FLAVOPROTEIN STRUCTURE AND MECHANISM 8

Structure-function relations for old yellow enzyme

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The old yellow enzyme of yeast is the first discovered and purified flavoprotein, with a history of more than 60 years. Its peculiar name is part of this history; when it was first discovered it was called "das gelbe Ferment"; when a few years later another yellow enzyme was isolated from yeast and called "das neue gelbe Ferment," the first was renamed the old yellow enzyme, a name that has stuck because its physiological function is still unknown. However, much is known about the physical and chemical properties of this abundant protein, including its facility for forming beautifully colored charge transfer complexes with a wide variety of phenolic compounds. The enzyme is encoded by at least two separate genes in both brewer's bottom yeast and in *Saccharomyces cerevisiae;* one of these gene products, OYE1, from brewer's bottom yeast has been characterized structurally at 2 Å resolution. This article summarizes the information available from the crystal structure and correlates it with information from solution studies of ligand binding and catalytic properties.

Vincent Massey, Coordinating Editor

ABSTRACT The past 5 years have seen tremendous progress in our knowledge of old yellow enzyme (OYE) as a number of OYEs have been cloned and expressed, a high-resolution crystal structure has been determined for one of these, and new substrates have been found that can be turned over by the enzyme. Together these studies do not yet define the physiological role of OYE, but they lead to significant new insights into the enzymatic properties and structure-function relations of OYE. Karplus, P. A., Fox, K. M., Massey, V. Structure-function relations for old yellow enzyme. *FASEB J.* 9, 1518-1526 (1995)

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THE FIRST CHARACTERIZED FLAVOENZYME was isolated from brewer's bottom yeast more than 60 years ago (1) and called "das gelbe Ferment" (the yellow enzyme). Two years later another yeast flavoenzyme was purified and named "das neue gelbe Ferment" (2), and the enzyme of Warburg and Christian was then creatively dubbed the "old yellow enzyme" (OYE).[2] The name still sticks today because, despite extensive characterization, the physiological function of OYE remains unknown.

OYE (E.C. 1.6.99.1) from brewer's yeast has the following three characteristic features: it is a dimer of ~45 kDa subunits with one noncovalently bound FMN per subunit; it is rapidly reduced by NADPH and can be reoxidized by oxygen (NADPH-dependent oxidase activity); and it binds phenolic compounds tightly and the complexes show dramatic changes in absorption spectra, involving the development of intense long wavelength absorbance bands. Because many flavoenzymes show NADPH oxidase activity, the unusual phenol binding activity is the characteristic that is most useful for defining an enzyme as an old yellow enzyme. An "old yellow enzyme" purified from *Gluconobacter suboxydans* has many characteristics of the yeast enzymes, but the development of charge transfer bands with phenols has not been reported (3). A recent review (4) details what was known about OYE as of 1989, summarizing the results of biochemical, kinetic, spectroscopic, and artificial flavin studies performed on OYE from brewer's yeast (*Saccharomyces carlsbergensis*). This review focuses on more recent results, but some key results from those early studies are repeated here.

OYE FROM NATURAL SOURCES

Extensive purification and characterization of OYE has only been carried out for the enzymes from *S. carlsbergensis*, on which most studies before 1992 were performed, and *Saccharomyces cerevisiae* (5, 6). Protein from these natural sources has been shown to be heterogeneous both in terms of chromatographic behavior and amino acid sequence. OYE purified from *S. carlsbergensis* shows several peaks in ion exchange chromatography (5, 7, 8) and isoelectric focusing (9) and at least two variants of amino acid sequence (5, 7, 10). It was also shown to be heterogeneous by nuclear magnetic resonance (NMR) studies in which the native FMN was replaced by FMN containing ^{13}C, ^{15}N, or ^{19}F at specific positions in the

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[2]Abbreviations: OYE, old yellow enzyme; NOX, NADH oxidase; CHX, cyclohexenone; NMR, nuclear magnetic resonance.

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flavin (7–9). OYE purified from *S. cerevisiae* shows two major ion exchange peaks and two amino acid sequences (5). For studies before 1976, partial limited proteolysis of OYE caused additional heterogeneity and complicated measured kinetic and binding properties (11).

CLONING AND EXPRESSION

Both to determine the protein sequence and to develop a source of homogeneous OYE, it was crucial that OYE be cloned. Using an *S. carlsbergensis* cDNA library probed with oligonucleotides based on partial amino acid sequence data, Saito et al. (12) concluded that there were two OYE genes in *S. carlsbergensis;* they isolated and sequenced one of them, which they designated *oye1*. The open reading frame encoded a 400 residue subunit of 44,890 daltons. Since then, three other OYE genes have been sequenced, designated *oye2*, *oye3*, and *kye1:* the *oye2* gene was isolated by screening a genomic library of *S. cerevisiae* using the *oye1* gene (5); the *oye3* gene was isolated in a similar manner after it was discovered that an *oye2* deletion mutant still produced a protein with the properties of OYE (6); and the *kye1* gene was recently found in the genome of *Kluyveromyces lactis* (13). Although the properties of the Kye1 protein have not been reported, the very high sequence similarity allows it to be tentatively classified as on OYE. The *oye2* gene was shown to be on chromosome 8 of *S. cerevisiae* (14). Oye1, Oye2, and Oye3 have been expressed in *Escherichia coli* to yield an abundant source of homogeneous enzyme. These expression systems yield nearly 30 mg/l culture. The N-(*p*-hydroxybenzoyl)-aminohexylagarose affinity column developed for rapid purification of OYE from brewer's yeast (11) also works well for purifying the recombinant protein. The four known OYE sequences are shown in **Fig. 1,** and their level of sequence similarity is shown in **Table 1.**

