REVIEW
ARTICLE

'New uses for an Old Enzyme' – the Old Yellow Enzyme family of flavoenzymes

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Overview

As the first flavin-dependent enzyme identified, and by virtue of its simplicity, the yeast Old Yellow Enzyme (OYE) has been characterized in detail by the whole gamut of physical techniques. Despite this scrutiny, the true physiological role of the enzyme remains a mystery. After 60 years in isolation, OYE has become the archetype of a growing family of flavoenzymes that have been discovered through studies of bacterial metabolism and genome sequencing projects.

Old Yellow Enzyme

Early studies of OYE demonstrated several important biochemical concepts, giving the enzyme a significant place in the history of enzymology. OYE was isolated from brewers' bottom yeast by Warburg & Christian (1932), during attempts to elucidate the nature of biological oxidations. Glucose 6-phosphate was oxidized by methylene blue in the presence of two components of erythrocytes, an enzyme ('Zwischenferment'), and a small heat-stable 'Coferment'. Following this, Warburg identified a yellow enzyme ('Gelbe Ferment') that permitted the system to form a complete respiratory chain reacting with molecular oxygen (Fig. 1). Following the isolation of a second, 'new', yellow enzyme from yeast by Haas (1938), Warburg's enzyme was termed 'Old Yellow Enzyme', a name that has persisted to this day. OYE was purified by Theorell in 1935, and shown to comprise a colourless apoprotein and a yellow dye, both essential for enzyme activity. This finding helped to establish the essential role of protein in enzyme catalysis. The yellow dye was similar in nature to vitamin B₂ (riboflavin). Thus OYE provided the first biochemical role for a vitamin. Theorell (1955) demonstrated that the yellow cofactor was in fact riboflavin 5'-phosphate, now also termed flavin mono-

nucleotide (FMN). Since then, OYE has been characterized in great detail by Vincent Massey's laboratory, and a considerable amount is now known about the mechanism of the enzyme.

The physiological reductant of OYE is assumed to be NADPH. Substrates capable of reoxidizing OYE include methylene blue, Fe³⁺, quinones, cytochrome *c* (Massey *et al.*, 1969) and ferricyanide. Reoxidation can be effected by molecular oxygen to yield hydrogen peroxide and superoxide; however, this reaction appears to be adventitious, as the rate observed is substantially lower than that observed with oxidase enzymes, and is lower than obtained with substrates such as quinones. Many different potential ligands have been screened in an attempt to identify possible physiological substrates (Schopfer & Massey, 1991; Vaz *et al.*, 1995). Examples of known substrates and inhibitors are shown in Fig. 2. Only α/β -unsaturated aldehydes and ketones were substrates for the reaction – acids, esters and amides did not react. Cyclic enones can also act as substrates for the reductive half-reaction of OYE – thus OYE catalyses the disproportionation of 2-cyclohexenone into phenol and cyclohexanone with no nicotinamide cofactor requirement.

The majority of known inhibitors contain a phenolic functional group. OYE in complex with phenolic ligands exhibits absorbance bands between 300 and 425 nm,

Fig. 1. Reaction system of Warburg and Christian.

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SUBSTRATES

cyclohexenone

duroquinone

menadione

INHIBITORS

p-hydroxybenzaldehyde

testosterone

Fig. 2. Some typical ligands and substrates of OYE.

and between 500 and 800 nm. The short wavelength absorbance is consistent with the absorbance of a phenolate anion, slightly red-shifted, and the long wavelength absorbance arises from a charge transfer interaction between the phenolate anion and neutral flavin (Abramovitz & Massey, 1976).

The observed similarity between the sequences of the gene encoding an OYE (Saito *et al.*, 1991) and a gene from the bile-acid-inducible operon in *Eubacterium* sp. suggested that OYE could also be involved in sterol metabolism. This would involve reduction of a 2-cyclohexenone functional group, and it was demonstrated that 2-cyclohexenone is a very good oxidant of OYE (Stott *et al.*, 1993), giving a turnover number in excess of that seen with quinones such as menadione. However, no evidence has been found for sterol metabolism by OYE *in vivo*.

