The Oxidative Half-reaction of Old Yellow Enzyme

THE ROLE OF TYROSINE 196*

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Tyrosine 196 in Old Yellow Enzyme (OYE) was mutated to phenylalanine, and the resulting mutant enzyme was characterized to evaluate the mechanistic role of the residue. The residue demonstrates little effect on ligand binding and the reductive half-reaction, but a dramatic slowing by nearly 6 orders of magnitude of its oxidative half-reaction with 2-cyclohexenone. Observation of the oxidative half-reaction with a series of substrates allows us to propose a model describing the mechanism of the oxidative half-reaction. In addition, the curtailed reactivity with enones allows for characterization of the manner in which reduced enzyme primes the substrate for the redox reaction by observation of the Michaelis complex with reduced enzyme bound to substrate.

Old Yellow Enzyme (OYE1, EC 1.6.99.1), revealed as the first protein requiring the use of an FMN prosthetic group by Theorell (1), opened a new chapter in the history of enzymology by unveiling the significance of small organic molecules in catalysis. Despite the enzyme’s antiquity, the physiological function of OYE has persisted as an unsolved question. OYE, originally isolated from brewer’s bottom yeast (2), has been shown to be a heterogeneous mixture consisting of several isoforms (3). The enzyme exists as a dimer of 49-kDa subunits containing one non-covalently bound FMN per subunit (4).

The gene for the isoform OYE1 from Saccharomyces carlsbergensis has been cloned (5), providing the opportunity to apply molecular biology techniques to probe enzyme function. In addition, a crystal structure of OYE1 has been solved at a resolution of 2.0 Å, revealing several residues likely involved in catalysis and ligand binding (6–8). The enzyme exists as a dimer of 49-kDa subunits containing one non-covalently bound FMN per subunit (4).

The role of tyrosine 196 (Tyr-196) in the protonation reaction. In addition, Y196F OYE1 is mutated to phenylalanine (Y196F) mutation of OYE1 was constructed. The reduction of the substrate double bond is demonstrated to consist of two phases, hydride transfer and proton uptake, which are coupled to varying degrees depending on the identity of the substrate. Kinetic characterization of the mutation with a variety of substrates reveals the role of Tyr-196 in the protonation reaction. In addition, Y196F OYE1 is used to probe the manner in which the enzyme primes the substrate for the oxidative half-reaction in the Michaelis complex. The Old Yellow Enzyme demonstrates that it has much new information to reveal regarding the nature of catalysis of redox reactions.

EXPERIMENTAL PROCEDURES

Mutagenesis for Y196F OYE1—OYE1 cloned in the pET expression system as described previously (5) was used in conducting a novel method for polymerase chain reaction-based mutagenesis (16). By substituting a silent mutation introducing a restriction site, two overlapping polymerase chain reaction fragments containing the desired mutation are fused and mutant fragment subcloned into the wild type vector. The generation of the mutation is confirmed by screening digestion for the incorporation of a CfoI restriction site encoded by the introduction of a silent mutation in the mutagenic primer and by sequencing with automated fluorescent sequencing carried out by the University of Michigan Biomedical Research Core Facility. E. coli strain

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‡ The abbreviations used are: OYE, Old Yellow Enzyme; OYE1, Old Yellow Enzyme isoform 1; GC/MS, coupled gas chromatography/mass spectroscopy; DMACNA, 4-dimethylaminocinnamaldehyde.
FIG. 1. Catalytic cycle for Old Yellow Enzyme.

BL21-DE3 harboring the Y196F OYE1-pET plasmid was used to express mutant OYE1 by induction with 400 μM IPTG 8 h after 1% inoculation of 10 L of LB/Amp. The cells were harvested 8 h after induction. Purification of the mutant enzyme was carried out as described previously (17, 18).