An additional gene, *ebp1* (for estradiol binding protein), which encodes a protein with 41% amino acid sequence identity to Oye1, was cloned as the major steroid binding protein of *Candida albicans* (15). Given the similarity to OYE, the authors showed that when the protein was expressed in *S. cerevisiae*, cytosolic fractions showed significant NADPH-dependent oxidase activity that was stimulated by 2-cyclohexen-1-one and inhibited by the steroid β-estradiol. Although this shows that Ebp1 has many properties in common with OYE, it cannot yet be classified as an OYE because it has not yet been shown whether Ebp1 binds phenols to give characteristic absorption changes. Another recently reported enzyme with OYE-like properties is the morphinone reductase isolated from *Pseudomonas putida* M10 (16). This protein is a dimer of 41.1 kDa subunits that binds FMN and exhibits NADPH-dependent reduction of morphinone and codei-

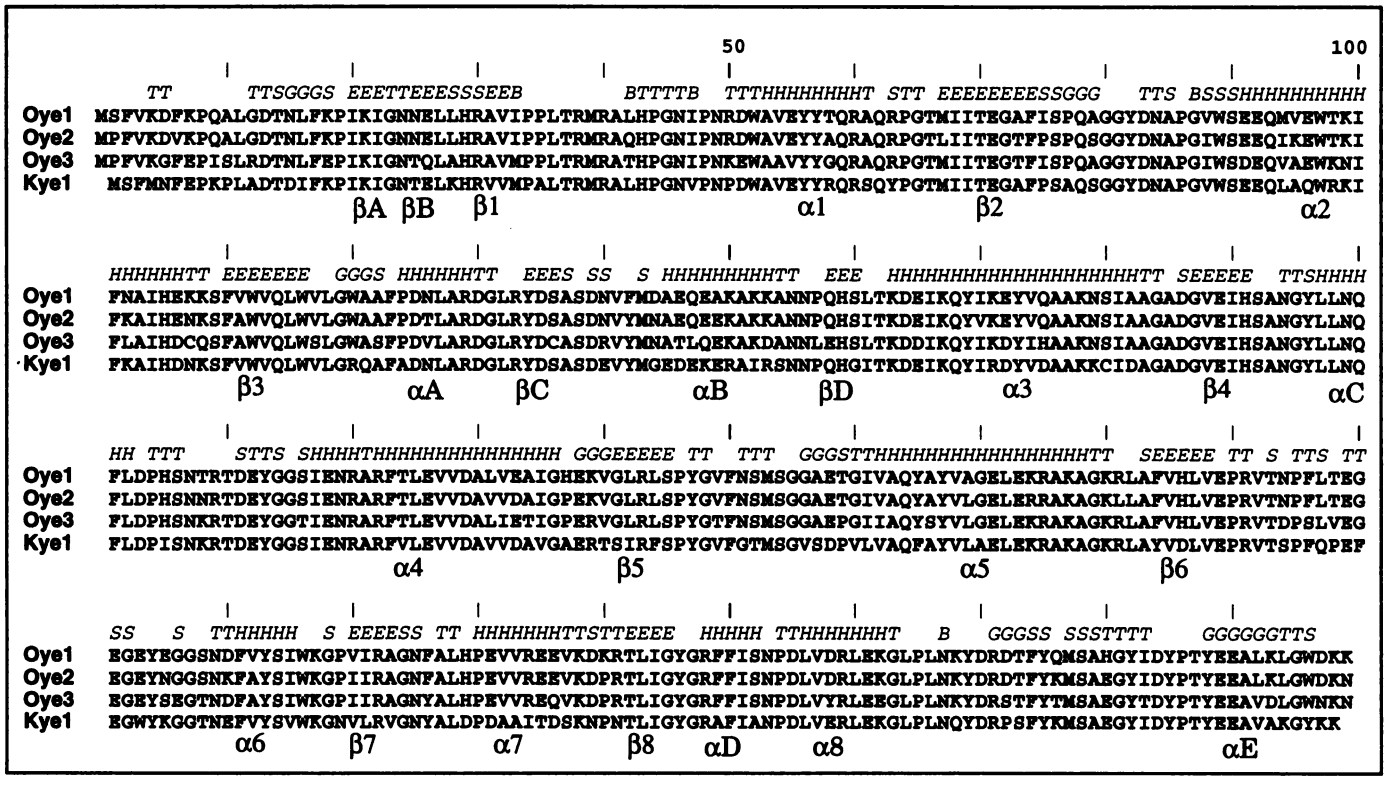


Figure 1. Amino acid sequence alignment for known OYEs. The sequences are shown for Oye1 from *S. carlbergensis*, Oye2 and Oye3 from *S. cerevisiae*, and Kye1 from *Kluyveromyces lactis*. Modified secondary structure assignments out of DSSP (38) are included in italics above the sequence for Oye1: abbreviations are E (β-sheet), H (α-helix), G (3_{10}-helix), T (H-bonded turn), S (bend or non-H-bonded turn). Short-hand names for each secondary structural element are shown in boldface below the last sequence. Hash marks are given every 10 residues. For Oye1 the initiator methionine residue is positioned as residue number 0, because the mature protein begins with the serine residue.