An Old Yellow Enzyme family of flavoproteins

A growing family of proteins possess amino acid sequences similar to that of OYE. Genomic sequencing predicts the existence of many more uncharacterized

relatives. In all, homologous proteins have been found in other yeasts, in bacteria, in plants and in the nematode *Caenorhabditis elegans*. Members of this OYE family have been identified as a consequence of their catalysis of a diverse range of transformations (Fig. 3). The α/β -unsaturated carbonyl functional group is present in many, but not all, of the substrates identified. No single physiological role has emerged to explain the relatively high degree of amino acid sequence similarity between the enzymes.

Both *Saccharomyces carlsbergensis* (Saito *et al.*, 1991) and *Saccharomyces cerevisiae* (Niino *et al.*, 1995) have been found to contain pairs of closely related OYE genes. Two *Schizosaccharomyces pombe* homologues have been identified during genomic sequencing (Wood *et al.*, 2002), and a further yeast OYE clone, from *Kluyveromyces lactis*, was isolated fortuitously (Miranda *et al.*, 1995). Also, recent biochemical characterization of the oestrogen-binding EBP1 protein of *Candida albicans* (Buckman & Miller, 2000) shows it to be similar in many ways to OYE.

Several bacterial enzymes with amino acid sequence homology to OYE have now been reported. Multiple independent studies of the bacterial degradation of nitrate ester explosives have identified very similar OYE-related proteins. The enzymes catalyse the nicotinamide-cofactor-dependent reductive cleavage of nitrate esters to give the alcohol and nitrite. Pentaerythritol tetranitrate (PETN) reductase from *Enterobacter cloacae* PB2 (Binks *et al.*, 1996) sequentially removes two of the four nitro groups of PETN, and two of the three groups of nitroglycerine (GTN). The crystal structure of this enzyme has now been elucidated (Barna *et al.*, 2001). Similar enzymes are responsible for nitrate ester degradation by *Agrobacterium radiobacter* (Snape *et al.*, 1997) – ‘nitrate ester reductase’ – and by strains of *Pseudomonas fluorescens* and *Pseudomonas putida* (Bleher *et al.*, 1999) – ‘xenobiotic reductases’. OYE also catalyses the cleavage of nitrate esters, albeit at a slower rate than these bacterial enzymes, probably via a radical-based mechanism (Meah *et al.*, 2001).

Research into the degradation of morphine alkaloids by a strain of *P. putida* led to the identification of a two-stage transformation sequence from morphine via morphinone to hydromorphone (Hailes & Bruce, 1993). The second enzyme in the pathway, morphinone reductase, is homologous to OYE (French & Bruce, 1994). This enzyme is responsible for the reduction of an α/β -unsaturated carbonyl functionality analogous to that of known OYE substrates. Another similar enzyme has been found to be cold-shock-induced in *Pseudomonas syringae*, a plant pathogen (Rohde *et al.*, 1999).

A close relative of PETN reductase, N-ethylmaleimide reductase (Miura *et al.*, 1997), has been identified in *Escherichia coli*. N-Ethylmaleimide is a variation on the α/β -unsaturated carbonyl functional group, and is also a substrate for OYE (Vaz *et al.*, 1995). Original characterization of the enzyme (Mizugaki *et al.*, 1979) described it

Fig. 3. Some of the reactions catalysed by members of the OYE family. (a) Reductive denitration of pentaerythritol tetranitrate by PETN reductase (Binks *et al.*, 1996). (b) Formation of a hydride-Meisenheimer complex of trinitrotoluene by PETN reductase (French *et al.*, 1998). (c) Reduction of morphinone ($\text{R}=\text{H}$)/codeinone ($\text{R}=\text{CH}_3$) to form hydromorphone ($\text{R}=\text{H}$)/hydrocodone ($\text{R}=\text{CH}_3$) by morphinone reductase (Hailes & Bruce, 1993). (d) Reduction of *N*-ethylmaleimide to *N*-ethylsuccinimide by *N*-ethylmaleimide reductase (Miura *et al.*, 1997). (e) Reduction of 9*R*,13*R*-12-oxophytodienoate by 12-oxophytodienoate reductase (Breithaupt *et al.*, 2001).

as a *cis*-enoyl-CoA reductase, involved in the β -oxidation of fatty acids such as linoleic acid.

