM) is used. In addition, VpYE has a preference for NADH over NADPH and a low diaphorase rate.

These observations led us to the question whether the active site of SYE1 has a ‘special’ significance. A BLAST search conducted on the NCBI website (www.ncbi.nih.gov) using SYE1 as the probe gave numerous homologues of SYE1 possessing Met25 as the Trp274 and Phe350 equivalents. The organisms in which these homologues occur are Vibrio, Photobacterium, Burkholderia, Ralstonia, Pseudomonas, Azotobacter, Colwellia, Photorhabdus, Chromobacterium,… species, all belonging to the β- and γ-class of proteobacteria. Since the simultaneous transition of three amino acids in a protein sequence is too much of a coincidence, this might indicate a common origin or function for these enzymes in the above noted organisms.

In addition, after a more extended characterisation of the VYE enzymes, one can proceed to the expression and characterisation of the glutaredoxin 2 and the glyoxalase enzymes.

**Fig. 6.4. Spectrum of the titration of VpYE with p-chlorophenol.**

**I.C. Conclusion**
II. Mutation studies

Mutational studies can be performed for several purposes, roughly divided in ‘economical’ ones (improving the catalytic properties of an enzyme, diminishing an unwanted side reaction, enhancing the temperature stability of an enzyme), and ‘theoretical’ ones (exploring the necessity of a specific amino acid in catalysis, in maintaining the structure). For every purpose there are different mutational techniques, ranging from point-mutations to random mutagenesis combined with family shuffling.

For many of the conserved amino acids of the OYE homologues the function has been determined by mutational studies. Mutations can enhance or completely abolish a reaction; they can have structural effects or even no effect at all. Structure determination is a great help in choosing which amino acids to mutate. In addition, comparison of the different homologues can give clues about the function of some residues. For example the HxxH sequence present in some OYE species confers the ability to reduce TNT to the H-Meisenheimer complex (Williams et al., 2004).

The SYE enzymes have some amino acids that differ from the conserved amino acids of the OYE family. For instance, SYE2 lacks Arg317 and it is replaced by Thr. This arginine normally makes contact with two phosphate oxygens of the FMN ribityl tail. Possibly, SYE2 has a lower affinity for FMN, resulting in a destabilisation of this protein.

SYE1 and SYE3 both have Leu25 replaced by Met25, the former is more common in OYE homologues. The structures of YqiM (Kitzing et al., 2005) and SYE1 (Chapter four, Section VI) reveal a possible involvement of this amino acid in bending the flavin. SYE1 and SYE3 also lack Tyr350; it is replaced by Phe350. A mutation in morphinone reductase has shown the importance of this residue in NADH binding (Messiha et al., 2005).

Based on these differences, mutational studies of the SYE proteins have been proposed.

The following point mutations are of interest:

(1) Thr317Arg in SYE2

In Chapter two, Section I.B different possibilities are mentioned to explain the insolubility of SYE2. One option discusses the Arg→Thr transition of a conserved residue. This residue corresponds to Arg323 of SYE1. By inspecting Fig.1.8 in Chapter one, one can see that Arg323 is a conserved amino acid that is part of the hydrogen bonding network surrounding the FMN ribityl tail. Important interactions are lost, due to the transition of arginine to threonine.

(2) Met25Leu in SYE1

When we solved the structure of SYE1 (Chapter four, section VLE) we observed very strong ‘butterfly bending’ of the flavin ring system in both the oxidised and the reduce form. This bending around the theoretical N5-N10 axis had thus far only been observed for Bacillus subtilis’ YqiM (Kitzing et al., 2005). Comparison of the YqiM and SYE1 structures showed us an amino acid that might be involved in this butterfly bending. The conserved Leu25 (SYE1 and YqiM numbering) in both SYE1 and YqiM is substituted by a methionine. In addition, the methionine side chain in both structures is positioned under the dimethylbenzene ring. So the question results: is Met25 the amino acid responsible for the butterfly bending of FMN?
(3) Trp274Phe and Phe350Tyr in SYE1

What are the influences of these mutations on the affinity of the enzyme for NADH, on the interaction with phenolic ligands, and the position of Loop 6 in the enzyme structure?