TABLE 1. *Amino acid sequence identities among possible OYEs*

Oye1	100				
Oye2	91	100			
Oye3	80	81	100		
Kye1	71	71	67	100	
Ebp1	41	43	43	43	100
	Oye1	Oye2	Oye3	Kye1	Ebp1

none. It also uses 2-cyclohexen-1-one as a substrate and is inhibitable by steroids. Sequence data will be required to more clearly define its relation to OYE.

HETEROGENEITY CLARIFIED

The homogeneous recombinant Oye1 and Oye2 preparations could be compared with the respective heterogeneous proteins purified from the natural sources to explain the observed natural heterogeneity. Both the chromatographic and sequence heterogeneity can be explained by assuming that two OYE isozymes in each species form both homodimers and heterodimers which are in a dynamic equilibrium. The $t_{1/2}$ values for the recombination at 4°C are on the order of 15 days. The chromatographic (5) and amino acid sequence (5, 7, 10) properties of brewer's yeast OYE imply that a second OYE gene exists in *S. carlsbergensis* that has not yet been isolated and is not identical with *oye2* or *oye3*. In addition to the formation of heterodimers within a species, Stott et al. (5) showed that mixing the Oye1 and Oye2 proteins (which come from different yeast species) in vitro led to a high yield (~70%) of the "unnatural" Oye1:Oye2 heterodimer.

CRYSTAL STRUCTURE OF OYE

Natural OYE from *S. carlsbergensis* would only yield reproducible crystals after the protein had been subjected to limited proteolysis; however, recombinant intact Oye1 yielded crystals diffracting to beyond 2 Å resolution (17). The structure of oxidized Oye1 has been solved at 2 Å resolution and refined to an R- factor of 17.9% (18). The structure shows that OYE folds into a single domain based on a $(\beta/\alpha)_8$ barrel (or TIM barrel) with the FMN binding within the barrel near the carboxy-terminal ends of the β-strands. **Figure 2** shows two views of the structure. The exact positions of the secondary structural elements are given along with the sequence in Fig. 1.

Nine secondary structural elements exist in addition to those of the main barrel: four β-strands and five α-helices. Two strands form an anti-parallel ββ-supersecondary structure that covers the base (amino-terminal end) of the barrel; the other seven elements decorate the carboxy-terminal end of the barrel (Fig. 2), forming some segments that help bind the flavin and contribute to a ligand binding pocket. The overall dimensions of the monomer are approximately 60 Å in diameter and 45 Å in height. The dimer is built through the association of helices α4, α5, and α6 of the TIM barrel with their own symmetry mates across a crystallographic twofold axis, which runs tangent to the surface along the equator of the barrel. This places the active sites on opposite sides of the dimer, so that in terms of function it seems that OYE can be conceptually described as a monomer.

FMN binds to OYE at the carboxy-terminal end of the TIM barrel in an extended conformation that lies roughly perpendicular to the barrel axis. Hydrogen bonds to FMN come from the side chains of Thr^{37} (to O4), Gln^{114} (to O2 and N3), Arg^{243} (to O2, O2′, and O3′), and Arg^{348} (to two phosphoryl oxygens) and from the main chains of Thr^{37} (to N5), Gly^{72} (to O4), and Gly^{324}, Asn^{325}, Phe^{326}, Gly^{345}, and Gly^{347} (to phosphoryl oxygens directly or through a water molecule). More details can be seen in Fox and Karplus (18). All of these residues are well conserved among the known OYE sequences (Fig. 1). The flavin itself is tilted about 45° from the barrel axis, with the C7 and C8 methyls being the farthest atoms from the barrel

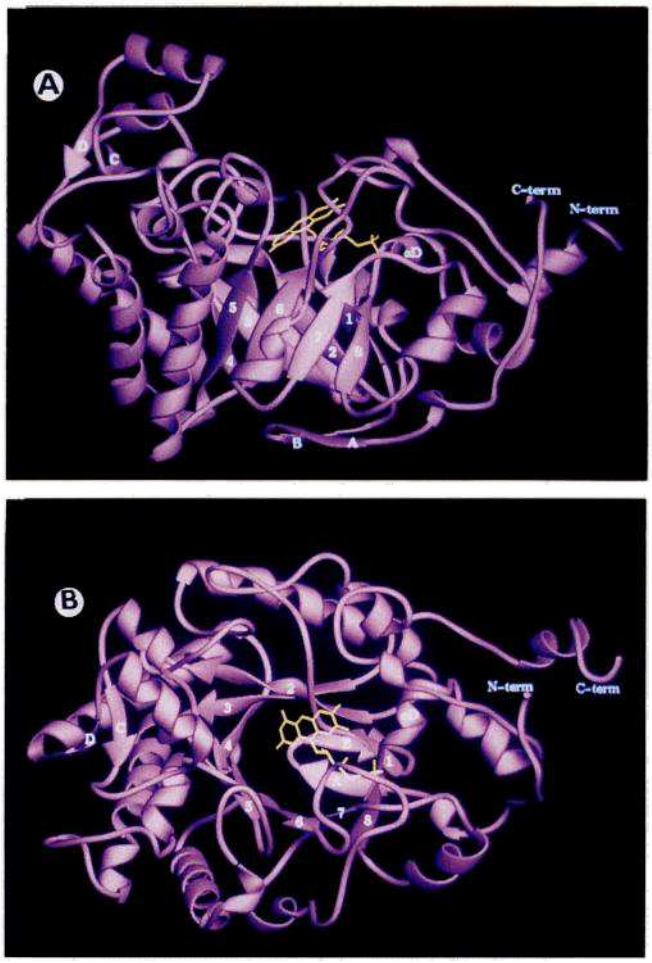


Figure 2. Ribbon diagrams of the Oye1 monomer. The protein chain is shown in violet and the FMN prosthetic group is shown in yellow. The amino- (N-term) and carboxy-(C-term) termini are labeled, as are most of the β-strands as well as αD, the helix involved in binding the FMN phosphate. *A)* A view perpendicular to the barrel axis with the carboxy-terminal end of the β-barrel pointing up; *B)* a view looking down the barrel axis. This figure and Fig. 5 were generated with Ribbons (39).