Unless otherwise noted, all studies with Y196F OYE1 were conducted at pH 7.0 and 25 °C in the presence of 0.1 M potassium P,

Ligand Binding Studies—Titrations by various ligands of OYE were recorded by following absorbance changes with a Varian Cary 3 UV-visible spectrophotometer. Titrations were conducted by addition of small volumes of concentrated ligand to oxidized enzyme in 1-ml quartz cuvettes. Dissociation constants were determined by assessing from the amount of spectral perturbation the relative amounts of bound versus unbound ligand relative to unbound enzyme.

Turnover Assay—Turnover assays were conducted with NADPH as the substrate for the reductive half-reaction and various substrates for the oxidative half-reaction. The standard assay was conducted in 1 ml with 100 μM NADPH, 1 mM substrate for the oxidative half-reaction, and 10 nM enzyme. Aerobic turnover reactions were conducted at air saturation (256 μM) of O2. Turnover numbers, reported as moles of pyridine nucleotide oxidized per molecule of FMN per minute, were determined by following the rate of change in absorbance at 340 nm as a function of [NADPH] and extrapolating to a theoretical maximum for [NADPH] by fitting a double-reciprocal plot. The reaction product was generated by addition of an NADPH-generating system containing 3,4-dihydroxybenzoate and protocatechuate-3,4-dioxygenase. All buffer substrates were made anaerobic by incubation overnight with an anaerobic solution of reduced ketone or aldehyde, or benzyl alcohol and substrate solutions were made anaerobic by bubbling argon through the solution for at least 15 min before use. For the oxidative half-reaction with oxygen, solutions of various concentrations of oxygen were made up by equilibrating 0.1 mM potassium P₅ solutions by bubbling with the desired concentration of oxygen. Enzyme solutions were made anaerobic in a tonometer by alternately applying a vacuum and purging with argon. For studies of the oxidative half-reaction, reduced enzyme was generated by addition of an NADPH-generating system containing 4 mM glucose 6-phosphate, 40 units of glucose-6-phosphate dehydrogenase, and 0.03 μM NADP⁺ from a sidearm after the tonometer had been made anaerobic. Analysis was conducted by fitting data to exponential equations using the Marquardt algorithm (20) with Program A, developed by C.-J. Chiu, R. Chung, J. Diverno and D. P. Ballou at the University of Michigan.

Reoxidation of OYE by 2-Cyclohexenone—The slow oxidative half-reaction with 2-cyclohexenone was studied with the Varian Cary UV-visible spectrophotometer by taking enzyme in an anaerobic cuvette with slightly less than a stoichiometric equivalent of NADPH in one sidearm and varying concentration of 2-cyclohexenone in the second sidearm. After making the cuvette anaerobic, the enzyme was first reduced by addition of the contents of the NADPH sidearm. After a stable reduced enzyme spectrum appeared, reoxidation was subse-

RESULTS

Generation of Y196F OYE1—Purification of Y196F OYE1 was conducted by affinity chromatography with N-(4-hydroxybenzoyl)aminohexyl-agarose (18). The successful isolation of the Y196F noted by the generation of a single band on SDS-polyacylamide gel electrophoresis suggests that ligand binding interaction with the p-hydroxybenzaldehyde analog remains tight with the mutant enzyme. The expression and purification system generated Y196F OYE1 in a yield of 26 mg/liter of culture. The spectrum of Y196F has a wavelength maximum identical to that of the wild type enzyme at 462 nm (Fig. 2) and an extinction determined as described previously (21) of ε₄₆₂ = 10,700 M⁻¹ cm⁻¹.