Besides the butterfly bended flavin of SYE1, another aspect specific for SYE1 was noted. The phenolic ligands were bound in a different way in the active site of SYE1 compared to e.g. OYE (Fox and Karplus, 1994). The amino acids responsible for this different interaction are Phe350 and Trp274. By constructing a Trp274Phe mutant one can explore the importance of this interaction on the affinity of the enzyme towards substrates, NADH and phenolic ligands. A Phe350Tyr mutation is expected to restore the enzymes’ affinity towards phenolic ligands of high pK_a. In addition, one should explore the influence of these mutations on the conformation of Loop6 (which comprises Trp274).

III. References


Messia, H.L., Bruce, N.C., Satelle, B.M., Sutcliffe, M.J., Munro, A.W., and Scrutton, N.S. (2005) Role of active site residues and solvent in proton transfer and the modulation of flavin reduction potential in bacterial morphinone reductase. J. Biol. Chem. 280, 27103-27110

CONCLUSION

Four Old Yellow Enzymes were identified for *Shewanella oneidensis*. Since this is an exceptionally high number of OYE homologues for one organism to have, we proceeded, in this thesis, to characterise these enzymes by a comparative analysis. Different characteristics for these enzymes might indicate a difference in function, so a divergence between the enzymes might explain the presence of four homologues in *S. oneidensis*.

In general, the conclusions of this work are the following:

1. We were able to divide the SYE enzymes in two subgroups, comprising SYE1 and SYE3 on the one hand, and SYE4 on the other, based on their biochemical characteristics.
2. Structural analysis of SYE1 and protein modelling of the different SYEs learned us that (i) SYE1 has some specific characteristics different from other OYE family members, and that (ii) the biochemical differences of SYE4 are manifested in structural differences of the capping subdomain.
3. By expression analysis we were only able to show the expression of SYE4 in vivo.

These observations indicate a divergence between the bacterial OYE s.

Characterisation

First, the sye1 and sye2 genes are present in *S. oneidensis* in an operon, the other genes are not. In addition, they have little sequence homology to each other. The sye1, sye2, and sye3 genes are preceded by a LysR type regulator, the sye4 gene by a TetR type regulator. A different regulation might be an indication for a different function.

We were able to express and purify three of the four enzymes. SYE2 could only be expressed as insoluble protein. Based on sequence alignments with other OYE homologues we observed an Arg→Thr substitution of a conserved amino acid necessary for the binding of FMN. By use of the pGEX-4T-2 vector, SYE2 could be expressed and purified as a GST-fusion protein (most GST-SYE2 protein was present in inclusion bodies, very little could be purified). The protein could, however, not be reconstituted by the addition of FMN, possibly the apoenzyme is unstable.

Biochemical characterisation of the other three SYE proteins showed that the characteristics of SYE4 were different than those of SYE1 and SYE3:

1. SYE4 had a slightly different UV-Vis spectrum than SYE1 and SYE3, showing a peak at 380 nm rather than at 370 nm. This resulted in a different colouration for SYE4 describing the protein as salmon instead of orange.
2. SYE4 was able to use both NADPH and NADH as a reductant, with a preference for the former, whereas SYE1 and SYE3 only accepted NADH.
3. SYE1, SYE3 and SYE4 were shown to reduce the small substrates NEM, acrolein and cyclohexenone; only SYE4 accepted in addition glycerol trinitrate and picric acid as a substrate.
4. Both SYE1 and SYE3 were unable to form charge transfer complexes with phenolic ligands of high pKₐ, a phenomenon thus far unencountered for OYE homologues.
In addition, some similarities could be observed between the three enzymes: they did not form semiquinone species upon photoreduction of the FMN, as was expected for bacterial members of the OYE family. Also the enzymes showed very low diaphorase rates, an observation which remains thus far unexplained.

Based on these observations, we divided the SYE enzymes in **two subgroups**, SYE4 constitutes one group and SYE1 and SYE3 make up the other group.