core and with the *si* face of the flavin exposed to a ligand binding pocket (see below). The structural changes seen upon reduction of OYE by 10 mM dithionite are a puckering at the N5 atom, a 15° butterfly bending of the flavin, the exchange of a chloride ion bound above the flavin for two water molecules, and a minor rearrangement of the ribityl side chain. The butterfly bending largely involves preferential movement of the less well-packed dimethylbenzene ring of the flavin. Overall, the flavin environment seen crystallographically is consistent with the results of numerous studies done with artificial flavins to characterize the enzyme-bound flavin environment (4).

The domain structure and the mode of FMN binding are highly similar to those of the flavoenzyme trimethylamine dehydrogenase (19), and somewhat less similar to the structures of glycollate oxidase (20) and flavocytochrome b_2 (21). With trimethylamine dehydrogenase, not only is the TIM barrel similar, but the placement of most of the additional secondary structural elements is also conserved. For the other two enzymes these excursions occur at different places relative to the TIM barrel. The levels of amino acid sequence similarity reflect this distinction, with trimethylamine dehydrogenase having a recognizable 24% sequence identity with OYE, and glycollate oxidase and flavocytochrome b_2 each having near 10% identity with OYE. Thus, within the FMN-binding TIM barrel family of enzymes, it is reasonable to speak of a OYE/trimethylamine dehydrogenase subfamily and a glycollate oxidase/flavocytochrome b_2 subfamily. Very few of the substrate binding residues are conserved between these enzymes and OYE, so despite their probable homology little reliable insight into the catalytic properties of OYE can be gained from these comparisons. Also, the three enzymes discussed here, other than FMN content, are not known to have any OYE-like properties.

ADDITIONAL DISTANT OYE FAMILY MEMBERS

Amino acid sequence database searches using the Oye1 sequence finds a number of proteins less similar than the other OYEs and Ebp, yet still recognizable as homologs. These include a NADH oxidase (NOX) from *Thermoanaerobium brockii* (22), a pair of proteins encoded by the C gene (BAIC) and the H gene (BAIH) of a bile acid inducible operon in *Eubacterium sp.* strain VPI 12708 (23, 24), and of course trimethylamine dehydrogenase from the bacterium W3A1 (25), for which we have already discussed the structural similarity. These proteins all have around 20–25% sequence identity with the various OYEs. The first three have not been characterized at the protein level, so their function is unknown, but as they have 30–40% amino acid sequence identity with each other, they may have more similarity in function with each other than they do with OYE.

The flavin binding residues of all of these enzymes are quite well conserved, but the most strongly conserved segment is a stretch after strand 4 of the TIM barrel in which 9/16 residues are conserved. An alignment of this segment, along with some surrounding sequence, is shown in **Fig. 3.** In OYE and TMADH, these residues form a complicated turn and part of helix 4 on the surface of the protein. The turn is about 15–20 Å from the flavin, and in OYE this segment is involved in the dimer interface. However, in TMADH this segment is not involved in such an interface, so if the segment has a conserved function it does not have to do with dimerization. An intriguing possibility is that it serves as part of a docking site for a common proteinaceous electron acceptor.

LIGAND BINDING

Studies during the past 30 years have identified many ligands that are specifically bound with millimolar to micromolar affinities to oxidized OYE. These include numerous nicotinamide dinucleotides (e.g., α-NADPH, β-NADPH, NADH) (26), simple anions (e.g., chloride, azide) (26), many phenols (e.g., *p*-hydroxybenzaldehyde, *p*-chlorophenol) (27), and, more recently, a number of steroids (e.g., β-estradiol, testosterone) (28, 29). There is strong biochemical evidence that they all bind to a common site near the flavin, as the nicotinamide dinucleotides serve to rapidly reduce the enzyme-bound FMN and the other compounds both act as competitive inhibitors of OYE reduction by NADPH and cause perturbation of the flavin spectral properties (26). None of the compounds bind well to reduced OYE (11, 27). Only a few of the best-studied ligands have had their affinities redetermined using the homogeneous recombinant enzymes, but these values agree fairly well with those reported earlier based on studies with natural OYE.

Many aromatic and heteroaromatic compounds that have an ionizable hydroxyl group (e.g., phenols) bind to OYE with micromolar dissociation constants and induce marked changes in the flavin absorption spectrum, including the development of strong absorbance maxima in the 500–800 nm range. A typical example is given in **Fig. 4** for the binding of *p*-chlorophenol. A positive cor-

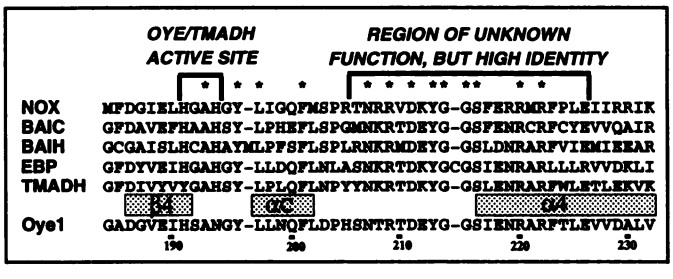


Figure 3. Amino acid sequence alignment for the OYE/TMADH subfamily members of the best conserved segment and a neighboring region containing the active site residues His191 and Asn194 of Oye1. OYE is the only member of the family that has an Asn at the position of residue 194, and as seen in Fig. 1, all of the OYE sequences have Asn at this position. All of the other subfamily members have a His residue at this position. The His could more easily act as an acid base, which suggests that OYE may catalyze somewhat different chemistry than the other enzymes in the subfamily.