Plant homologues of OYE were first identified during various screens for genes induced under different circumstances. OYE-related messenger RNAs are produced during cytokinin induction in *Chenopodium rubrum* (Peters *et al.*, 1996), sulphate starvation in *Catharanthus roseus* (GenBank accession no. AF005237) and dehydration stress in *Vigna unguiculata* (Iuchi *et al.*, 1996). Three *Arabidopsis* OYE homologues have been identified as 12-oxophytodienoate reductases (Schaller & Weiler, 1997), which can catalyse the reduction of an α/β -unsaturated carbonyl intermediate in the synthesis of jasmonic acid, a wound-response hormone. Only one of the paralogues appears to be involved in the wound-response pathway *in vivo* (Schaller *et al.*, 2000). Interestingly, other lipids with α/β -unsaturated carbonyl functionality appear to be produced in response to physical wounding and *P. syringae* infection, being implicated both in signalling and as causative agents of tissue damage (Vollenweider *et al.*, 2000). The smaller, volatile, α/β -unsaturated carbonyl compound *trans*-2-hexenal is also involved in response to bacterial pathogens (Farmer, 2001). A tomato orthologue of 12-oxophytodienoate reductase has been compared to OYE in some detail (Strassner *et al.*, 1999), and the structure of this enzyme has very recently been determined (Breithaupt *et al.*, 2001).

A series of more distantly related proteins is present in the sequence databases. A barrel domain related to OYE forms part of trimethylamine dehydrogenase (Lim *et al.*, 1986), and is used as a module in several other multi-domain proteins (Scrutton, 1994), such as *E. coli* 2,4-

dienoyl-CoA-reductase (He *et al.*, 1997) and enoate reductases of clostridia (Rohdich *et al.*, 2001). Whilst some of the putative active site residues differ in these distant relatives of OYE, there is a strong sequence conservation in a core region of around 40 amino acids. The second domain in trimethylamine dehydrogenase is also redox-active, being related to glutathione reductase and dihydrolipoamide dehydrogenase. There is clear evidence for intra-protein electron transfer from the OYE-related domain to an iron-sulphur cluster located in the second domain (Falzon & Davidson, 1996). Thus rather than having a common binding site for both the reducing and oxidizing substrates (as in OYE), these enzymes would appear to have separate sites, permitting optimization of each site for the half-reaction it catalyses. However, a two-domain 2-aminobenzoate monooxygenase/reductase with an OYE-related domain as the C-terminus has recently been reported (Schuhle *et al.*, 2001), in which each domain would appear to act independently, albeit to catalyse consecutive steps in a metabolic pathway.

Structure and mechanism of OYE family members

Despite the crystallization of OYE by Theorell in 1955, it has required the cloning of the gene encoding OYE to produce X-ray quality crystals from recombinant enzyme. This was probably a result of the heterogeneity of native OYE arising from the two genes present in *S. carlsbergensis*. There are now published coordinates refined to 2.0 Å for the oxidized and reduced forms of OYE, and for the complex with the phenolic inhibitor *p*-hydroxybenzaldehyde (Fox & Karplus, 1994).

Fig. 4. Ribbon diagram of the structure of OYE. Flavin mononucleotide and *p*-hydroxybenzaldehyde ligands are shown in ball-and-stick form. Figure drawn using MOLSCRIPT (Kraulis, 1991) using co-ordinates from Fox & Karplus (1994) deposited as 1oyb in the Protein Databank (PDB; <http://www.rcsb.org/>).

Fig. 5. Active site of OYE in complex with *p*-hydroxybenzaldehyde. Figure drawn using MOLSCRIPT (Kraulis, 1991) using co-ordinates from Fox & Karplus (1994) deposited as 1oyb in the Protein Databank (PDB; <http://www.rcsb.org/>).