Ligand Binding—Through site-directed mutagenesis, we seek to specifically isolate and alter the residue Tyr-196 while leaving other aspects of the system unchanged. The concern regarding undesired structural rearrangements was allayed through several lines of evidence. The interaction of Y196F with a series of phenolic ligands provides ample data regarding the nature of the FMN and conformation of the active site. Ligand binding as measured by absorbance detection as described previously (19). The system was conducted by affinity chromatography with N-(4-hydroxybenzoyl)aminohexyl-agarose (18). The successful isolation of the Y196F noted by the generation of a single band on SDS-polyacylamide gel electrophoresis suggests that ligand binding interaction with the p-hydroxybenzaldehyde analog remains tight with the mutant enzyme. The expression and purification system generated Y196F OYE1 in a yield of 26 mg/liter of culture. The spectrum of Y196F has a wavelength maximum identical to that of the wild type enzyme at 462 nm (Fig. 2) and an extinction determined as described previously (21) of ε₄₆₂ = 10,700 M⁻¹ cm⁻¹.

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flavin potential for Y196F of approximately 10–15 mV (11, 22).

**Turnover**—Initial efforts to characterize the kinetic parameters of the reaction of Y196F were attempted though analysis of the aerobic steady state turnover assays with NADPH. Assays of the wild type enzyme, conducted by following oxidation of NADPH at saturating conditions and air saturation of oxy-

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appearance of long-wavelength absorption in the course of the reaction. As with the wild type enzyme, the oxidant of choice for Y196F. When the turnover was

over was unchanged by the mutation as the enzyme fails to

Given that

TABLE I

| Ligand         | WT OYE1 | Y196F OYE1 |
|----------------|---------|-----------|
|                | K_d (μM) | K_d (μM) |
| p-Chlorophenol | 1 (4400) | 1.5 (4100) |
| p-Fluorophenol | 380 (3000) | 164 (4000) |
| p-Cyanophenol  | 0.08 (4500) | <1 (5000) |
| Pentafluorophenol | 0.02 (4700) | 532 (4700) |

* Data are from Ref. 21, except for p-fluorophenol.

kinetics of the reductive half-reaction were not responsible for the dramatic change in rates with the aerobic turnover in the presence of 2-cyclohexenone, we turned our attention toward the oxidative half-reaction. The oxidative half-reaction with 2-cyclohexenone proved to be amenable to study by monitoring the absorbance at 462 nm, corresponding to the slow appearance of oxidized enzyme, after mixing various concentrations of substrate with reduced enzyme in an anaerobic cuvette.

The reduced enzyme shows minimal absorbance in the region of oxidized enzyme. Upon mixing with 2-cyclohexenone, there is an immediate increase in absorbance with a maximum of 455 nm (Fig. 3). The initial burst in absorbance is followed by the very slow generation of a final spectrum, which is that of oxidized OYE bound to 2-cyclohexenone. The spectrum initially formed upon mixing seems deceptively like the oxidized enzyme spectrum. However, a closer examination reveals that the sharp flavin peak is replaced instead by a broader absorbance spectrum reminiscent of the charge-transfer complexes seen with oxidized enzyme and phenolic compounds. The spectrum initially observed after mixing is tentatively assigned to the formation of a complex between reduced enzyme and 2-cyclohexenone. The rate of oxidation was measured by monitoring the disappearance of the reduced enzyme complex and the appearance of the spectrum of oxidized enzyme bound to substrate. Wild type enzyme in the presence of 2-cyclohexenone typically undergoes a dismutation reaction whereby oxidized enzyme oxidizes 2-cyclohexenone to phenol, which successively forms a long-wavelength absorbing charge-transfer complex (14). The lack of the appearance of such a long-wavelength species even after following the oxidation over the period of many hours suggests that the dismutation reaction occurs at an undetectable rate with Y196F OYE1. This is consistent with the concept that oxidation of the 2-cyclohexenone would require Tyr-196 to act as an active site base and deprotonate the non-vinyllic \( \alpha \)-carbon to the carbonyl.