It is to note that, the initial characterisations made us conclude that SYE4 might be interesting for bioremediation. If it can be shown that SYE4 not only reduces picric acid but also TNT to the hydride-Meisenheimer complex, this would be the first enzyme without a HxxH sequence to catalyse this reaction.

**Structure**

The structure of native SYE1 was solved by X-ray crystallography, in addition to the pHBA- and pACE-complexed and NADH-reduced forms. Although the overall structure shows good similarity to the other bacterial homologues PETN reductase and morphinone reductase, some special properties were observed for this enzyme.

1. The FMN ring was butterfly bended as noted for *Bacillus*’ YqjM. The significance of this bending is as yet unknown. We propose that Met25 might be the determinant of the bending.
2. A different binding mode was observed with phenolic ligands. The carbonyl oxygen is not bound to Tyr350, which is not present in SYE1, but to Trp274. Possibly this different binding mode is the reason why SYE1 only binds a limited range of phenolic ligands.
3. Since Trp274 is present in the active site of SYE1 to accommodate for substrate binding, Loop 6 is folded towards the centre of the enzyme, thereby causing the enzyme to have a very narrow and shallow active site tunnel and thus putting a restraint on the range of possible substrates.

Because of the ‘special’ properties observed for the **SYE1 structure** (the butterfly bended flavin, the special binding mode of phenolic ligands in the active site, and the presence of a secondary binding site for phenolic ligands) a BLAST search was conducted on the *nr* database of NCBI using SYE1 as the probe. The search gave us numerous enzymes with high homology to SYE1, possessing all the amino acids needed for the ‘special’ properties. In addition, all homologues found were enzymes of β- or γ-proteobacteria. Therefore, we believe the special properties observed for SYE1 may be significant.

In addition, structural models were calculated by computational means. These theoretical models indicated quite similar structures for SYE1, SYE2 and SYE3; **SYE4** differed by displaying a very small **capping subdomain**. This small subdomain might have impact on substrate recognition and substrate access, evidenced by the fact that SYE4 can accept more bulky substrates. A comparison of the theoretical model and the experimental SYE1 structure, however, indicated that care must be taken in drawing conclusions from these models.
Based on comparisons of the primary structure, the biochemical characteristics and the structural models of SYE1, SYE3 and SYE4, we pose that the structure of SYE3 will be very similar to SYE1, whereas SYE4 is expected to have a structure more similar to YqjM.

**Physiological function**

We performed two different sets of experiments to gain more insight in the physiological function of the SYE enzymes. Since no expression of SYEs occurred under ‘normal’ growth conditions, we assayed in the first set different growth conditions under which the SYE proteins could be induced in *S. oneidensis*. The proteins were shown to be induced by

1. the addition of iron and manganese to the growth medium,
2. by exerting oxidative stress on the cells by for example the addition of peroxides,
3. by exposing the cells to a heat shock and
4. by adding the substrates N-ethylmaleimide, acrolein and cyclohexenone to the cells.

Partial purification combined with N-terminal sequencing made us conclude that the enzyme induced was SYE4. In addition, a study by Gao *et al.*, 2004 indicated that SYE4, but not SYE1, SYE2 nor SYE3 was induced in *S. oneidensis* under conditions of heat stress. These results indicate a function for SYE4 in an oxidative stress related response. Since SYE4 could also be expressed by the addition of substances that target free sulfhydryl groups, it is possible that the function of SYE4 is linked to disulfide (glutathione) related detoxification mechanisms. It has been shown for OYE2 of bakers’ yeast that the enzyme is indeed able to reduce a protein-protein disulfide bridge.

Since the SYE1 and SYE3 protein were not expressed under the growth conditions tested, this indicates a difference in the mechanism by which these proteins are induced and/or that the induction condition is not yet found.

In a second study we tried to identify the physiological protein partner for SYE1 and SYE4. We observed a 43 kDa protein able to form a strong interaction with SYE1 but the protein has not been identified thus far. This protein was also able to form a less specific interaction with SYE4.

**Future perspectives**

This thesis work can be used a starting point for numerous studies. Different lines of investigation can be pursued.

