On the Active Site of Old Yellow Enzyme

ROLE OF HISTIDINE 191 AND ASPARAGINE 194*

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Old Yellow Enzyme (OYE) binds phenolic ligands forming long wavelength (500–800 nm) charge-transfer complexes. The enzyme is reduced by NADPH, and oxygen, quinones, and α,β-unsaturated aldehydes and ketones can act as electron acceptors to complete catalytic turnover. Solution of the crystal structure of OYE1 from brewer’s bottom yeast (Fox, K. M., and Karplus, P. A. (1994) Structure 2, 1089–1105) made it possible to identify histidine 191 and asparagine 194 as amino acid residues that hydrogen-bond with the phenolic ligands, stabilizing the anionic form involved in charge-transfer interaction with the FMN prosthetic group. His-191 and Asn-194 are also predicted to interact with the nicotinamide ring of NADPH in the active site. Mutations of His-191 to Asn, Asn-194 to His, and a double mutation, H191N/N194H, were made of OYE1. It was not possible to isolate the N191H mutant enzyme, but the other two mutant forms had the expected effect on phenolic ligand binding, i.e. decreased binding affinity and decreased charge-transfer absorbance. Reduction of the H191N mutant enzyme by NADPH was similar to that of OYE1, but the reduction rate constant for NADH was greatly decreased. The double mutant enzyme had an increased rate constant for reduction by NADPH, but the reduction rate constant with NADH was lower by a factor of 15. The reactivity of OYE1 and the mutant enzymes with oxygen was similar, but the reactivity of 2-cyclohexenone was greatly decreased by the mutations. The crystal structures of the two mutant forms showed only minor changes from that of the wild type enzyme.

Old Yellow Enzyme (OYE; EC 1.6.99.1) is an NADPH oxidoreductase that contains flavin mononucleotide (FMN) as the prosthetic group. OYE was initially isolated from brewer’s bottom yeast (1, 2) and was the first enzyme in which a vitamin-prosthetic group. OYE was initially isolated from brewer’s bottom yeast (Fox, K. M., and Karplus, P. A. (1994) Structure 2, 1089–1105) made it possible to identify histidine 191 and asparagine 194 as amino acid residues that hydrogen-bond with the phenolic ligands, stabilizing the anionic form involved in charge-transfer interaction with the FMN prosthetic group. His-191 and Asn-194 are also predicted to interact with the nicotinamide ring of NADPH in the active site. Mutations of His-191 to Asn, Asn-194 to His, and a double mutation, H191N/N194H, were made of OYE1. It was not possible to isolate the N191H mutant enzyme, but the other two mutant forms had the expected effect on phenolic ligand binding, i.e. decreased binding affinity and decreased charge-transfer absorbance. Reduction of the H191N mutant enzyme by NADPH was similar to that of OYE1, but the reduction rate constant for NADH was greatly decreased. The double mutant enzyme had an increased rate constant for reduction by NADPH, but the reduction rate constant with NADH was lower by a factor of 15. The reactivity of OYE1 and the mutant enzymes with oxygen was similar, but the reactivity of 2-cyclohexenone was greatly decreased by the mutations. The crystal structures of the two mutant forms showed only minor changes from that of the wild type enzyme.

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Phenolic ligands bind to Old Yellow Enzyme with perturbation of the flavoprotein spectra and formation of striking long wavelength (500–800 nm) absorbance bands (6, 10). This binding is affected by substituents on the phenol and a correlation between the energy of the charge-transfer absorption, and the Hammett para constant has been demonstrated. This correlation and an associated one with the redox potential of the flavin (11) have been used as evidence that the phenolate ion is the charge-transfer donor in the enzyme-phenol complex and the isoalloxazine ring of FMN is the acceptor (5, 12).

OYE is prepared from brewer’s bottom yeast using a phenol affinity column (13). This protein preparation ran as one 45-kDa band on SDS-PAGE. Subsequent experiments demonstrated that this enzyme preparation could be separated into multiple peaks using high performance liquid chromatography (14) and FPLC (8). Amino terminal sequence determination of the FPLC peaks suggested that more than one isozyme of OYE exists and that the enzyme as prepared was a mixture of homodimers and heterodimers derived from separate genes. OYE1 was cloned from brewer’s bottom yeast, and the expressed protein coeluted with one of the bands from the FPLC separation of the isozymes. Southern blotting confirmed at least one more gene for OYE in brewer’s bottom yeast (15). Another clone of OYE was isolated from a CenA library of Saccharomyces cerevisiae. The expressed protein had 91% sequence identity with OYE1 and was somewhat displaced from any of the FPLC peaks seen with the protein from brewer’s bottom yeast (8). Deletion of the coding region for OYE2 from the genome of S. cerevisiae had no readily detectable phenotype (16), and it was possible to isolate a second OYE by affinity chromatography, which was more anionic than OYE2. This second isozyme from S. cerevisiae was cloned and designated OYE3 (17).

The cloning of the isozymes of OYE resulted in the expression and isolation of large quantities of homodimeric protein, making it possible to crystallize OYE in a form suitable for structural determination. The crystal structure of OYE1 was solved at 2-Å resolution (18), and amino acid residues that influence the binding of FMN to OYE were identified. The crystal structure was also solved with p-iophenol and p-hydroxybenzaldehyde bound to the enzyme. The phenolic ligand was positioned parallel to the si face of the isoalloxazine ring of FMN, and the phenolate oxygen was within hydrogen-bonding distance of His-191 and Asn-194. Solution of the crys...
tal structure with an NADPH analogue showed the amide oxygen of the nicotinamide ring was positioned approximately the same as the phenolate oxygen with the C-4 position of the nicotinamide ring close to N-5 of FMN, suggesting optimal positioning of NADPH for hydride transfer to the flavin (19).

In order to evaluate the effect of Asn-194 and His-191 on ligand binding and enzyme function, these residues were mutated in OYE1. His-191 was changed to Asn, Asn-194 was changed to His, and the two residues were exchanged to yield OYE1 H191N/N194H. These mutated proteins have been expressed in Escherichia coli and procedures for isolation developed. The ligand binding properties, the enzymatic properties, and the crystal structures of the variants of the mutations have been studied and compared with those of native OYE1.