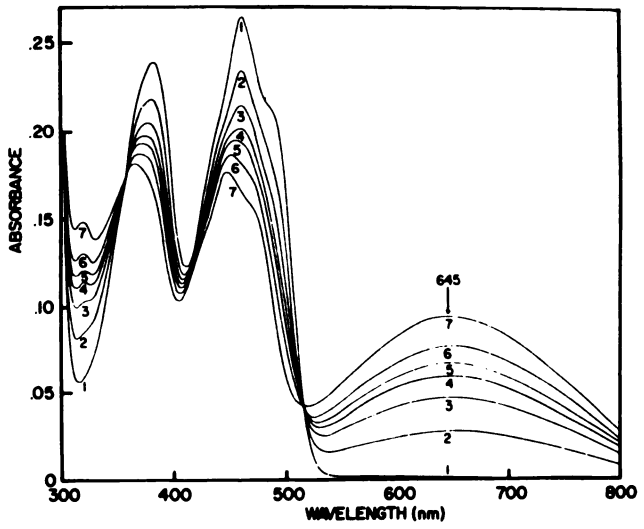


Figure 4. Titration of OYE with *p*-chlorophenol. Natural Brewer's yeast OYE at a concentration of 25 μM (curve 1) in 0.1 M phosphate, pH = 6.5, 25°C was titrated with the following concentrations of p-chlorophenol: 10.7 μM (curve 2), 21.4 μM (curve 3), 31.9 μM (curve 4), 42.4 μM (curve 5), 66.8 μM (curve 6), 700 μM (curve 7). The data are well fit with a dissociation constant of 10.8 μM. From ref 27.

relation between the energy of the long wavelength transition, as measured by its wave number, and the Hammett *para* constant for a series of *p*-substituted phenols, in addition to the pH dependence of the binding strength, provided the first experimental evidence that the long wavelength absorption bands were due to charge transfer transitions between a phenolate anion as charge transfer donor and the oxidized flavin as acceptor (27). In addition to the marked perturbation of the flavin absorbance and the long wavelength band, a perturbation of the phenolate absorbance also occurs in all cases. This is evident with *p*-chlorophenol in the spectra of Fig. 4. In addition to the long wavelength band, a new absorbance band develops at 316 nm due to the ionization of the phenol on binding to the protein. For all phenols, the extinction change observed with the enzyme-bound form is approximately the same as that expected for free phenolate, except there is a relatively constant shift in the energy of the transition to lower values, in the range of 1900–2500 cm^{-1} (30). The pH- behavior of the binding of *p*-chlorophenol and other ligands showed not only that the long wavelength absorbance requires ionization of the phenol, but that the enzyme induces the ionization, lowering the pK of some enzyme-bound phenolates by at least four pH units (27). These data also implied that a protein residue with a pK of 8.4 was involved in ligand binding and that its pK was raised to higher values on binding phenolates.

Further strong support for the charge transfer nature of OYE long wavelength bands came from studies where the native FMN was replaced by a series of artificial flavins with different redox potentials (27, 30, 31). Flavins with higher redox potential than FMN should be better charge transfer acceptors, and as was expected, they resulted in transitions of lower energy (i.e. with maxima at longer wavelength) than those with native enzyme. Consistent with this concept, enzyme containing a flavin of lower potential than that of FMN gave charge transfer of higher energy, i.e., with maxima at shorter wavelengths. As expected, the best correlation was with the first 1-electron potential of the enzyme-bound flavin, that between oxidized flavin and anionic semiquionone (31). Additional support came from resonance Raman studies, where excitation in the long wavelength band clearly showed resonances originating from both the flavin and the phenol (32–34). Flavin vibrations associated with the N(5)-C(4a) locus were particularly enhanced, suggesting that this region of the flavin is involved in the complex. The resonance Raman results describe a charge transfer interaction with significant π-orbital overlap between the bound phenolate and the pyrimidine moiety of the flavin. A similar conclusion was reached from ^{13}C and ^{15}N-NMR studies (9, 35).

The crystal structure (18) beautifully confirms the interpretation of the biochemical studies summarized above. First, the structure of the oxidized enzyme, which is stored in mother liquor containing 400 mM chloride, appears to have a chloride ion bound in a pocket just above the flavin. It is 3.5 Å from the flavin and is held by hydrogen bonds from His^{191} (3.5 Å), Asn^{194} (3.3 Å), and possibly Tyr^{196} (3.7 Å). Second, the structure determined for dithionite-reduced OYE shows that, in addition to changes in the geometry of the flavin, the major change is the dissociation of the chloride ion. Finally, structures determined for oxidized OYE crystals that have been soaked with either *p*-hydroxybenzaldehyde, β-estradiol, or (c-THN)TPN (an NADPH analog that does not reduce the flavin) show that all of these ligands displace the chloride upon binding and enter into a stacking interaction 3.2–3.5 Å from the flavin (**Fig. 5**). In all cases, His^{191} and Asn^{194} donate two hydrogen bonds to the phenolic oxygen or for (c-THN)TPN, to the amide oxygen. The phenolic oxygen interaction with two hydrogen bond donors in the plane of the ring confirms that it is bound as a phenolate ion.