The oxidation of enzyme-bound reduced flavin was examined at a series of concentrations of substrate to assess the complete kinetics of the oxidative half-reaction. At all concentrations, the oxidation is seen to fit a single exponential, which represents the slow oxidation rate. The \( k_{\text{obs}} \) includes the typically rapid rate of product dissociation and binding of oxidized enzyme to excess substrate. Ligand binding and dissociation typically occur on the millisecond time scale and thus do not affect the observed rate. A double-reciprocal plot of the data gives an oxidation rate constant of \( (1.4 \pm 0.2) \times 10^{-2} \text{ min}^{-1} \) which represents nearly 6 orders of magnitude decrease in the oxidation rate constant of wild type enzyme with 2-cyclohexenone (21). The individual data points show significant scatter, which allow only an approximate estimate of the enzyme affinity for substrate in the low micromolar range.

**Oxidative Half-reaction with O$_2$**—The reaction with molecular oxygen as a substrate for the oxidative half-reaction was studied by rapid reaction methods and the results are presented in Fig. 4. The reaction is second order with respect to O$_2$ concentration. The \( k_{\text{ox}} \) value obtained for O$_2$ is double that of the wild type enzyme (Table II). Given the slowing in oxidation with 2-cyclohexenone, the increased rate with molecular oxy-
The existence of a charge-transfer complex between reduced enzyme and 2-cyclohexene is supported by the presence of the initial phase in the reoxidation by molecular oxygen in the presence of the enone. As a test of the accuracy of the model of inhibition and the value for the \( K_d \) of 2-cyclohexene generated by the model, we observed the rapid reaction mixing of reduced enzyme with low concentrations of 2-cyclohexene. With saturating enone (500 \( \mu \)M) and no molecular \( O_2 \) present, there is a dead-time increase in absorbance which remains unchanged with time. This gives a measure for a fully bound reduced enzyme complex with an extinction of 2700 M\(^{-1}\) cm\(^{-1}\). The change in absorbance to a value two-thirds between the reduced and fully bound spectra suggests that the determined \( K_d \) is reason-
ably accurate and that the dead-time absorbance increase can be confidently assigned to 2-cyclohexenone bound to the reduced enzyme. We may speculate that a similar complex to that detected with Y196F forms with wild type enzyme, but passes through an undetectable dead-time binding event due to the rapid rate of the subsequent reoxidation.

**Oxidative Half-reaction with Various Substrates**—Given that kinetic evidence suggests that Tyr-196 acts as a necessary active site acid for the reduction of 2-cyclohexenone, the effect of the residue upon other substrates was examined by observation of their oxidative half-reactions (Table II). Methyl vinyl ketone was selected as a representative straight chain ketone, cinnamaldehyde as a representative extensively conjugated aldehyde and 1-nitrocyclohexene as a member of the class of newly discovered unsaturated nitro substrates for the oxidative half-reaction. The rates were measured by following the reoxidation of the enzyme by monitoring the absorbance at 460 nm with a stopped-flow spectrophotometer. The oxidative half-reaction with methyl vinyl ketone occurs largely in the dead time with wild type enzyme, giving statistically difficult to measure rate constants. For the half-reactions with cinnamaldehyde and methyl vinyl ketone, the phase corresponding to the largest change in absorbance was assigned to the oxidation of flavin and used to determine the $k_{ox}$ and $K_d$ through a double-reciprocal plot. For the half-reactions with 1-nitrocyclohexene and oxygen, the reaction appears second order with relation to concentration of substrate up to the highest concentrations used (800 $\mu$M 1-nitrocyclohexene and 620 $\mu$M O$_2$). Across the series of substrates for the enzyme, reduced Y196F binds all substrates slightly more weakly than wild type enzyme with the exception of 2-cyclohexenone. The rate of olefinic bond reduction varies significantly across the series of compounds. In all cases with organic substrates, the oxidative half-reaction is impaired in the mutant Y196F.