**Experimental Procedures**

Aagar, yeast extract, and tryptone were from Difco Laboratories. *E. coli* BL21(DE3) strains and *E. coli* HMS174 were from Novagen, as was the plasmid vector, pET3b. The plasmid vector, pGEM3Zf(+) – *E. coli* JM109-competent cells, Helper phage R408, and T4 ligase were from Promega. Trypsin, pepsin, alkaline phosphatase, isopropyl-1-thio-β-D-galactopyranoside (IPTG), and the restriction enzymes *Kpn*I and *Apa*I were from Boehringer Mannheim. Phenylmethylsulfonylfluoride, 2-cyclohexenone, NADPH, NADH, DEAE-Sepharose, and Q-Sepharose were from Sigma. The restriction enzymes *Alu*I and *Vsp*I were from Life Technologies, Inc.

**Mutation of OYE1—Sequence alignments and restriction endonuclease mapping** was done using the GCG (Genetic Computer Group) program maintained by the Clinical Research Core of the University of Michigan. Oligonucleotides were designed to introduce a restriction endonuclease site along with a point mutation and were synthesized by the University of Michigan Molecular Biology Core Facility. The Oligonucleotide primers had the following sequence: H191N (GGTGTTGA-

AATTAGTGGCATAACGGT), N194H (GAAATCCAGTCGACACCGGTAC-GGTTACTG), and the 750-base pair *Apa*I fragment from the native OYE1 expression plasmid with the equivalent fragment from the mutated pGEM plasmid. The sequence of the mutant plasmid was confirmed by the University of Michigan Molecular Biology Core Facility using the T7 primer and three oligonucleotides designed to span the total coding region of the OYE.

Calcium-competent *E. coli* BL21(DE3) cells were transformed with the OYE plasmid for expression of the protein. Growth was in Luria Broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) containing ampicillin (50 µg/ml) at 30 °C. After 10–12 h, IPTG was added to a concentration of 0.4 mM and growth allowed to proceed for an additional 10–12 h.

Native OYE1 was isolated by a phenol affinity column as described previously (13). It was not possible to isolate the mutated proteins with this procedure, and a combination of anion exchange and gel exclusion chromatography was used. The first anion exchange column was DEAE-Sepharose, which was loaded in 40 mM Tris, pH 8.0. The second anion exchange column was a Centrprep 30 (Amicon), and dialyzed overnight against 10 mM potassium Pi, pH 8.0 containing phenylmethylsulfonyl fluoride (1% tryptone, 0.5% yeast extract, 0.5% NaCl) containing ampicillin (50 µg/ml) at 30 °C. After 10–12 h, IPTG was added to a concentration of 0.4 mM and growth allowed to proceed for an additional 10–12 h.

The extinction coefficient of protein-bound FMN was evaluated. In all cases, the spectrum of protein-bound FMN was recorded, the protein was denatured by the addition of 0.1 volume of 50% trichloroacetic acid, and the precipitated protein removed by centrifugation. The supernatant was neutralized with solid sodium bicarbonate and the spectrum determined against a reagent blank containing everything but the flavoprotein. In an alternate method, the protein was denatured by the addition of 0.05 volume of 10% SDS, with spectra recorded until no further changes occurred due to release of FMN. The extinction coefficient of the enzyme bound flavin at its wavelength maximum was calculated from the known extinction coefficient of 12,500 M⁻¹ cm⁻¹ of FMN (21).

The oxidation of the OYE1, H191N, and the double mutant H191N/N194H by NADPH was determined by stopped flow spectrophotometry, but the reduction of the H191N mutant was determined under anaerobic conditions using the diode array spectrophotometer. In all cases data were acquired at pH 7.0 in 0.1M potassium phosphate at 25 °C.

The kinetics of the reduction of the enzyme and mutants by NADPH were measured using a Cary 219 stopped flow spectrophotometer as described previously (17). Reduction of OYE1 and the double mutant H191N/N194H by NADPH was determined using the stopped flow spectrophotometer, but the reduction of the H191N mutant was determined under anaerobic conditions using the diode array spectrophotometer.

**Steady State Turnover**—Turnover of OYE1 and the mutants by NADPH and oxygen was determined by stopped flow spectrophotometry. The initial rates of reaction monitoring absorbance change at 340 nm. The turnover of OYE1 and the double mutant H191N/N194H by NADPH and cyclohexenone was also determined using the stopped flow spectrophotometer. The turnover of OYE1 and the double mutant H191N/N194H by NADPH and cyclohexenone was also determined using the stopped flow spectrophotometer. The initial rates of reaction monitoring absorbance change at 340 nm.

**X-ray Crystallographic Methods**—Single crystals of mutants H191N and H191N/N194H were grown from the same conditions used for wild type OYE1 (18), except the precipitant concentrations were: 30%PEG400 (v/v) for H191N and 28%PEG400 (v/v) for H191N/N194H. These crystals were isomorphous, belonging to space group P4_2_2_1 with unit cell parameters a = b = 143.3 Å, c = 43.0 Å for H191N, and a = b = 143.6 Å, c = 42.8 Å for the double mutant. X-ray diffraction data were collected on a Siemens SMART diffractometer. The unit cell parameters were a = b = 143.3 Å, c = 43.0 Å for H191N, and a = b = 143.6 Å, c = 42.8 Å for the double mutant. X-ray diffraction data were collected on a Rigaku RU-200 rotating anode (Cu-Kα) as described previously (18).

The data were then merged using SCAIPEPACK (24), and their qualities were evaluated using program Rmeas (25). All crystallographic refinements and difference Fourier syntheses were carried out using the program X-PLOR (26). The data were collected to 2.0 Å resolution for the starting model, with residues 191 and 194 changed appropriately, as guided by the electron density maps. These initial models were then refined against their corresponding diffraction data between 8 Å and the highest resolution limits, using conventional positional refinement and then restrained individual B-factor refinement, followed by manual adjustments guided by 2Fo - Fo (ac) and Fo - Fc (ac) maps. Several
rounds of refinement yielded the final models. The data collection statistics, crystallographic refinement statistics, and the final model quality are shown in Table I. Soaking trials with various ligands (including p-hydroxybenzaldehyde) were also carried out, but crystals were unstable in the x-ray beam and no usable data were collected.