(1) physiological function

After more then 70 years of investigation, the first functions for OYE homologues are being discovered. Initial studies showed that the different enzymes in one organism, although quite alike, can have different functions. Here we propose two different function for the *Shewanella* OYE homologues SYE1 and SYE4. The elucidation of SYE4s function could provide new insights in the way bacteria cope with oxidative stress. Possible studies concerning this enzyme are a structural study and the construction and characterisation of a knock-out mutant.
For SYE1, on the contrary, we focus on the ‘special’ properties observed during biochemical characterisation. In addition, we believe that the determination of the identity of a partner might be crucial in the elucidation of SYE function.

(2) reduction of TNT
TNT is a very recalcitrant and toxic compound. Because of widespread contamination of the environment with TNT, numerous studies have been performed regarding its detoxification. Full detoxification relies on the specific reduction and opening of the aromatic ring. Recent studies showed that only OYE homologues possessing the HxxH sequence are able to catalyse the ring reduction. Our studies concerning SYE4 and the TNT analogue picric acid indicate that SYE4, might be able to reduce TNT. If this could be confirmed, SYE4 would be the first enzyme possessing a HxxN sequence to catalyse such a reduction.

(3) study of the regulator
A topic that is never considered regarding OYE homologues is the functionality of the regulator that precedes many bacterial OYE homologues. It would be very interesting to know if the induction of the different OYEIs is effectively controlled by these regulators, and to identify the molecule that controls the switch of the regulator. Possible studies concern the construction and characterisation of knock-out mutants of the regulator, purification and structure determination, determination of the molecule that operates ‘the switch’,…
Old Yellow Enzyme (OYE) werd voor het eerst aangetroffen in brouwers gist. Het enzyme werd geïsoleerd in 1932 door Christian and Warburg. Theorell (1935) toonde vervolgens aan dat het enzyme een flavine groep bevatte die absoluut noodzakelijk was voor katalyse. Deze ontdekking was het begin van een lange en uitgebreide studie naar de eigenschappen van flavoproteinen.

OYE’s komen voor in gisten, planten, bacteriën,.. De structuur van het eiwit bestaat steeds uit een 8 strengige α,β-ton die aan de onderzijde wordt afgesloten met een kleine β-plaat en waar aan de bovenzijde zich een niet covalent gebonden FMN groep bevindt. Een active site tunnel reikt van het oppervlak van het enzyme tot de si-zijde van de flavine. Katalyse door een OYE homoloog verloopt via een Ping-pong mechanisme. Eerst wordt flavine gereduceerd door NADPH of NADH, vervolgens bindt een substraat in de active site dat op zijn beurt wordt gereduceerd. Het exacte fysiologische substraat van OYE is niet gekend en de zoektocht wordt bemoeilijkt doordat de enzymes verschillende substraten zoals onverzadigde aldehyden en ketonen, steroïden, nitro-olefinen, nitro-esters, nitro-aromaten,… kunnen reduceren. Het voordeel van deze brede substraatsvoorkeur is dat er enkele biotechnologische toepassingen voor OYE’s bestaan. Ze worden o.a. gebruikt voor de productie van actinol and hydromorphon, maar worden ook onderzocht voor de reductie van het explosieve TNT.

De fysiologische functies van OYE’s waren lange tijd onbekend. Bovendien is er tot nu toe geen overkoepelende functie gevonden tussen gisten, planten, bacteriën,… In planten en in de parasiet Trypanosoma cruzi vervullen OYE homologen respectievelijk een rol in de jasmonaat en de prostaglandine pathway. In bakkergist zijn 2 OYE homologen aanwezig. OYE2 heeft een functie in het beschermen van het actine-cytoskelet tegen oxidatieve schade door de reductie van een disulfidebrug die gevormd kan worden tussen 2 actine-filamenten. Deze ontdekking reikte ons een nieuw type substraat aan voor OYE homologen. OYE3 wordt geïnduceerd in condities van oxidatieve stress die teweeggebracht worden door het toevoegen van waterstofperoxide aan de cellen. Bovendien zijn er aanwijzingen dat het enzyme een rol heeft in de Bax-gemediëerde geprogrammeerde celdood. Hiernaast werd ook voor het OYE homoloog van de Gram-positieve bacterie Bacillus subtilis aangetoond dat het enzyme wordt opgereguleerd onder oxidatieve stress. De exacte functie, echter, is voor bijna alle homologen nog niet eenduidig bepaald.