OYE is a single-domain protein of around 45 kDa, with an α/β barrel fold (Fox & Karplus, 1994), as shown in Fig. 4. There is a β -hairpin structure covering the base of the barrel, whilst the FMN is bound at the top of the barrel, with the *si*-face of the flavin accessible to the solvent, where it forms the bottom of the active site. From representation of the active site of OYE in complex with *p*-hydroxybenzaldehyde in Fig. 5, it can be seen that the phenolic ring (white bonds) undergoes a stacking interaction with the flavin (grey bonds). The

phenol oxygen forms two hydrogen bonds to His-191 and Asn-194, displacing a chloride ion bound in the empty oxidized enzyme structure. Binding studies with a variety of substituted phenols suggest that it is the phenolate anion that is a ligand for the enzyme (Abramovitz & Massey, 1976; Brown *et al.*, 1998). The crystal structure of OYE soaked with the non-substrate analogue of NADPH, (c-THN)TPN, suggests a novel cofactor binding arrangement where only the nicotinamide moiety of the cofactor is bound tightly, whilst the

Fig. 6. Overlaid C α backbones of OYE, 12-oxophytodienoate reductase and PETN reductase. OYE, 12-oxophytodienoate reductase and PETN reductase are shown in blue, green and red, respectively. The FMN cofactor of OYE is shown in ball-and-stick representation. Figure drawn using MOLSCRIPT (Kraulis, 1991) using co-ordinates deposited as 1oyb (Fox & Karplus, 1994), 1icq (Breithaupt *et al.*, 2001) and 1h50 (Barna *et al.*, 2001) in the Protein Databank (PDB; <http://www.rcsb.org/>).

adenine phosphate is not fixed. This fits the observations that the α -anomer of NADPH reacts as well or even slightly better than the usual β -anomer, and that 2'-phospho-5'-AMP is not a competitive inhibitor. However, it makes the observed selectivity for NADPH over NADH hard to explain.

The combination of site-directed mutagenesis studies of OYE (Brown *et al.*, 1998; Kohli & Massey, 1998) and crystal structures of two more members of the OYE family, PETN reductase (Barna *et al.*, 2001) and 12-oxophytodienoate reductase (Breithaupt *et al.*, 2001), has given significant insight into the catalytic mechanism and substrate specificity across the OYE family as a whole. Site-directed mutagenesis of His-191 and Asn-194 in OYE has confirmed their importance in binding the oxygen atom of substrate molecules (Brown *et al.*, 1998). If an α/β -unsaturated carbonyl substrate binds with the carbonyl oxygen atom positioned to hydrogen bond to His-191 and Asn-194, the β carbon atom can be aligned above the flavin N-5 atom. This is consistent with earlier experiments suggesting hydride transfer from the flavin N-5 atom to the β carbon. Site-directed mutagenesis has implicated Tyr-196 in transfer of a proton to the α carbon (Kohli & Massey, 1998), completing the saturation of the double bond. The rate of 2-cyclohexenone reduction catalysed by the Y196F mutant of OYE is significantly lower than wild-type. Hydride transfer and proton transfer to 2-cyclohexenone need to be concerted, as it is not possible to form a stable carbanion intermediate. Reduction of 1-nitrocyclohexene to nitrocyclohexane proceeds via a comparatively stable nitronate intermediate. Whilst the Y196F mutant can catalyse hydride transfer to 1-nitrocyclohexene, it is unable to protonate the nitronate formed (Kohli & Massey, 1998), providing strong evidence for the role of Tyr-196 as an active site acid.

In the three enzymes for which crystal structures are available, the FMN cofactor is bound in a very similar

manner (Barna *et al.*, 2001; Breithaupt *et al.*, 2001; Fox & Karplus, 1994). Where the side-chains of residues contact the cofactor, these residues are conserved across the family. The residues lying immediately above the plane of the flavin ring, whether involved in catalysis as described above, or forming the hydrophobic substrate-binding site, are also highly conserved. Asn-194 in OYE is replaced by histidine in PETN reductase and 12-oxophytodienoate reductase; however, this does not significantly alter the position of ligand binding in the structures. This substitution might account for some difference in reactivity and ligand binding within the OYE family (Strassner *et al.*, 1999). The major structural differences between the enzymes occur in the loop regions at the top of the barrel (Fig. 6). Such differences are likely to be more significant when considering the binding of larger ligands, such as the steroids and 12-oxophytodienoate.