**RESULTS**

**Mutagenesis**—The mutagenesis primers were designed to introduce restriction endonuclease sites concomitantly with the amino acid substitution. The oligonucleotide primers introduce the mutations H191N and N194H and the double mutation H191N/N194H, as well as the silent mutation, which introduced an otherwise unaltered coding region. It was established that the DNA sequence contained the 750-base pair type OYE1 (15). The mutation was introduced by replacing the H191N/N194H—

| Mutant form | Resolution limit | No. of reflections measured | No. of unique reflections | Completeness | $R_{	ext{free}}$ | $R_{	ext{free}}$ | Bond length | Bond angles |
|-------------|------------------|-----------------------------|--------------------------|--------------|----------------|--------------|-------------|-------------|
| H191N       | 2.3 | 76,546 | 20,012 | 97.1 (96.1) | 8.4 (38.3) | 9.6 (49.1) | 18.0 | 25.4 | 0.01 | 1.5 |
| H191N/N194H | 2.7 | 42,885 | 11,781 | 91.4 (82.1) | 11.7 (30.3) | 13.6 (39.1) | 19.0 | 27.3 | 0.01 | 1.6 |

* Numbers in parentheses are completeness or $R_{	ext{free}}$ values for the highest resolution bins.

The expression plasmid for the mutant proteins was constructed in pET3b, which was the expression vector for wild type OYE1 (15). The mutation was introduced by replacing the 750-base pair Kpn1-ApaI segment of the coding region with the mutated coding region. The complete plasmid was sequenced, and it was established that the DNA sequence contained the expected mutation in an otherwise unaltered coding region.

**Expression of Mutants**—E. coli BL21(DE3), E. coli BL21(DE3)pLysS, and E. coli BL21(DE3)pLysE were transformed with plasmid DNA for the expression of OYE1 and the mutants of OYE1. The expression of OYE1 and the mutant enzymes was highest when E. coli BL21(DE3) was the host and this strain was used for the expression of all OYE clones. At 37 °C some of the expressed protein was associated with the pellet of the cell lysate, but the yield of soluble protein increased at 30 °C. Time of induction with IPTG was 10–12 h with growth allowed to proceed for an additional 12 h.

There was a large fluctuation in the expression level of the different OYE mutants. H191N was expressed at levels that appeared to be equivalent to that of wild type OYE1. The double mutant H191N/N194H was expressed less efficiently, and the N194H mutant was expressed only at low levels. It is not clear if this was a result of failure to express the mutant protein or an increased lability of the expressed protein.

**Isolation of the Expressed Proteins**—Old Yellow Enzyme from brewer’s bottom yeast and the cloned isozymes of OYE are isolated using a phenol affinity column (13). Oxidized OYE binds to the phenolic ligand attached to a Sepharose support. After thorough washing to remove other proteins in the cell extract, the purified protein is eluted with a reducing buffer containing sodium dithionite. When this procedure was used with the expressed mutant proteins, they did not bind to the phenol affinity column, which was a first reflection of the fact that phenol ligand binding affinity was severely decreased.

The isolation procedure used for the mutants was a modification of the one which had been used originally to isolate OYE (10). The cell extract was first applied to a DEAE-Sepharose column and eluted with a Tris, pH 8.0 gradient (40–250 mM). The second anion exchange resin was Q-Sepharose eluted with a sodium chloride gradient (0–125 mM) as described for the separation of the isozymes of OYE from brewer’s bottom yeast on FPLC (8). The final step in the isolation procedure was size exclusion chromatography with a S-200 Sephacryl column (0.9 × 180 cm) eluted with potassium phosphate buffer (pH 7.0, 0.1 M). The yield of OYE1 using the phenol affinity column is approximately 60 mg of protein/liter of culture. Using the anion exchange columns and gel exclusion chromatography, the yield of H191N was 20–25 mg/liter of culture and the yield of the double mutant was 6–8 mg/liter. In all cases the resulting product ran as a single band on SDS-PAGE and had a ratio $A_{280}/A_{460}$ of less than 10. The yield of OYE1 N194H was so low that it was not possible to isolate pure material.

**Spectral Properties of OYE1, OYE1 H191N, and OYE1 H191N/N194H**—The spectra of FMN, OYE1, and the mutant enzymes are slightly different. Free FMN has wavelength maxima at 370 and 445 nm. These maxima were shifted to higher wavelengths when bound to all enzyme forms (Fig. 1). The extinction coefficient of the protein-bound FMN was determined for OYE1 and the mutant enzymes. Even though SDS binds to OYE, when added to a final concentration of 0.5% denaturation with complete liberation of FMN occurs over a period of 10–30 min, allowing a direct calculation of the extinction coefficient of the enzyme-bound flavin at its wavelength maximum using the known extinction of 12,500 $\text{M}^{-1}\text{cm}^{-1}$ for free FMN (21). In a second method, trichloroacetic acid was used to denature the protein and release FMN. The denatured protein was removed by centrifugation and the supernatant neutralized with sodium bicarbonate. The sample was read against a blank containing everything except the enzyme, and the concentration of free FMN was determined. The wavelengths of the absorbance maxima as well as the extinction coefficients for the protein bound FMN are summarized in Table II.

The extinction coefficient used for the mixture of OYE isozymes from brewer’s bottom yeast has been reported previously as 10,600 $\text{M}^{-1}\text{cm}^{-1}$ (4, 10). The value determined for the cloned isozyme, OYE1, is higher. This higher value for the extinction coefficient of FMN bound to OYE1 was also confirmed by titrating FMN with apo-OYE1 and following the change in absorption spectrum as the holoenzyme was formed (results not shown).

**Binding of Phenolate Ligands**—Mutation of His-191 and Asn-194 was expected to alter phenol binding and development of long wavelength charge-transfer absorbance. Both amino acid residues are within H-bonding distance of the bound phenol oxygen and would be expected to stabilize the phenolate anion involved in charge-transfer complex formation. From the crystal structure of OYE1, it is evident that His-191 and Asn-194 could stabilize the phenolate anion form of phenols by...
hydrogen-bonding (18), effectively lowering the pK\textsubscript{a} of the bound phenol by several pH units (5). Thus it was not surprising that the H191N and double mutant forms failed to give the normal intense charge-transfer bands at pH 7.0 when the pK\textsubscript{a} of the free phenol is 8.0 or greater. However, with phenols possessing a low pK\textsubscript{a}, such as pentafluorophenol (pK\textsubscript{a} 5.3), charge-transfer absorbance bands are still observed with the mutant enzymes. In Fig. 2 the binding of p-hydroxybenzaldehyde to OYE1 is compared with the binding to H191N and H191N/N194H. There is a marked decrease in the extinction of the charge-transfer complex and a decreased perturbation of the flavoprotein spectra for the mutant enzymes compared with OYE1. The absorbance maxima for the charge-transfer bands of H191N and the double mutant with p-hydroxybenzaldehyde are shifted to longer wavelengths with a decrease in extinction and a decreased phenol binding affinity. Comparison of dissociation constants for OYE1 and the two mutant forms with p-chlorophenol, p-cyanophenol, p-hydroxybenzaldehyde, and pentafluorophenol are summarized in Table III. As expected, the mutant enzymes bind all phenols tested much more weakly than OYE1, consistent with the proposed hydrogen-bonding to His-191 and Asn-194.