Shewanella oneidensis is een Gram-negatieve bacterie met een heel divers metabolisme. Omwille van dit metabolisme wordt ze wereldwijd bestudeerd voor mogelijke toepassingen in de bioremediatie. In dit kader werden 4 Old Yellow Enzyme homologen geïdentificeerd in het Shewanella oneidensis genoom. We noemden ze de Shewanella Yellow Enzymes (SYE). Ze zijn aanwezig op drie verschillende locaties in het genoom en worden steeds vooraf gegaan door een transcriptionele regulator (LysR of TetR type). Gezien dit een bijzonder hoog aantal OYE homologen is om aanwezig te zijn in één organism, besloten we deze homologen te karakteriseren. Verschillen in de eigenschappen tussen de verschillende OYE homologen zou duiden op een verschillende functie in de cel. We expresseerden de 4 eiwitten als GST-fusie proteïnen in E. coli. Grote hoeveelheden van zuiver eiwit zijn immers noodzakelijk voor karakterisatie. Het SYE2 enzyme bleek heel slecht oplosbaar en was aanwezig als apo-enzyme. Vermoedelijk is de affinitie van SYE2 voor FMN verminderd door een Arg→Thr transitie van een geconserveerd aminozuur dat mee instaat voor de binding van FMN, en is
het enzyme onstabiel. SYE1, SYE3 en SYE4 waren wel oplosbaar en werden op verschillende manieren gekarakteriseerd.

Een eerste karakterisatie betrof de flavine groep. De identiteit van de flavine werd bevestigd als zijnde FMN en er werd een UV-Vis spectrum opgenomen tussen 300 en 800 nm. Flavine vertoont 2 karakteristieke pieken bij ongeveer 360 en 450 nm. De drie enzymen vertoonden een ‘red shift’ van de flavine absorbantie ten opzichte van vrij FMN. Deze was net iets groter voor SYE4 dan voor SYE1 en SYE3, waardoor de kleur van dit enzyme eerder als ‘zalm’ dan als ‘oranje’ kon beschreven worden. Hiernaast werd het flavine gereduceerd door fotoreductie om na te gaan of de SYE’s flavine semiquinonen kunnen stabiliseren. Tot nog toe is aangetoond dat enkel OYE homologen uit eukaryote organismen hiertoe in staat zijn. De SYE’s weken niet af van deze regel.

Als een tweede karakterisatie werd de mogelijkheid tot vorming van ‘charge transfer’ complexen tussen de flavine en fenolische liganden onderzocht. Dit is een heel bijzondere eigenschap van OYE homologen. Fenolische liganden binden in de active site van OYE’s parallel met de flavine. Een interactie wordt aangegeven tussen hun π-systemen wat resulteert in specifieke verschuivingen in het UV-Vis spectrum: er verschijnt een nieuwe absorbantieband tussen 500-800 nm, de pieken bij 360 en 450 verminderen in absorbantie en in het gebied < 360 nm gebeuren ook verschuivingen van het spectrum. Hier werd echter iets uitzonderlijks waargenomen: SYE1 en SYE3 waren maar in staat interacties aan te gaan met liganden met een pK_a ≤ 8.05.