**Interaction of Cinnamaldehyde Derivatives with Reduced Enzyme**—The tight binding of cinnamaldehyde to reduced enzyme without the rapid reduction of the double bond offered a unique opportunity to study the Michaelis complex for the oxidative half-reaction. The cinnamaldehyde analog, 4-$N,N$-dimethylamino cinnamaldehyde (DMACNA), introduces an electron-donating dimethylamino group to the cinnamaldehyde chromophore, giving free substrate an absorbance maximum at 398 nm. When reduced Y196F OYE1, generated by the use of an NADPH-generating system, is mixed with DMACNA in the stopped flow diode array spectrophotometer, the initial spectra produced demonstrate a maximum at 428 nm (Fig. 5). This red shift may be attributed to enzyme-induced changes in the chromophore upon binding. In addition, as a stoichiometric equivalent bound to enzyme gives a large spectral shift with one clear peak rather than two distinct overlapping curves, it is evident that substrate binds to reduced Y196F quite tightly.

The reduction of the olefinic bond, which is evidenced by the disappearance of the chromophore due to the disruption of conjugation, occurs quite slowly as turnover of a single equivalent of the substrate proceeds over the course of more than 10 h when followed with a Varian/Cary spectrophotometer. The product of the turnover reaction was analyzed by GC/MS and shown to be 4-$N,N$-dimethylaminohydrocinnamaldehyde.

The effects of systematic variation of the chromophore upon kinetics of the oxidative half-reaction with cinnamaldehyde and wild type enzyme also revealed much about the nature of substrate binding. A series of cinnamaldehyde derivatives with variable electron-donating and -withdrawing abilities was used as substrates in a rapid reaction study of the oxidative half-reaction. The appearance of absorbance at 460 nm of oxidized enzyme fits well to a single exponential, and clear saturation of the observed rate constant with increasing substrate concentration demonstrates that the mechanism proceeds through a binding equilibrium followed by a step where the cinnamaldehyde is reduced. The data for the binding step (Table III) suggest a distinct trend. The electron-donating substituents, 4-dimethylamino and 4-methoxy, are seen to lead to significantly tighter binding to the reduced enzyme, while the electron-withdrawing 4-nitro substituent leads to weaker binding as compared with the unsubstituted cinnamaldehyde.

![Fig. 5. The interaction of 4-$N,N$-dimethylaminocinnamaldehyde with reduced enzyme.](http://www.jbc.org/)

**TABLE II**

| Substrate                  | WF OYE1 $k_{ox}$ $K_d$ | Y196F OYE1 $k_{ox}$ $K_d$ | Ratio of $k_{ox}$ (Y196F/WT) |
|----------------------------|------------------------|---------------------------|-----------------------------|
| 2-Cyclohexenone            | $102 \pm 5$ s$^{-1}$   | $2.4 \pm 0.3 \times 10^{-4}$ s$^{-1}$ | 13                          |
| Methyl vinyl ketone        | $660 \pm 170$ s$^{-1}$ | $0.59 \pm 0.06$ s$^{-1}$   | 105                         |
| Cinnamaldehyde             | $17 \pm 1$ s$^{-1}$    | $0.48 \pm 0.02$ s$^{-1}$   | 180                         |
| 1-Nitrocyclohexene         | $(6.1 \pm 0.2) \times 10^{3}$ M$^{-1}$ s$^{-1}$ | $(5.2 \pm 0.2) \times 10^{3}$ M$^{-1}$ s$^{-1}$ | 0.85                        |
| Molecular O$_2$            | $(3.5 \pm 0.1) \times 10^{3}$ M$^{-1}$ s$^{-1}$ | $(7.0 \pm 0.1) \times 10^{3}$ M$^{-1}$ s$^{-1}$ | 1.85                        |

a Data for 2-cyclohexenone and molecular oxygen are from Ref. 21.

![Image](http://www.jbc.org/)

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2 Y. Meah and V. Massey, manuscript in preparation.
Michael type acceptor, such an enolate-like intermediate is the likely form that substrate takes upon binding to the reduced enzyme. No trend is apparent in the $k_{\text{ox}}$ values with varying $\sigma_p$ (23). Observing the chemical reaction of reduction as two steps, one involving hydride transfer to the $C_\beta$ of the olefinic bond and one involving proton uptake at $C_a$, allows for the reduction to be viewed through a push-pull electron model. Pulling electrons through the carbonyl primes $C_\beta$ for hydride transfer, while pushing them back through the carbonyl bond promotes uptake of a proton from Tyr-196 at $C_a$. In this sense, electron-withdrawing and -donating substituents will differentially affect these two aspects of the overall reaction in opposing manners, thus convoluting the overall picture of the oxidative half-reaction by showing no apparent trend with $\sigma_p$.