Looking more widely across the family, it is possible to interpret primary structures in the light of the secondary and tertiary structure of OYE. As expected, regions of amino acid sequence predicted to form loops between secondary structure elements show most sequence variability. However, one loop region, corresponding to residues 206–216 of OYE, is conserved throughout the family, even in the more distant, multi-domain, homologues of OYE. This loop forms part of the dimer interface in OYE (Fox & Karplus, 1994); however, morphinone reductase has a different dimer interface (Moody *et al.*, 1999), and many other members of the family are known to be monomers. It is possible that the loop forms the binding site for an unknown protein partner. However, the size of the conserved region in the OYE family is fairly small, and is located at considerable distance from the substrate-binding site. Rather than providing a binding site, this region might be critical in determining the overall fold of the protein, as it contains a Tyr-Gly-Gly-Ser motif which forms a tight turn secured by interactions with nearby residues. A similar

explanation would account for the observed conservation of amino acid residues forming the β hairpin at the base of the barrel structure.

Towards an appreciation of the true physiological roles of the enzymes

Application of the standard strategy knockout mutagenesis to the study of OYE gene function was initially frustrated by the presence of two paralogous genes in *S. cerevisiae*. A double knockout mutant (Δ OYE2: Δ OYE3) has been reported (Brown & Massey, 1999). Unfortunately, no phenotypic difference from wild-type *S. cerevisiae* could be found. *S. cerevisiae* is the focus of many large-scale functional genomics studies at present. These have suggested a number of tentative functions for OYE, from assembly of the yeast cytoskeleton (Amberg *et al.*, 1995) to oxidative stress response (Lee *et al.*, 1999).

The high degree of conservation of regions of primary and tertiary structure across the family, at least within the 'close' family of single-domain yeast, plant and Gram-negative bacterial enzymes, would suggest that the enzymes are orthologous. If this is the case, the conserved functional role is still to be discovered. The suggestion of a role in the general detoxification of a broad spectrum of electrophilic molecules is attractive; however, it is not clear why a 'general purpose' enzyme should resist evolutionary change. Small α/β -unsaturated carbonyl compounds are substrates for all family members; however, activity towards these compounds varies considerably [e.g. 2-cyclohexenone: OYE has a $K_m = < 10 \mu\text{M}$, and $k_{\text{cat}} = 4.2 \text{ s}^{-1}$ (Brown *et al.*, 1998); morphinone reductase has an apparent $K_m = 5.5 \text{ mM}$, and an apparent $k_{\text{cat}} = 0.67 \text{ s}^{-1}$ (French *et al.*, 1998)].

Plant homologues of OYE are increasingly implicated in the metabolism of larger lipid molecules with α/β -unsaturated carbonyl functionality (Schaller & Weiler, 1997; Strassner *et al.*, 1999). These compounds are made during insect attack (Farmer, 2001), and, perhaps more interestingly, during bacterial pathogenesis (Vollenweider *et al.*, 2000). The presence of an extremely similar enzyme in both the plant pathogen *P. syringae* (Rohde *et al.*, 1999) and the host plants raises the possibility of horizontal transfer of a detoxification enzyme from host to enemy.

Biotechnological applications of the enzymes

Enzymes from the OYE family catalyse a range of oxidations that are of potential industrial importance. A reaction of particular interest is the hydrogenation of nitroalkenes, since it involves the intermediate formation of a nitronate, followed by slow protonation of the intermediate to the nitroalkane. Accumulation of the nitronate can be forced by the Y196F mutation of OYE (Meah & Massey, 2000), providing the attractive prospect of biocatalytic production of a nitronate. When integrated with chemical alkylation, this could provide a

route to nitroalkanes with two chiral centres. This would be a useful alternative to the more complex transformation needed to provide the same products following complete biocatalytic nitroalkene reduction.