Hierna werd overgegaan naar de kinetische karakterisatie. De voorkeur voor NADPH of NADH, evenals het pH optimum werden bepaald. Daarinboven werden verschillende substraten van de hierboven vernoemde types getest voor katalyse. SYE1 en SYE3 hadden een voorkeur voor NADH als reductans en SYE4 voor NADPH. Het pH optimum voor SYE1 en SYE4 lag respectievelijk rond pH 7.75 en 7.25. Het pH optimum van SYE3 kon niet eenduidig bepaald worden (het enzyme was actief in een breed pH gebied). De drie SYE’s waren in staat de onverzadigde aldehyden/ketonen N-ethylmaleimide, cyclohexenone and acroleine te reduceren. Hiernaast kon ook activiteit aangetoond worden voor SYE4 met het nitro-ester nitroglycerine. Geen van de enzymen reduceerde steroïden onder de geteste condities noch de nitro-aromaat picraat. Hierbij moet echter wel opgemerkt worden dat indien SYE4 lange tijd (16 uur) werd geïncubeerd met picraat, er een verkleuring optrad, wat wijst op een mogelijk reductie van de aromatische ring. Op basis van de bekomen resultaten kunnen we de drie homologen indelen in 2 groepen.

<table>
<thead>
<tr>
<th>Eigenschap</th>
<th>SYE4</th>
<th>SYE1 en SYE3</th>
</tr>
</thead>
<tbody>
<tr>
<td>gen wordt vooraf gegaan door TetR / LysR type regulator</td>
<td>TetR</td>
<td>LysR</td>
</tr>
<tr>
<td>enzyme gereduceerd door NADH/NADPH</td>
<td>NADPH</td>
<td>NADH</td>
</tr>
<tr>
<td>enzyme kan nitroglycerine reduceren</td>
<td>JA</td>
<td>NEE</td>
</tr>
<tr>
<td>enzyme aanvaart ook picraat als substraat</td>
<td>JA</td>
<td>NEE</td>
</tr>
<tr>
<td>vormt CT complexen vormen met fenolische liganden met pK_a ≤ 8.05</td>
<td>NEE</td>
<td>JA</td>
</tr>
</tbody>
</table>
Naast biochemische karakterisatie was het eveneens de bedoeling om de structuur te bepalen van de verschillende SYE’s. Helaas ondervonden we enkele problemen bij kristallisatie. De keuze om de eiwitten te expresseren als GST-fusie gaf geen problemen voor karakterisatie maar echter wel voor kristallisatie. Het was noodzakelijk deze ‘tag’ te verwijderen; hiertoe gebruikten we trombine. SYE1 en SYE4 bleken echter een interne herkenningssequentie te bezitten voor trombine, wat resulteerde in extra verknipping. SYE3 en GST, aan de andere kant, konden nauwelijks van elkaar gescheiden worden door verknipping met trombine. Hierdoor waren we (mits aanpassing van de klonerings- en expressiecondities) enkel in staat kristallen te bekomen voor SYE1. Deze werden opgenomen aan de synchrotron in Hamburg in hun natieve toestand, als complex met de fenolische liganden p-hydroxybenzaldehyde (pHBA) en p-hydroxyacetophenone (pACE) en in de NADH gereduceerde toestand. De algemene structuur van SYE1 was heel sterk gelijktijdig op deze van de bacteriële homologen PETN reductase en morphinone reductase. Er waren echter enkele duidelijke verschillen die vermoedelijk significant zijn.

(1) SYE1 heeft een gebogen flavine ring
(2) SYE1 heeft een heel nauwe active site tunnel
(3) SYE1 vertoont een andere bindingsmanier voor de fenolische liganden; een vermoedelijke verklaring voor bovenvermelde fenomeen van beperkte interactie
(4) SYE1 kan een tweede pHBA molecule binden, wat een conformationele verandering veroorzaakt in het ‘capping’ subdomein

Via een BLAST analyse waarbij SYE1 als probe werd gebruikt waren we in staat tientallen OYE homologen aan te tonen die over de aminozuren noodzakelijk voor de speciale kenmerken van SYE1 beschikken. Deze enzymen waren steeds afkomstig van β- of γ-proteobacteria. Hiernaast werden ook theoretisch modellen berekend voor de overige drie enzymen. Door de intrinsieke eigenschap van OYE’s, met name dat de lussen aan de bovenzijde mobiel zijn en verschillende structuren aannemen, was het onmogelijk eenduidige conclusies te trekken uit deze modellen aangaande de toegang tot de active site. We konden uit deze modellen echter wel besluiten dat SYE4 een ‘capping’ subdomein heeft duidelijk verschillend dan dit van SYE1, SYE2 en SYE3.