**DISCUSSION**

The Crystal Structure and the Role of Tyr-196—An examination of the active site of OYE1 suggests a place for Tyr-196 in the mechanism of the oxidative half-reaction with reduced flavin and $\alpha,\beta$-unsaturated compounds. The structure of the oxidized enzyme with $p$-hydroxybenzaldehyde in the active site (7) provides a system by which to model the location of the substrate upon binding to the enzyme (Fig. 6). The carbonyl bond of the $\alpha,\beta$-unsaturated substrate, with hydrogen bonds between the oxygen and His-191 and Asn-194, would be positioned in an analogous way to the phenolate oxygen of $p$-hydroxybenzaldehyde. The $C_\alpha$ carbon analogous to the $C_\beta$ of the enone substrate (the site of hydride transfer) is located 3.5 Å from $N_5$ of the flavin. Tyr-196 is situated in the plane above the substrate with its phenolate oxygen to $C_\alpha$ distance of 3.4 Å reflecting the proximity of the residue to the $C_\alpha$ of the enone. The relative orientation of the $N_\delta$ hydride donor and the Tyr-196 proton donor are in agreement with a trans addition stereocchemistry observed in the product (14). Further examination of the crystal structure reveals that the residue Asn-251 has an $R$-group amidic proton situated at 2.7 Å from the tyrosine oxygen, perhaps serving to increase the acidity of Tyr-196 from the normal $pK_a$ of 10.1.

**TABLE III**

Reactivity of series of 4-substituted CNA compounds with wild type OYE1

| Substrate          | $K_d$  | $k_{\text{ox}}$ |
|--------------------|--------|-----------------|
| 4-dimethylamino-CNA | $\sigma_p=1.7$ | very tight (≈1 µM) 0.47 s$^{-1}$ |
| 4-methoxy-CNA      | $\sigma_p=0.78$ | 60 µM 265 s$^{-1}$ |
| 4-CNA              | $\sigma_p=0$ | 125 µM 17 s$^{-1}$ |
| 4-nitro-CNA        | $\sigma_p=0.79$ | 165 µM 4.8 s$^{-1}$ |

FIG. 6. The active site of oxidized OYE1 with $p$-hydroxybenzaldehyde bound from the data of Fox and Karpplus (7) (Protein Data Bank identification code 10YB). The phenolic anion is hydrogen-bonded to the His-191 and Asn-194 residue. Hydrogen bonding distances given are between donor and acceptor atoms, ignoring the speculated location of the hydrogen atoms.
Tyr-196 is also well conserved across the family of related proteins. In best-fit alignment, a Tyr residue is situated in an analogous position in all of the isozymes of OYE, N-ethylmaleimide reductase (24), pentacyrthritol tetranitrate reductase (25), glycerol trinitrate reductase (26), estrogen-binding protein (27), and trimethylamine dehydrogenase (28). In the nitrate reductases, this suggests the interesting mechanistic possibility that hydride transfer to the carbon bonded to a nitrate is followed by a breaking of the carbon to nitrate oxygen bond, which is likely facilitated by the corresponding Tyr residue. In morphinone reductase, a member of the OYE family of enzymes, a conservatively substituted residue Cys-191, with its more acidic sulhydryl side chain, aligns with Tyr-196 (29).