Catalytic Turnover with NADPH and Oxygen—Turnover measurements of OYE1, H191N, and H191N/N194H with NADPH and oxygen were carried out by measuring initial rates of NADPH oxidation at 340 nm using the stopped flow spectrophotometer as a convenient tool for monitoring the reaction at different oxygen concentrations. Enzyme at approximately 100 nM concentration was mixed with NADPH at various concentrations between 50 and 500 \(\mu M\) and oxygen at 10, 21, 50, and 100% saturation, and the oxidation of NADPH was followed at 340 nm. Plots of 1/turnover number versus 1/\([\text{NADPH}]\) resulted in a series of parallel lines with a slope close to zero for OYE1 and the double mutant and an increased slope with H191N. Secondary plots of primary intercepts versus the reciprocal of the oxygen concentration resulted in finite values of \(k\text{cat}\) for OYE1 of 165 min\(^{-1}\) and H191N of 105 min\(^{-1}\) with similar \(K_m\) values for both NADPH and oxygen. The kinetics with the double mutant (H191N/N194H) were almost second order in oxygen dependence. These results are summarized in Table IV.

Catalytic Turnover with NADPH and Cyclohexenone—Turnover of OYE1 with NADPH and cyclohexenone was determined under anaerobic conditions in the stopped flow spectrophotometer using 50, 500, and 1500 \(\mu M\) starting concentrations of cyclohexenone and 60, 120, 250, and 400 \(\mu M\) NADPH. The turnover number at saturating concentrations of both substrates was 250 min\(^{-1}\). At the lowest concentration of cyclohexenone, turnover was maximal even at the lowest concentration of NADPH tested, and increasing concentrations of

### Table II

| Absorbance peaks | Extinction coefficients (\(\mu^2 cm^{-1}\)) |
|-----------------|---------------------------------|
| \(nm\)          | \(\mu^2 cm^{-1}\)                |
| Free FMN        | 370 445 10,800 12,500          |
| OYE1            | 384 462 10,500 11,700          |
| H191N           | 387 456 11,000 11,800          |
| H191N/N194H     | 385 461 10,600 11,200          |

### FIG. 1. Flavoprotein spectra of OYE1, FMN, and H191N. Spectra of FMN (solid line), OYE1 (thin line), and H191N (short dashed line) were recorded using a Cary 3 Spectrophotometer.

### FIG. 2. Titration of OYE1, H191N, and H191N/N194H with p-hydroxybenzaldehyde. Spectra were recorded in 0.1 M potassium Pi, pH 7.0 at 25 °C. For OYE1 (17.9 \(\mu M\)) spectra shown are after the addition of 0, 2, 6, 7.9, 11.9, 15.7, 19.6, and 228.2 \(\mu M\) p-hydroxybenzaldehyde. Spectra for H191N (14.7 \(\mu M\)) were recorded after the addition of 0, 9.9, 19.8, 39.2, 67.6, 104.3, 139.4, and 219 \(\mu M\) p-hydroxybenzaldehyde. Spectra for H191N/N194H (15.2 \(\mu M\)) were recorded after the addition of 0, 9.9, 19.8, 39.2, 48.8, 86.1, 139.5, and 219 \(\mu M\) p-hydroxybenzaldehyde. In all cases the effect of increasing ligand concentration is shown by the increase in the long wavelength absorbance, accompanied by a corresponding decrease in the 450 nm region.

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Old Yellow Enzyme: His-191 and Asn-194

TABLE III
Comparison of binding of substituted phenols by OYE1, H191N, and H191N/N194H

| Ligands       | OYE1          | H191N mutant | H191N/N194H |
|---------------|---------------|--------------|-------------|
|               | $K_d$ (µM)    | $C_{T_{\text{max}}}$ (µM) | $K_d$ (µM)    | $C_{T_{\text{max}}}$ (µM) | $K_d$ (µM)    | $C_{T_{\text{max}}}$ (µM) |
| p-Cl-phenol   | 1 ± 0.2       | 645 nm (4400) | 2400 ± 120  | None                      | 620 ± 85       | None                      |
| $pK_a$ = 9.4  |               |              | $pK_a$ = 7.9 | (4500)                    |               | (1600)                    |
| p-CN-phenol   | 0.08 ± 0.03   | 576 nm       | 270 ± 20    | 622 nm (2200)             | 200 ± 25       | 588 nm (1600)             |
| $pK_a$ = 7.9  |               |              | $pK_a$ = 7.6 | (3500)                    |               | (1100)                    |
| p-Hydroxybenzaldehyde | 0.2 ± 0.1 | 585 nm       | 25 ± 4      | 599 nm (1600)             | 45 ± 5         | 589 nm (1100)             |
| $pK_a$ = 7.6  |               |              | (4000)      | (1600)                    |               |                           |
| (F)-phenol    | 0.02 ± 0.01   | 550 nm       | 77 ± 1      | 600 nm (1400)             | 120 ± 6        | 520 nm (1500)             |
| $pK_a$ = 5.4  |               |              | (4700)      | (1400)                    |               |                           |

TABLE IV
Comparison of NADPH/oxygen and NADPH/cyclohexenone turnover by OYE1, H191N, and H191N/N194H

|                     | OYE1          | H191N        | H191N/N194H |
|---------------------|---------------|--------------|-------------|
| NADPH/oxygen        |               |              |             |
| $k_{\text{cat}}^{\text{NADPH}}$ | 2.75 s$^{-1}$ | 1.75 s$^{-1}$ | Second order with [oxygen] |
| $K_{d}^{\text{oxygen}}$ | 1.8 x 10$^{-5}$ M | 3.3 x 10$^{-5}$ M |
| NADPH/2-cyclohexenone |             |              |             |
| $k_{\text{cat}}^{\text{NADPH}}$ | 4.2 s$^{-1}$ | 1.4 s$^{-1}$ | Second order with [2-cyclohexenone] |
| $K_{d}^{\text{NADPH}}$ | -9.0 µM | 5.5 x 10$^{-5}$ M |
| $K_{d}^{\text{CHX}}$ | - Very low$^a$ | 1 x 10$^{-2}$ M |

$^a$ Value indeterminable because of inhibition with increasing concentration of 2-cyclohexenone.