Upon the binding of *p*-hydroxybenzaldehyde and β-estradiol, OYE undergoes minor conformational changes at Phe^{250}, Phe^{296}, and Tyr^{375}, but upon the binding of (c-THN)TPN a more major shift occurs for the segment, including residues 291 to 305 (18). Another striking feature of (c-THN)TPN binding is that the 2-phospho-5′-AMP portion of the dinucleotide analog appears to be disordered rather than entering into specific interactions with the enzyme. This is unprecedented in other NAD(P)H-dependent enzymes, where the AMP and/or 2′-phosphoryl portions of the ligand are very important for binding. The observation that NADPH binding seems to depend mainly on the nicotinamide helps to explain the surprising fact that the unnatural form of NADPH, the α-anomer, can also reduce the enzyme-bound flavin, and is in fact a slightly better substrate than the normal β-NADPH (26).

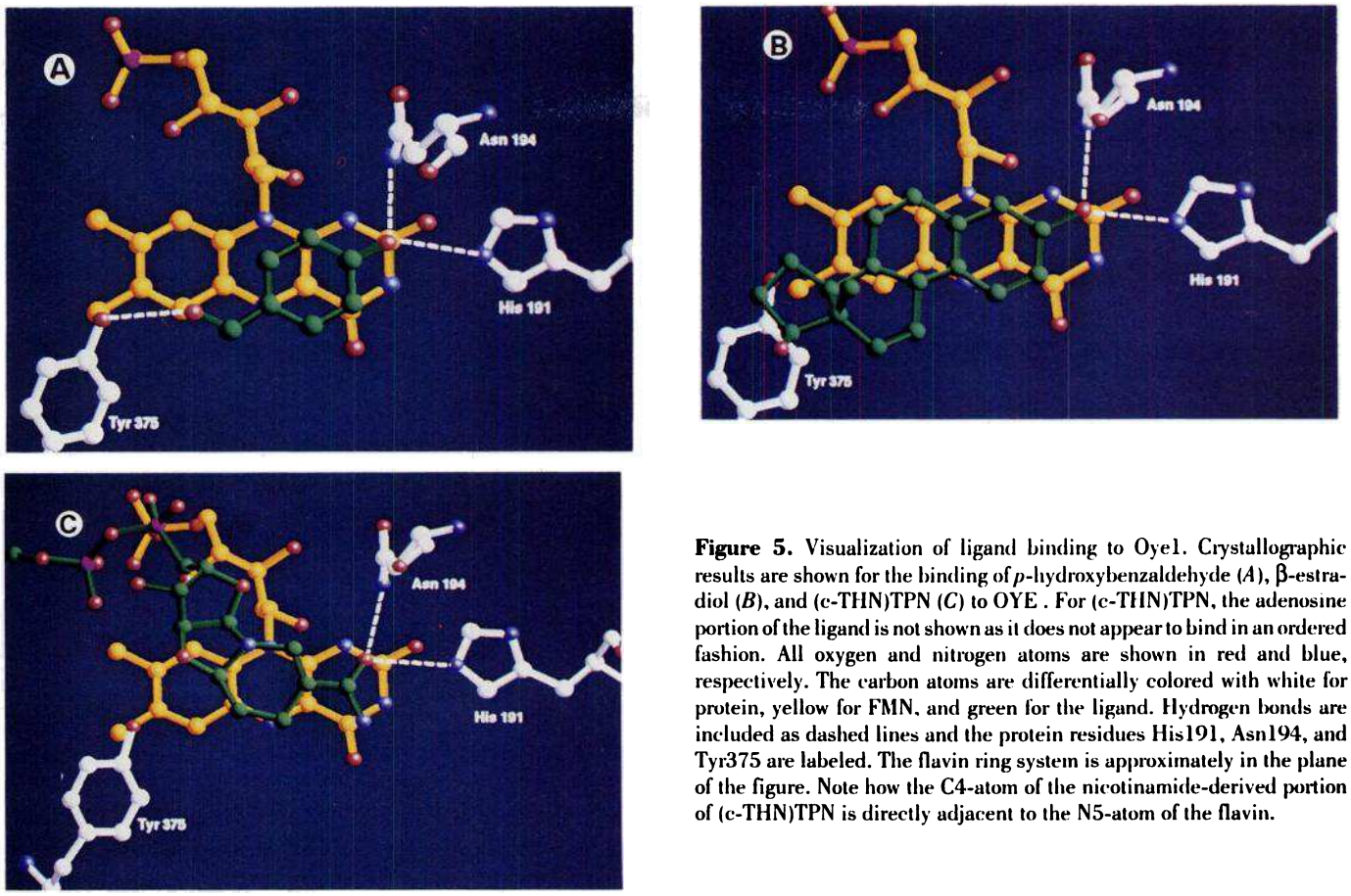


Figure 5. Visualization of ligand binding to Oye1. Crystallographic results are shown for the binding of *p*-hydroxybenzaldehyde (*A*), β-estradiol (*B*), and (c-THN)TPN (*C*) to OYE . For (c-THN)TPN, the adenosine portion of the ligand is not shown as it does not appear to bind in an ordered fashion. All oxygen and nitrogen atoms are shown in red and blue, respectively. The carbon atoms are differentially colored with white for protein, yellow for FMN, and green for the ligand. Hydrogen bonds are included as dashed lines and the protein residues His191, Asn194, and Tyr375 are labeled. The flavin ring system is approximately in the plane of the figure. Note how the C4-atom of the nicotinamide-derived portion of (c-THN)TPN is directly adjacent to the N5-atom of the flavin.