Isolating the Kinetic Effects of Y196F—To explore the hypothesis that Tyr-196 serves as an active site acid in reduction of unsaturated ketones, aldehydes and nitro compounds, a detailed investigation of effects of mutation of Tyr-196 to Phe on the catalytic cycle of OYE was conducted. While aerobic turnover with only molecular oxygen as the substrate for the oxidative half-reaction remains unaffected, the aerobic turnover reaction in the presence of 2-cyclohexenone proved to be dramatically slowed. The cumulative ligand binding data suggest that the active site and the distribution of electrons in the FMN are only perturbed in a minor way with the Y196F mutation. Thus, any structural change should not significantly alter undesired aspects of the thermodynamics of the reaction catalyzed by OYE. The reductive half-reaction for Y196F is not perturbed, in fact, there is a minor increase in \( k_{\text{red}} \). Our characterization of the oxidative half-reaction with molecular oxygen gives a second order rate constant double the wild type value. 2-Cyclohexenone is shown to inhibit oxidation by molecular oxygen by forming a spectrally distinct complex with the reduced enzyme which is both unreactive to molecular oxygen and which itself undergoes oxidation at a rate slowed almost 6 orders of magnitude. The almost complete loss of catalysis with Y196F speaks to its significance in the mechanism of reduction of 2-cyclohexenone.

Decoupling the Oxidative Half-reaction—A fuller mechanistic picture of the oxidative half-reaction was given by observing the reaction with various substrates. The character of the substrate significantly affected the extent to which the reaction was slowed. The examples of 1-nitrocyclohexene and 2-cyclohexenone illustrate two extreme situations, which may be integrated into a single model to explain the differential results with wild type enzyme and Y196F and elucidate the nature of the oxidative half-reaction. While with 2-cyclohexenone enzyme oxidation is slowed by nearly 6 orders of magnitude, with 1-nitrocyclohexene the rate constant only falls to 0.85 that of the wild type value. The reduction of the \( \alpha,\beta \)-unsaturated bond requires both hydride transfer from the flavin to the \( C_\beta \) and proton uptake from Tyr-196 to the \( C_{\alpha} \). Whether these components of the reaction occur in a stepwise or concerted fashion has remained an open question.

The differential reoxidation rate constants across the series of compounds examined suggests a model for the oxidative half-reaction (Fig. 7). With Y196F, since no proton donor is oriented toward \( C_{\alpha} \) and the enzyme is only equipped to perform hydride transfer, catalysis of the two components of the half-reaction is likely decoupled with all substrates. In the case of 1-nitrocyclohexene, where the reaction is decoupled in the wild type enzyme (as evidenced by the biphasic generation of product), in Y196F the intermediate aci-nitro compound is rapidly generated (1A), while decay of the aci-nitro intermediate to its tautomeric product proceeds at the uncatalyzed rate (1B). For 2-cyclohexenone, the fact that hydride transfer is not observed to occur with Y196F suggests that the intermediate enolate anion is less thermodynamically accessible. Thus, Y196F remains tightly bound with 2-cyclohexenone while oxidation of the enzyme is slowed by 6 orders of magnitude. By comparison, this suggests that wild type enzyme may be able to catalyze the reaction through a different mechanism than that observed with the 1-nitrocyclohexene. The results can be explained by noting that the presence of the Tyr-196 activated for proton transfer to \( C_{\alpha} \) either allows for a concerted transfer of hydride and proton or stabilizes the transition state for the transfer of hydride (2). The model describing these two reactions may be applied to other substrates. The extent of the reduction in rate with Y196F versus the wild type enzyme would be expected to depend upon the thermodynamic accessibility of the intermediate species resulting from hydride transfer decoupled from proton uptake. Applied to cinnamaldehyde, the extensive conjugation in the substrate is expected to make the intermediate more thermodynamically accessible than for 2-cyclohexenone, as the energy difference between the \( \pi \) and \( \pi' \) orbitals is reduced by conjugation. The greatest effect on catalysis is shown with 2-cyclohexenone and methyl vinyl ketone, both of which lack conjugated double bonds as present in cinnamaldehyde or lack a resonance-stabilized intermediate as with 1-nitrocyclohexene.