NADPH did not result in an increased rate of turnover. At the higher concentrations of cyclohexenone, saturation with NADPH was reached at the same limiting turnover rate, but the apparent $K_m$ of NADPH was higher, indicating inhibition by high concentrations of cyclohexenone. This is consistent with the fact that OYE has one active site in which turnover occurs by a ping-pong mechanism and that cyclohexenone also binds tightly to oxidized enzyme effectively competing with NADPH. Turnover of H191N with NADPH and cyclohexenone was too slow for determination in the stopped flow spectrophotometer. Turnover was followed at 340 nm and was measured under anaerobic conditions with a Cary 219 spectrophotometer. In previous studies with the mixture of isozymes of OYE1, H191N, and the double mutant, H191N/N194H, by NADPH were measured by stopped flow spectrophotometry. In previous studies with the mixture of isozymes from brewer’s bottom yeast, it was possible to demonstrate at least two oxidized enzyme intermediates before the reduction of enzyme-bound flavin, as shown in Scheme 1 (6, 7).

Reactive Half-reaction with NADPH—The kinetics of reduction of OYE1, H191N, and the double mutant, H191N/N194H, by NADPH were measured by stopped flow spectrophotometry. In previous studies with the mixture of isozymes from brewer’s bottom yeast, it was possible to demonstrate at least two oxidized enzyme intermediates before the reduction of enzyme-bound flavin, as shown in Scheme 1 (6, 7).

![FIG. 3. NADPH/2-cyclohexenone turnover by H191N/N194H.](http://www.jbc.org/)

After mixing H191N/N194H (13.8 µM) with NADPH (50 µM) and 2-cyclohexenone (100 µM) in the stopped flow spectrophotometer, spectra were recorded on mixing, at 30 and 90 s, and at 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 16, 19, and 23 min after mixing (shown by decreasing absorbance at 340 nm followed by flavin reoxidation after NADPH was exhausted). Turnover as a function of cyclohexenone concentration is plotted in the inset for cyclohexenone at 300, 400, 750, and 1500 µM.

The binding of OYE and NADPH took place in the 3-ms dead time of the stopped-flow instrument and could be followed by the change in flavin absorbance. The initial binding step was followed by the rapid appearance of a long wavelength absorbing charge-transfer complex of NADPH as charge-transfer donor and oxidized flavin as acceptor (steps $k_5$ and $k_3$ in Scheme 1)
zyme were calculated from reaction traces. The spectra of OYE1 are shown in Fig. 4. The rapid change of absorbance measured at 460 nm and in the 520–600 nm range for OYE1 \((k_3 + k_4)\) demonstrated that the binding of NADPH to the enzyme is concentration-dependent while the rate of reduction of the enzyme \((k_3 + k_4)\) appears to be independent of NADPH concentration at 5.1 s\(^{-1}\). In the case of the H191N mutant, both the formation of the charge-transfer complex and the reduction of the flavin varied with the concentration of NADPH. A double-reciprocal plot of NADPH concentration versus observed reduction rate constant gives a \(k_{\text{red}}\) of 5.4 s\(^{-1}\) for the H191N mutant. Both the formation of the charge-transfer complex and reduction of the flavin in the double mutant were dependent on the concentration of NADPH. The rate constant for reduction of the double mutant was approximately 15-fold higher than for OYE1 or H191N at 75 s\(^{-1}\).

Reductive Half-reaction with NADH—It had been established previously for the mixture of OYE isozymes from brewer’s bottom yeast that reduction of the enzyme by β-NADH proceeded with a limiting rate constant similar to that of OYE by β-NADPH (7). This was only marginally the case with cloned OYE1, one of the isozymes from brewer’s bottom yeast. The reduction phase with NADPH was independent of pyridine nucleotide concentration at all concentrations studied with a rate constant of 5.1 s\(^{-1}\). With NADH as the reducing agent, the observed rate constant of reduction was quite dependent on pyridine nucleotide concentration and a double-reciprocal plot gave a saturating \(k_{\text{red}}\) of 0.9 s\(^{-1}\), one-fifth that for NADPH reduction. In the case of the H191N mutant, reduction of the enzyme by NADPH was dependent on the NADPH concentration, but the limiting value of \(k_{\text{red}}\) was similar to that of OYE1. The NADPH reduction of the H191N mutant was so slow that it was more convenient to determine it under anaerobic conditions without the use of a stopped-flow spectrophotometer. The resulting \(k_{\text{red}}\) was 0.03 s\(^{-1}\), a decrease of approximately 150-fold from that of NADPH reduction of H191N. The \(K_d\) for NADPH is of the same order of magnitude as that for NADPH. The reduction of the double mutant by NADH was studied in the stopped-flow spectrophotometer and yielded \(k_{\text{obs}}\) values that did not reach saturation up to \(8 \times 10^{-4}\) M NADH. A double-reciprocal plot of the data resulted in a \(K_d\) of approximately \(5 \times 10^{-3}\) M and \(k_{\text{red}}\) of approximately 4 s\(^{-1}\). This value is higher than that for reduction of OYE1 by NADH, but \(k_{\text{red}}\) for NADPH with this enzyme form was also surprisingly higher than that found with OYE1. Results for all three enzyme forms are summarized in Table V.

Oxidative Half-reaction with Oxygen—Reduced OYE is oxidized by oxygen to yield H\(_2\)O\(_2\). The kinetics of oxidation of OYE1, H191N, and the double mutant by oxygen were determined using the stopped flow spectrophotometer and in each case found to be second order in oxygen concentration. The rate constants are similar in magnitude and are not markedly affected by alterations of the amino acid residues His-191 and Asn-194. The rate constants for OYE1, H191N, and H191N/N194H were found to be \(3.8 \times 10^5\) M\(^{-1}\) s\(^{-1}\), \(7.2 \times 10^5\) M\(^{-1}\) s\(^{-1}\), and \(6.4 \times 10^5\) M\(^{-1}\) s\(^{-1}\) respectively.

Oxidative Half-reaction with 2-Cyclohexene—Reaction of reduced OYE with cyclohexeneone yields cyclohexanone as depicted in Reaction 1 (8).