CATALYTIC PROPERTIES OF OYE

In addition to the long-known NADPH-oxygen oxidoreductase activity, OYE has been shown to catalyze the NADPH-dependent reduction of quinones (4), and more recently, the reduction of many α, β-unsaturated carbonyl compounds, such as 2-cyclohexenone, in which the olefinic bond is reduced, but not the carbonyl function (5, 29). Many of these reactions are considerably faster than the NADPH oxidase activity and are limited in rate by the reduction of the enzyme flavin by NADPH. In all cases so far studied, systematic variation of the concentrations of NADPH and the acceptor results in sets of parallel Lineweaver-Burk plots typical of a ping-pong mechanism in which the first product, $NADP^+$, leaves the enzyme before reaction with the acceptor. The ping-pong kinetics is consistent with the structural studies, which suggest that the nicotinamide and the electron acceptors bind in the same pocket, and thus cannot be bound simultaneously. Such situations, common among flavoproteins, are amenable to detailed study by rapid reaction techniques, where the reduction of the enzyme flavin by NADPH can be studied separately from the reoxidation of the reduced flavin enzyme by the acceptor. Such studies have been done with the enzyme from brewer's yeast and its separated isozymes, and with the recombinant enzymes Oye2 and Oye3 from *S. cerevisiae* (6, 26).

With all enzyme forms, reaction with NADPH under anaerobic conditions results in rapid equilibrium binding, followed by formation of long wavelength absorbance and a pronounced perturbation of the flavin absorption spectrum. The spectral changes accompanying this phase are typical of those of charge transfer complexes with NADPH as donor and oxidized flavin as acceptor (26). The K_d for binding of NADPH before development of the charge transfer band is difficult to determine accurately, but is in the region of 10 μM at pH 7.0, 25°C. Under these conditions the limiting rate of development of the charge transfer band is between 500 and 1000 s^{-1} for Oye2 and ~200 s^{-1} for Oye3, followed by reduction of the enzyme flavin at 3.9 s^{-1} for Oye2 and 18 s^{-1} for Oye3 (6). Most of the rapid reaction kinetics studies with OYE from brewer's yeast have been carried out at 4°C so that a direct comparison is not possible with the *S. cerevisae* enzymes. At 4°C the rate of formation of the charge transfer band was 21 s^{-1}, independent of NADPH concentration, and was followed by a biphasic reduction of the enzyme flavin, with approximately 60% occurring at a rate of 1.2 s^{-1} and 40% at a rate of 0.9 s^{-1}, both independent of NADPH concentration but showing a 9- to 12-fold deuterium isotope effect with [4R-^{2}H]NADPH (26). The biphasic reduction is, of course, consistent with the enzyme from brewer's yeast being a mixture of two kinds of active sites. This explanation finds support in studies with recombinant Oye1 from brewer's yeast, where the develop-

ment of the charge transfer band and the reduction are both monophasic and occur at rates of ~16 s^{-1} and 0.95 s^{-1} (reported in ref 6). The NADPH-dependent reduction of OYE thus reflects the following reaction scheme, with EFl_{ox} and EFl_{red} representing oxidized and reduced enzyme, and the form with the asterisk (*) denoting the nicotinamide-flavin charge transfer interaction:

$$EFl_{ox} + NADPH \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EFl_{ox} \cdot NADPH \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EFl^{*}_{ox}\,NADPH \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EFl_{red} + NADP^{+}$$

These studies provide convincing evidence for the binding of NADPH to the enzyme even though there is no recognizable binding site involving the AMP moiety of the pyridine nucleotide (18). The fast measurable development of the charge transfer band presumably involves the optimal positioning of the pyridinum ring of NADPH over the flavin for π-electron overlap and subsequent hydride transfer. There is no corresponding charge transfer band involving reduced flavin and oxidized pyridine nucleotide, presumably because the latter is expelled rapidly from the reduced enzyme. This would imply the existence of a positively charged residue in the vicinity of the flavin, as is also implied by the stabilization of the benzoquinoid anion form of 8-mercapto-FMN bound to the enzyme in place of the native FMN (36). However, there are only two basic residues close to the flavin: Arg^{243}, which interacts with the 2′-hydroxyl of the flavin N(10) side chain; and His^{191}, which is close to the flavin pyrimidine ring but appears to be in the neutral unprotonated form. The protonation state of His^{191} is well defined because the Nδ atom accepts a strong hydrogen bond from the peptide amide of Ala^{193}.

Studies of the oxidative reaction, i.e., the reaction of reduced enzyme with electron acceptors, are very instructive. As with many flavoproteins, the reaction with molecular oxygen is a simple second-order process, with no evidence for formation of an intermediate complex. What is particularly remarkable is how slow the reaction is: 2400 M^{-1} s^{-1} for Oye2 and only 570 M^{-1} s^{-1} for Oye3. On the other hand, reaction of reduced enzyme with cyclohexenone under anaerobic condition shows clear saturation kinetics, with a limiting rate of 73 s^{-1} and a K_d of 10 μM for Oye2. For Oye3, the limiting reoxidation rate is 20 s^{-1}; the K_d for binding of cyclohexenone to reduced enzyme is much higher—500 μM (6). The OYE-dependent reductions of O_2 and cyclohexenone (CHX) can thus be described by the following two reaction schemes:

$$EFl_{red} + O_2 \xrightarrow{k_{ox}} EFl_{ox} + H_2O_2$$

O_2 reduction

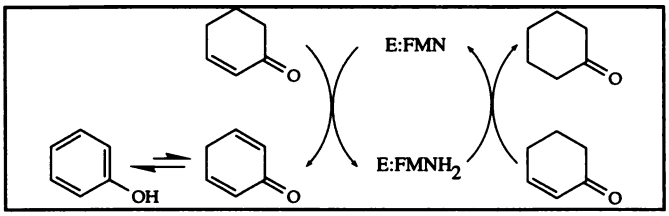
$$EFl_{red} + CHX \underset{k_8}{\overset{k_7}{\rightleftharpoons}} EFl_{red} \cdot CHX \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} EFl_{ox} + CHXH_2$$

cyclohexenone reduction

The steady-state kinetic constants, K_m and k_{cat}, are well-defined combinations of the rate constants shown in the schemes above, and the individual rate constants or ratios of selected rate constants determined by rapid reaction techniques give satisfactory fits to the experimentally determined steady-state kinetics constants (for details, see ref 6).

In addition to cyclohexenone, many α,β-unsaturated carbonyl-containing compounds have been found to be reduced by OYE. For these compounds the olefinic bond is reduced by a sequence of hydride transfer from NADPH via the FMN-N5 atom to the β-carbon and solvent proton uptake by the α-carbon (29). The NADPH-dependent reduction of the olefinic bond appears to be limited to aldehydes and ketones because α,β-unsaturated acids, esters, amides, and nitriles are not reduced. The finding that OYE catalyzes the NADPH-dependent reduction of the olefinic bond of selected α,β-unsaturated cyclic ketones suggests that this may also be the site of reduction of quinones rather than the carbonyl oxygen, as is commonly assumed for flavoenzymes.

In addition to being good electron acceptors from reduced enzyme flavin, some α,β-unsaturated cyclic ketones can also act as electron donors, resulting in reduction of the enzyme flavin and aromatization of the donor. Thus, 3-oxo-decalin-4-ene and 3-oxo-decalin-4-ene-10-carboxaldehyde are oxidized to 3-hydroxy-6,7,8,9-tetrahydronapthalene and 2-cyclohexenone is oxidized to phenol. The aromatization is stereospecific and involves the *trans*-dehydrogenation of the saturated C-C bond adjacent to the carbonyl (29). Thus, OYE can catalyze a dismutation reaction of compounds such as 2-cyclohexenone, in which one molecule is oxidized, forming phenol and reduced enzyme; the enzyme in turn is reoxidized by a second molecule of cyclohexenone, which is converted to cyclohexanone:



This reaction and the aromatization of tetrahydropyrimidines by monoamine oxidase (37) are, to our knowledge, the only examples of a flavoprotein catalyzing such an aromatization reaction.

Both the oxidative and reductive enzymatic reactions seen with the simple substrate cyclohexenone are also observed for some steroids (29), but with fascinating selectivity properties. For instance, some steroids such as testosterone undergo no reaction in the presence of OYE, whereas $\Delta^{1,4}$-androstadien-3,17-dione is selectively reduced at the Δ^{1}-double bond. Also, although 19-nor-testosterone cannot be reduced in an NADPH-dependent fashion, it is oxidized (and aromatized) to β-estradiol by OYE (29). These selective oxidation/reduction properties

are nicely explained by the structural results for β-estradiol binding, which showed that steroids bind in a unique orientation that would place the C1,C2 positions of the steroid nucleus near the redox active flavin N5-atom (Fig. 5). In the specific cases mentioned, the reducible Δ^1-double bond of $\Delta^{1,4}$-androstadien-3,17-dione, as well as the site of oxidation of 19-nor-testosterone, are near the flavin N-5, but the double bond of testosterone is near the flavin N-10 where it could not be easily reduced. The structure also helps explain why α,β-unsaturated acids, esters, amides, and nitriles are not reduced, as those groups cannot interact as appropriately with the binding pocket comprised by His^{191} and Asn^{194}.

ROLES OF SPECIFIC AMINO ACID RESIDUES IN LIGAND BINDING AND CATALYSIS

In addition to providing a framework for explaining many of the enigmatic properties of OYE, knowledge of the crystal structure of OYE permits the rational construction of site-directed mutant forms of the enzyme to determine the role of specific residues. Clearly, the first residues to be targeted include the active site residues His^{191}, Asn^{194}, Tyr^{196}, and Tyr^{375} (Fig. 5). Work of this kind has only recently begun, but has already proved to be instructive. Consistent with binding of the phenolate oxygen by hydrogen bonds from the $N_ε2$-proton of His^{191} and the $N_δ2$-proton of Asn^{194} (18), the Oye1 mutant $His^{191}Asn$ binds *p*-chlorophenol (pK_a 9.2 in free solution) with a K_d of 2.4 mM at pH 7.0 and without the development of a long wavelength absorbance band (B. J. Brown and V. Massey, unpublished results). This is some three orders of magnitude weaker than the binding to wild-type Oye1. Significantly, the mutant enzyme also fails to undergo the dismutation reaction with 2-cyclohexenone, and it has lost its ability to act as an NADPH-cyclohexenone reductase even though the NADPH-oxidase activity is unaffected. Thus, His^{191} is implicated as having an important role not only in the lowering of the phenolate pK_a of bound phenols, but in the hydride transfer reactions with α,β-unsaturated enones. Continued mutagenesis, kinetic, and structural studies promise to reveal further secrets of this enigmatic enzyme. FJ

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