Whilst the OYE-related nitrate ester reductases were isolated on the basis of their ability to liberate nitrite from the high explosives PETN and GTN (Fig. 3a), it has become apparent that they might also share an ability to reduce trinitrotoluene (TNT; Fig. 3b), which is a significant environmental pollutant. *Enterobacter cloacae* PB2 is able to grow very slowly on TNT as a sole nitrogen source (French *et al.*, 1998), and incubation of *E. cloacae* PETN reductase with NADPH and TNT resulted in the conversion of TNT to a mixture of reduced products. Many flavin-containing enzymes act as nitroreductases, and hydroxylamino- and aminodinitrotoluenes are produced from TNT by PETN reductase. However, additional strongly coloured products are observed with PETN reductase (French *et al.*, 1998) and the *P. fluorescens* nitrate ester reductase (Bleher *et al.*, 1999). These coloured products arise from hydride addition to the aromatic ring, yielding hydride- and dihydride-Meisenheimer complexes (Vorbeck *et al.*, 1998). Thus at least some nitrate ester reductases appear to reduce both the nitro groups and the aromatic ring of TNT. Nitrite is formed by the action of these nitrate ester reductases on TNT (French *et al.*, 1998), but the nature of other end products and the mechanism of their formation is as yet unknown. Phytoremediation, the use of plants to remediate contaminated land, is the subject of considerable research at present. Plants are fundamentally self-sustaining, and transpire large volumes of groundwater. Phytoremediation is therefore an attractive option for the remediation of large expanses of land and groundwater containing moderate concentrations of explosives. The success of such an approach relies upon the ability of the plant to metabolize the target compounds. Genetic modification of plants could permit the combination of the metabolic abilities of soil microbes with the high biomass and deep root systems of plants. PETN reductase has been introduced into tobacco plants (French *et al.*, 1999), and the resulting transgenic lines show considerably increased tolerance to GTN and TNT in growth media.

Morphine and its derivatives provide some of the most potent analgesic compounds in clinical use today. Small changes in the chemical structure of the morphine alkaloids can significantly alter the pharmaceutical properties of the drug. The semi-synthetic opiates are prepared by chemical modifications from the naturally occurring alkaloids morphine, codeine and thebaine isolated from the opium poppy. These processes are often difficult to achieve due to the complexity of the molecules and the abundance of functional groups. There has therefore been considerable interest in the use of biocatalysts to produce semi-synthetic derivatives (Rathbone *et al.*, 2001). As shown in Fig. 3(c), morphinone reductase catalyses the reduction of morphinone and codeinone to the potent analgesics hydromorphone and hydrocodone, respectively (French &

Bruce, 1994). Coexpression of morphinone reductase with a morphine dehydrogenase in *E. coli* resulted in efficient biocatalysts that transformed morphine and codeine to hydromorphone and hydrocodone, with high yields (Boonstra *et al.*, 2001).

Conclusions

Despite the failure to solve the mystery of OYE's physiological oxidant, the wealth of work on the OYE family of proteins has enhanced understanding of flavoenzyme catalysis. Comparisons of members of the OYE family show that there is considerable variation in the activities of the enzymes towards a diverse range of substrates. It is unclear whether such variation arises from the use of unphysiological substrates, which might be expected to reveal differences between enzymes that are irrelevant to the true physiological role. Alternatively, the differences observed could result from a genuine divergence in metabolic function between organisms. With a view towards enzyme engineering, it is clear that residues at some distance from the active site are involved in the functional differences between the family members. Targeting random mutagenesis to loop regions at the top of the barrel could be very productive, particularly when attempting to improve the binding of bulky substrates. The key role for such loops in substrate recognition by α/β -barrel proteins has been recently highlighted by a directed evolution study (Altamirano *et al.*, 2000). It is likely that for certain substrates, the redox potential of the flavin or the degree of semiquinone stabilization could be highly significant. Alteration of the protein scaffold of OYE has been shown to alter the flavin redox potential (Xu *et al.*, 1999), and the enzyme has been converted to a desaturase by substitution with modified flavins (Murthy *et al.*, 1999). The difficulties of engineering a redox enzyme with a common binding site for substrates of both half-reactions should not be underestimated – both substrate binding and the control of flavin redox potential involve a compromise between the demands of the two half-reactions. However, it is clear that the diverse range of reactions catalysed by the OYE family will ensure an active research field for many years. The long-term benefit of this work should hopefully manifest itself in providing novel biocatalysts for the production of fine chemicals, pharmaceuticals and environmental biotechnology.

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