This oxidation reaction was dramatically less efficient with the mutant enzymes than with the wild type enzyme. Oxidation of OYE1 by cyclohexeneone reaches saturation at micromolar concentrations of cyclohexeneone as indicated in Fig. 5. From the double-reciprocal plot, a \(k_{\text{ox}}\) of \(10^5\) s\(^{-1}\) and a \(K_d\) of \(3 \times 10^{-5}\) M for cyclohexeneone was determined. It was necessary to use millimolar concentrations of cyclohexeneone to approach saturation in the oxidation of the H191N mutant, giving a \(K_{\text{ox}}\) of 5.6 mm and a limiting rate constant of 1.1 s\(^{-1}\). The rate of oxidation of the double mutant by 2-cyclohexeneone was also very much lower than that of OYE1 and was directly proportional to the

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**Table V**

Comparison of the reductive and oxidative half-reactions of OYE1, H191N, and H191N/N194H

|           | OYE1    | H191N   | H191N/N194H |
|-----------|---------|---------|-------------|
| **Reductive half-reaction** |         |         |             |
| With NADPH |         |         |             |
| \(k_{\text{red}}\) NADPH | \(5.1 \pm 0.1\) s\(^{-1}\) | \(5.4 \pm 0.4\) s\(^{-1}\) | \(76 \pm 6\) s\(^{-1}\) |
| \(K_d\) NADPH | <10\(^{-5}\) M | \(1.5 \times 10^{-9}\) M | 2.6 \times 10\(^{-5}\) M |
| With NADH |         |         |             |
| \(k_{\text{red}}\) NADH | \(0.9 \pm 0.08\) s\(^{-1}\) | \(0.03 \pm 0.002\) s\(^{-1}\) | \(4\) s\(^{-1}\) |
| \(K_d\) NADH | \(1 \times 10^{-4}\) M | \(5 \times 10^{-9}\) M | \(5 \times 10^{-9}\) M |
| **Oxidative half-reaction** |         |         |             |
| Oxygen |         |         |             |
| \(k_{\text{ox}}\) | \(3.8 \times 10^5\) M\(^{-1}\) s\(^{-1}\) | \(7.2 \times 10^5\) M\(^{-1}\) s\(^{-1}\) | \(6.4 \times 10^5\) M\(^{-1}\) s\(^{-1}\) |
| Cyclohexeneone | \(102 \pm 4.5\) s\(^{-1}\) | \(1.4 \pm 0.12\) s\(^{-1}\) | \(100\) s\(^{-1}\) |
| \(K_d\) cyclohexeneone | \(3.2 \times 10^{-5}\) M | \(5.6 \times 10^{-7}\) M | \(\sim 2\) cyclohexeneone |

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**Fig. 4. Reaction of OYE1 with NADPH.** OYE1 was mixed in the stopped-flow spectrophotometer with NADPH to give an enzyme concentration of 14.7 \(\mu\)M and an NADPH concentration of 150 \(\mu\)M. The initial spectrum of OYE1 is given by the continuous line. Reduction was followed at the wavelengths shown, and the spectra for the EFNM-NADPH charge-transfer complex (filled circles) and the reduced enzyme (open circles) were calculated from reaction traces.
cyclohexenone at all concentrations of cyclohexenone tested (0.1–
1.5 mM), indicating a dramatically decreased binding affinity for
cyclohexenone. The rate constants determined for the oxidative
and reductive half-reactions are summarized in Table V.

Structures of H191N and H191N/N194H—Crystal struc-
tures of the H191N and H191N/N194H variants have been
determined at 2.3- and 2.7-Å resolutions, respectively. In each
case the mutated residues are clearly defined by electron den-
sity maps. Neither variant shows global perturbation of the
protein structure; however, small but significant shifts are
observed in the active site (Fig. 6). Aside from the mutations
themselves, the most notable difference in each variant struc-
ture is the absence of the chloride ion that in the structure of
wild type OYE1 interacts with His-191 (3.4 Å), Asn-194 (3.2 Å),
and Tyr-196 (3.5 Å) and is displaced in a charge-transfer inter-
action of phenols with the flavin (18).

In the H191N structure, the Asn-191 side chain is oriented
such that the Asn-191 Oδ atom is equivalent to the wild type
His-191 Nδ atom (−0.6 Å), to conserve the hydrogen bond
donated by the main chain nitrogen of Ala-193. This places the
Asn-191 Nδ atom at the position occupied by His-191 Cε2,
where it has no hydrogen bond partners. The chloride ion
present in the wild type OYE1 is replaced by an ordered water
molecule (WAT661) in a nearby position. The shift is such that
the water molecule can form reasonable hydrogen bonds with
the side chain of Tyr-196 (3.1 Å), but makes no interaction with
either Asn-191 (6.3 Å) or Asn-194 (4.3 Å). In addition to these
changes, the isoalloxazine ring slightly relaxes toward Asn-
191, with the flavin N3 atom moving as much as 0.6 Å. This
movement fills some of the void left by the loss of the chloride
and His-191 side chain. Interestingly, at the other end of the
flavin ring, the side chain Tyr-375 shifts slightly (~0.6 Å) in
the same direction as it shifts upon ligand binding (Fig. 6).
Finally, the loop between β6 and α6 (residues 293–306) has
very weak electron density and has presumably become more
disordered in the mutant structure: the average main chain B
factor of this segment is 43 Å² for H191N, compared with 20 Å²
for the wild type.

The double mutant, H191N/N194H, has Asn-191 situated as
in the H191N variant, and His-194 is positioned similar to
Asn-194, but with two additional atoms extending its reach.
The conformation of the His-194 side chain appears to place its
Nδ atom at the position occupied by the wild type Asn-194 Oδ,
where it can hydrogen bond to WAT522 (2.5 Å), which is pres-
ent in wild type OYE1. The Ca atom is situated only about 2 Å
from the wild type WAT615 position and that water shifts 1.0
Å to a more reasonable distance of 2.9 Å. Since the additional
length of the His-194 side chain points away from the Asn-191
side chain, the N194H mutation cannot compensate for the
H191N mutation to recreate the ligand binding site unless it is
reoriented upon ligand binding. Aside from the mutated resi-
dues, most of the structural changes are similar but in general
larger than those seen in the H191N variant: the loss of the
chloride, a shifting of the hydroxyl of Tyr-375 by ~1 Å, and the
increased disorder of the loop between β6 and α6 (residues
293–306), which has even weaker electron density than that of
the H191N variant. The average main chain B factor of this
section is 51 Å², despite the fact that its overall main chain
atom B factor for the rest of the structure is even smaller than
the wild type. As in H191N, the FMN cofactor also shifts, but in
the double mutant the shift is more significant, with the di-
methylbenzene ring, the ribityl group and the phosphate all
further away from the substrate binding pocket. The C-7 atom
of the isoalloxazine ring moves by as much as 0.75 Å. The shift
of the FMN appears most directly related to the 1 Å displace-
ment of WAT615, which in turn pushes the O-3 hydroxyl of the
ribityl by 0.50 Å.