Characterization of the Michaelis Complex of the Oxidative Half-reaction—Our analysis of the altered kinetics of Y196F allows for insight into the chemical step(s) of the oxidative half-reaction but fails to provide a description of the interaction of substrate with reduced enzyme upon binding. The slow catalysis of the oxidative half-reaction, however, offered the
unique opportunity to examine the manner in which enzyme primes substrate for the oxidative half-reaction. The Michaelis complex with reduced enzyme bound to substrate was examined by the use of 4-DMACNA, which contains a chromophore amenable to study. Upon binding to reduced enzyme, a 30-nm bathochromic shift is observed in the spectrum of the substrate with little change in extinction. Several precedents exist for such an observation. In liver alcohol dehydrogenase, a ternary complex formed between enzyme, NADH, and DMACNA, which results in a 66-nm red shift and marked increase in extinction is due to coordination of the carbonyl oxygen of substrate with a Zn$^{2+}$ active site metal (30). In enoyl-CoA hydratase, the presence of a strong polarizing electric field is used to explain the 90 nm red shift and marked increase in extinction upon binding of 4-N,N-dimethylaminocinnamoyl-CoA to enzyme (31). In the case of OYE, the lack of appropriately oriented oppositely charged residues in the active site makes the possibility of the such a polarizing electric field unlikely. When DMACNA was examined in a series of solvents of varying polarity, the $\lambda_{\text{max}}$ of the free substrate was correlated with solvent polarity (32). The trend is consistent with a narrowing of the gap between the $\pi$ and $\pi'$ orbitals in a more polar environment causing a lower energy transition (33) due to the greater asymmetry of the $\pi'$ orbital. A 30-nm shift upon binding to enzyme can be explained by noting that the active site is designed with a polar pocket with His-191 and Asn-194 residues, which can polarize the carbonyl and stabilize the enolate resonance form of the DMACNA. In addition, the importance of this hydrogen bond on substrate binding and priming for catalysis is revealed with the wild type enzyme by the trend toward tighter binding of 4-substituted cinnamaldehyde compounds having electron-donating substituents as compared with those having electron-withdrawing substituents. The polarizability of substrate upon binding, as observed in Y196F where catalysis is slowed, is likely an integral part of the means of activating the substrate for its reduction.

**Speculations on a Physiological Role for OYE**—The model for the mechanism of the oxidative half-reaction also allows for speculation regarding the nature of the physiological oxidant. Under the assumption that the enzyme is designed to meet the needs of the natural substrate or substrates, a substrate with a less thermodynamically accessible intermediate would justify the presence of Tyr-196. Substrates that can stabilize the product of hydride transfer, as with the resonance stabilization of cinnamaldehyde or the aci-nitro intermediate of 1-nitrocyclohexene, would be less likely to require nature to engineer OYE as it has. Several straight chain and cyclic enones, which lack such resonance stabilization have proven to be among the best substrates for the oxidative half-reaction. These include many of the possible breakdown products of lipid peroxidation, which can have toxic effects on cells (34). Studies of the effect on colony forming efficiency in *S. cerevisiae* in the presence of various aldehydes have suggested that the effectiveness of inhibition of cell proliferation decreased in the order: 2,4-aldehydes > 4-hydroxyalkenals > 2-alkenals > alkanes (35). In other species, inhibition of cell proliferation is seen to be absent or low in alkanals (36). OYE, by catalyzing the reduction of the $\alpha$, $\beta$-olefinic unit, thus could serve in a defensive role against these substrates by reducing their toxicity. Bakers’ yeast, established to be effective in antioxidant defense, has several known defense systems including glutathione, superoxide dismutase, catalase, and peroxidase (37). Enzymes involved in such detoxification pathways are typically marked by wide species differences, several isoforms of differing kinetics, broad specificity, and high catalytic efficiency (38). OYE, with several isoforms marked by their broad substrate specificity and efficiency, is a prime candidate for involvement in such detoxification pathways.

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