In een derde luik werd onderzoek gedaan naar de fysiologische functie van de SYE’s. Door middel van inductie experimenten gecombineerd met Western analyse waren we in staat aan te tonen dat

(1) SYE’s niet constitutief geëxprimeerd worden in S. oneidensis

en dat ze geïnduceerd kunnen worden

(2) door tweewaardig ijzer of mangaan toe te voegen aan het groeimedium
(3) door oxidatieve stress in de vorm van bvb. peroxiden toe te dienen aan de cellen
(4) door de cellen bloot te stellen aan een hitte schok
(5) door de cellen de substraten NEM, acrolein en cyclohexenone toe te dienen.

Het enzyme dat geïnduceerd wordt werd geïdentificeerd als SYE4. Dit is gebaseerd op het molecule gewicht van de geïnduceerde eiwitten op SDS-PA gel, N-terminale sequentiebepaling en peptide mapping van partiel gezuiverd eiwit en door vergelijking met literatuurgegevens (een micro-array studie). Onze resultaten zijn vergelijkbaar met deze
bekomen in de studie van de bacterie *Bacillus subtilis*; namelijk, er treedt inductie op van een OYE homoloog onder condities van oxidatieve stress. De inductie door het toevoegen van ijzer en mangaan kan ook onder deze noemer geklasseerd worden, gezien door de Fenton reactie reactieve zuurstof species gevormd kunnen worden in de aanwezigheid van deze metalen. Hiernaast was er nog de belangrijke vaststelling dat de substraten NEM, acroleïne en cyclohexenone ook aanleiding gaven tot inductie. Deze substraten zijn sterke electrofielen die in staat zijn oxidatieve stress uit te oefenen op de cel door reacties aan te gaan met de vrije sulfhydryl groepen van eiwitten, maar ook door de glutathione voorraad van de cellen uit te putten, waardoor ze gevoeliger worden voor oxidatieve stress. Een gelijkaardig fenomeen werd waargenomen voor OYE2 uit gist, een enzyme dat niet enkel werd geïnduceerd door oxidatieve stress, maar ook door een diamide behandeling van de cellen. Diamide heeft een gelijkaardige werking als bovenvernoemde substraten. Bovendien is OYE2 in staat de disulfidebrug tussen 2 actine filamenten te reduceren. Een gelijkaardige functie voor SYE4 is dus niet uitgesloten. Maar samenvattend, kunnen we besluiten dat OYE’s zowel in gisten als bacteriën opgereguleerd worden onder condities van oxidatieve stress en glutathione-depletie en mogelijk optreden als een ‘reserve’ defensiemechanisme tegen stress.

Hiernaast werd ook onderzoek verricht naar een mogelijke partner voor SYE1 en SYE4. Met behulp van ProteinChip technologie konden we een mogelijke partner aantonen voor SYE1: een 43 kDa eiwit. We waren helaas nog niet in staat het eiwit te identificeren. SYE4 was ook in staat interacties aan te gaan met hetzelfde eiwit, echter alleen wanneer het 43 kDa eiwit was aangerijkt. Dit duidt op een minder stringente binding.

Het antwoord op de vragen die aan begin van deze thesis gesteld werden: ‘Waarom heeft *S. oneidensis* 4 OYE homologen, hebben ze een verschillende functie en wat is deze functie?’ kan nu ten dele gegeven worden.

Om te beginnen hebben we nooit het SYE2 eiwit kunnen expresseren in oplosbare vorm. Vermoedelijk is het eiwit onstabiel door een verminderde bindingsaffiniteit voor de FMN groep. Hiernaast werd aangetoond, zowel door biochemische karakterisatie, structuurbepaling en studies naar fysiologische functie dat de SYE1, SYE3 en SYE4 enzymen in twee groepen te verdelen zijn: met name SYE1 en SYE3 in de ene groep en SYE4 in de andere. Deze 2 enzym-groepen zouden mogelijk instaan voor een specifieke functie in *S. oneidensis*. Voor SYE4 wordt een functie in algemene stress responsen vermoed.