DISCUSSION

Old Yellow Enzyme is historically significant as the enzyme
in which it was first demonstrated that a vitamin-derived co-
factor is required for the catalytic function and thus is also the
first discovered member of the large family of flavoprotein
enzymes. In the last few years, many NAD(P)H oxidoreductase

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**FIG. 5. Oxidation of OYE1 by 2-cyclohexenone.** OYE1 was re-
duced by an NADPH-generating system consisting of glucose-6-phos-
phate (0.625 mM), glucose-6-phosphate dehydrogenase (5 µl), and
NADP (0.625 µM). Reduction of the enzyme was followed spectrally
and was complete at 84 min. The reduced enzyme was mixed in the stopped
reaction traces at 460 nm were used to calculate the values of k_{obs} shown.

**FIG. 6. Active site of OYE1.** Overlay of four structures around the active sites: PHB bound wild type OYE1 as the **thickest lines** (PDB entry
code 1OYB), empty wild type structure (PDB entry code 1OYA) as **medium thickness lines**, H191N as **dashed lines**, and H191N/N194H as **thin lines**.
homologues of OYE have been cloned from plant, fungal, and bacterial sources. The sequences around His-191 and Asn-194, the residues involved in orientation of the active site ligand, are compared in Fig. 7. All of the homologues have a histidine at position 191 and an asparagine or histidine at position 194. Of the homologous proteins for which function has been measured, all, like OYE1, are able to reduce a double bond α,β to an aldehyde or keto. Two of the OYE homologues for which function is known, morphine reductase from Pseudomonas putida and 12-oxophytodienoate reductase from Arabidopsis thaliana, have the physiological function of reducing the olefinic bond of an α,β unsaturated ketone. The conservation of His-191 and Asn-194 throughout the large group of proteins indicates the importance of these residues to the protein function.

Solution of the crystal structure of Old Yellow Enzyme (18) made it possible to explain the positioning of the phenol ligands, which are known to act as electron donors in forming long wavelength (500–800 nm) charge-transfer complexes with OYE (5). Fig. 6 depicts OYE1 with p-hydroxybenzaldehyde positioned parallel at 3.6 Å from the plane of the FMN ring overlaid with the structures of OYE1, H191N, and H191N/N194H. The effect of this mutation is to exchange the donor in the formation of the charge-transfer complex, a shift in the local pH of the ligand binding pocket would be expected to have the greatest effect on substituted phenols with high pKₐ values, such as p-chlorophenol, which has a pKₐ of 9.4. As expected, neither the H191N-OYE1 nor the double mutant form a charge-transfer complex with p-chlorophenol. Substituted phenols with lower pKₐ values still form charge-transfer complexes at pH 7, presumably because of binding of the predominant phenolate ion.

The crystal structure of OYE1 shows hydrogen-bonding of the aldehyde group in p-hydroxybenzaldehyde to tyrosine 375. This could explain the less severe alteration in binding affinity of p-hydroxybenzaldehyde to H191N-OYE1, since there is still one unaltered residue to position the ligand. The Kₐ for p-hydroxybenzaldehyde binding to H191N-OYE1 is 25 μM, a 125-fold decrease in binding strength compared with native OYE1, while the binding of p-cyanophenol, p-chlorophenol, and pentafluorophenol is more severely compromised.

The asparagine 194 residue also is positioned to affect the pyrimidine moiety of the isoalloxazine ring. The amide nitrogen of Asn-194 is 3.3 Å from N-1 and 3.5 Å from C-2–O of the flavin. These distances are not altered greatly by p-hydroxybenzaldehyde binding (18). It is a surprising result that replacement of Asn-194 with His results in the expression of a protein that is difficult to isolate, especially since some of the OYE homologues are able to accommodate two histidine residues in equivalent positions and since the double mutant H191N/N194H could be isolated. It appears that FMN is not bound well to the N194H variant. The structure of the double mutant clearly shows that the N194H mutation disturbs FMN binding by pushing the ribityl side chain. We speculate that the impairment is partially offset by the H191N mutation, which provides room for the FMN to shift in a way that can relax the strained interaction between His-194 and the ribityl. Other factors such as electrostatic effects and the binding of water (38) may also contribute to the instability of N194H, but no clear evidence for this is visible in the available structures.

The general trend toward longer wavelength charge-transfer complex absorbance is not seen with the double mutant (H191N/N194H). The effect of this mutation is to exchange the two amino acid residues involved in phenolate anion stabilization, resulting in the same amino acid residues being present. However, as the crystal structures show, the residues are in
The amount of the probability that a charge-transfer transition will occur. For both the H191N and the double mutant, this value is decreased for all the ligands that form charge-transfer complexes. This is probably an indication that the highest occupied molecular orbital of the donor and the lowest unoccupied molecular orbital of the acceptor are not as favorably oriented in the mutants as in the native OYE1, again consistent with the importance of these residues in the interaction of the ligand with the enzyme-bound flavin.

The steady state turnover of NADPH with oxygen was not greatly affected by the H191N mutation, a result consistent with the similar values of $k_{\text{cat}}$ and $k_{\text{m}}(O_2)$. The $k_{\text{cat}}$ for the NADPH/oxygen turnover was 64% that of OYE1, and the effect was considerably greater than the corresponding values for NADPH. At various oxygen concentrations, a series of parallel Lineweaver-Burk plots resulted similar to that previously found for all OYE preparations, showing a small dependence on NADPH concentration at all experimentally available NADPH concentrations. The double mutant also had a small dependence on NADPH concentration similar to that of native OYE1, but for this mutant enzyme the secondary plot of intercept versus the reciprocal of the oxygen concentration of the parallel Lineweaver-Burk plots was second order with respect to oxygen concentration over the experimentally available range of oxygen concentrations. Again, this result is consistent with the values of $k_{\text{cat}}$ and $k_{\text{m}}$ (Table V), since the $K_m$ for $O_2$ is given by $k_{\text{cat}}/k_{\text{m}} = 1.18 \times 10^{-2}$ M (17), a concentration greatly in excess of the solubility of oxygen in water.

Turnover of NADPH with cyclohexenone was strongly compromised by the H191N mutation. While the NADPH dependen-

dence of the reaction was not greatly altered, the effectiveness of cyclohexenone as an electron acceptor was greatly reduced. Proper alignment of cyclohexenone to receive a hydride from N-5 of the flavin at the $\beta$-position of the unsaturated carbonyl compound requires that His-191 interact with the carbonyl oxygen in much the same way as the $\beta$-hydroxybenzaldehyde phenolate oxygen interacts with His-191 (9, 18). If the mutation to Asn results in the lack of a hydrogen-bonding entity to interact with the oxygen, orientation of cyclohexenone would not be as well controlled by the mutant and loss of optimal hydrogen bonding would result in weaker binding, as found experimentally. NADPH/cyclohexenone turnover with the double mutant is also decreased seriously at most concentrations of NADPH and cyclohexenone. Over the range of cyclohexenone concentrations tested, the steady state turnover of NADPH by the double mutant was directly proportional to cyclohexenone concentration.

Study of the oxidative and reductive half-reactions was done independently by rapid reaction techniques. Reduction of native OYE1 and of the H191N mutant enzyme by NADPH were similar. NADPH is thought to align parallel to the isooxazoline ring of FMN with the amide oxygen positioned similarly to the oxygen atom of the phenolate ligand and C-4 of the nicotinamide ring properly orientated for hydride transfer to N-5 of FMN (18, 19). It would be expected that His-191 would have an orienting function and its replacement by another amino acid residue would alter the efficiency of hydride transfer, but this does not seem to be the case. Other forces such as $\pi-\pi$ interactions between the isooxazoline ring and the nicotinamide ring and the contribution of other amino acid residues to the binding of NADPH must be adequate enough that a noticeable difference in reduction rate does not occur. In addition, as proposed by Fox and Karplus (18), the binding of NADPH involves significant rearrangement of the loop between B6 and A6 (residues 290–310), which makes extensive interactions with NADPH. Interestingly, in both mutant structures this loop becomes more flexible, and the change in its mobility may have an enhancing effect on NADPH binding, compensating for the loss of other interactions. The formation of the transient FMN-NADPH charge-transfer complex was observed during the re-
duction of the H191N mutant as it has been for all wild type forms of OYE. The formation of the charge-transfer complex of NADPH with OYE1 occurred with a rate constant of 350 s$^{-1}$ and was dependent on the concentration of NADPH ($k_2/k_1 \sim 100 \mu M$) while the reduction of the enzyme-bound FMN was independent of NADPH concentration ($k_3 + k_4 = 5.1 s^{-1}$; $k_4$ small but indeterminate). The rate constant for formation of the charge-transfer transition between the oxidized H191N mutant and NADPH was lower, approximately 70 s$^{-1}$, and there was a dependence on NADPH concentration for both formation of the charge-transfer complex and subsequent reduction of the enzyme. The loss of an orienting residue in the active site of OYE could result in a decrease in the rate of the forward reaction, resulting in a situation where $k_3$ and $k_4$ in Scheme I are approximately equal. Because of the resulting two-step mobile equilibrium preceding flavin reduction, the observed dependence of the reduction of the H191N mutant on the NADPH concentration would be explained ($K_{eq}$ overall = $k_{4}/k_{3}/k_{1}/k_{2}$).

It is surprising that the double mutant, H191N/N194H, is reduced by NADPH with a rate constant that is so much greater than that for native OYE1 or H191N. The reduction is so fast that no formation of a charge-transfer complex between the oxidized enzyme and NADPH was discernible in the time frame of the stopped-flow experiments. To explain this, we suggest that the exchange of the two amino acid residues alters the orientation of the flavin (as seen in the structure) and the NADPH to more effectively align the C-4 of the NADPH nicotinamide and the N-5 of the FMN for hydride transfer. This surprising 15-fold increase in the rate of FMN reduction suggests that wild type OYE1 is not optimized for this process alone. In retrospect, this conclusion makes sense because the single active site pocket of OYE is designed to carry out two different redox reactions; the NADPH-dependent reduction of the FMN and the FMNH$_2$-dependent reduction of an enone (for which 2-cyclohexenone is an analog), and thus it cannot be fully optimized for either reaction. Indeed, the mutations enhancing the rate of the first redox reaction have highly detrimental effects on the second redox reaction so that overall function is diminished.

Another surprising result of the comparison of OYE1 with the two mutant enzymes was that the rate of reduction of the mutant enzymes by NADH was so much lower than that by NADPH. For the mixture of isozymes from brewer’s bottom yeast, the rate constant for reduction of the enzyme by NADH was approximately the same as that for NADPH. For the OYE1

isozyme the rate constant for reduction by NADH is approximately one-fifth that with NADPH, but reduction rate constants of the mutants are decreased more significantly by replacing NADPH with NADH. For the H191N mutant the decrease in rate constant is approximately 180-fold, and for the double mutant the rate decrease is approximately 20-fold. Although there is no apparent binding pocket for the additional phosphate on NADPH, as is usual for enzymes that are specific for NADPH(18), it is probable that the additional phosphate of NADPH interacts with the protein. The crystal structure with an analogue of NADPH in the active site showed the nicotinamide ring positioned such that the amide oxygen was oriented toward His-191 and Asn-194 with C-4 positioned adjacent to N-5 to FMN, but no fixed location for the remainder of the molecule was determined (18). The additional phosphate on NADPH is some distance from the functional nicotinamide ring and the N-5 position of FMN which receives the hydride from NADPH, so the functional difference could be due to the cumulative effect of mutation of the His-191 and Asn-194 amino acid residues and the loss of the additional phosphate on binding of the NADH cofactor to the enzyme.

Oxidation of the reduced OYE by oxygen was unaffected by the mutations, as demonstrated by oxidation rate constants that are approximately equivalent. This is not surprising since accessibility to the active site for the oxygen molecule should be unaffected by the mutations. Oxidation of reduced OYE by cyclohexenone would be affected by the orientation of this electron acceptor in the active site. Presumably the carbonyl oxygen of cyclohexenone is positioned much like the phenolate oxygen of the phenolic ligands and would therefore be greatly affected by a change in the amino acid residues which would orient the molecule in the active site. This orientation positions the β-carbon of the double bond of the substrate adjacent to the N-5 position of FMN from which hydride transfer can occur. Hydride addition to the β-carbon would result in an enolate anion form, which could be stabilized by hydrogen bonding to His-191 and Asn-194. Evidence that the α-hydrogen is derived from solvent via the adjacent tyrosine residue, Tyr-196, is presented in the accompanying paper (39). If the orienting residues are changed, the oxidation reaction would be expected to be altered, as is found. The oxidation of the single mutant, H191N, by cyclohexenone is more strongly compromised than is the oxidation of the double mutant enzyme. This effect is also reflected in the decreased affinity of H191N for binding of cyclohexenone.
On the Active Site of Old Yellow Enzyme: ROLE OF HISTIDINE 191 AND ASPARAGINE 194

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