Old Yellow Enzyme: Aromatization of Cyclic Enones and the Mechanism of a Novel Dismutation Reaction†

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ABSTRACT: The origin of charge transfer bands that develop on reaction of Old Yellow Enzyme with α,β-unsaturated cyclic ketones such as 3-oxodecalin-4-ene (ODE, numbered according to the convention for steroids), 3-oxodecalin-4-ene-10-carboxaldehyde (ODEC), and 2-cyclohexenone is shown to be due to the aromatization of ODE and ODEC to 3-hydroxy-6,7,8,9-tetrahydronaphthalene (HTN) and of 2-cyclohexenone to phenol. The aromatization of ODEC to HTN is stereospecific and involves the *trans* dehydrogenation of the 1β,2α hydrogens. The aromatization occurs under aerobic as well as anaerobic conditions. With the exception of ODEC under aerobic conditions, the aromatization of these substrates is accompanied by a dismutation reaction in which the olefinic bond of a second molecule of each substrate is reduced to give the saturated cyclic ketone. Molecular oxygen may serve as the electron acceptor with ODEC and some other substrates under aerobic reaction conditions. The dismutation reaction involves an overall sequence of hydride transfer from one substrate molecule to the β-carbon of a second substrate molecule along with a solvent proton uptake by the α-carbon. 19-Nortestosterone is aromatized to β-estradiol; however, other 3-oxo-Δ^4-steroids such as progesterone, testosterone, and androstene-3,17-dione bind tightly to the enzyme but are not aromatized. The NADPH-dependent reduction of the olefinic bond of α,β-unsaturated carbonyl compounds is limited to aldehydes and ketones. α,β-Unsaturated acids, esters, amides, and nitriles are not reduced. The reduction of the olefinic bond of ODE or cinnamaldehyde by NADPH occurs by an overall sequence of hydride transfer from the reduced pyridine nucleotide to the β-carbon of the α,β-unsaturated carbonyl compound and a solvent proton uptake by the α-carbon. The 4-pro-*R* hydride of NADPH is transferred in the reduction reaction. Structure−function relationships in the NADPH-dependent reduction of α,β-unsaturated aldehydes or ketones indicate that increasing alkyl substitution at the β-carbon results in marked decrease in the rate of reduction of the olefinic bond, consistent with a steric hindrance to hydride transfer at the β-carbon.

Old Yellow Enzyme (OYE),[1] the first discovered flavoprotein, isolated originally from Brewer's Bottom Yeast (Warburg & Christian, 1933) has had a long and illustrious history in the evolution of our understanding of proteins as enzymes and the role of cofactors in enzymology. OYE has in recent years been shown by protein purification and molecular biological techniques to consist of several isoforms, encoded by separate genes (Miura *et al.*, 1986; Saito *et al.*, 1991; Stott *et al.*, 1993). The crystal structure of OYE 1 has been solved at a resolution of 2.0 Å (Fox & Karplus, 1993, 1994). Despite an extensive knowledge of the physical and biochemical properties of this enzyme, its physiological function has remained elusive. While NADPH has come to be the accepted physiological reductant of the enzyme, the true physiological oxidant (electron acceptor) remains an enigma. A number of electron acceptors are known including molecular oxygen, methylene blue, ferricyanide, and quinones such as *p*-quinone and menadione; more recently, the olefinic bond of several α,β-unsaturated carbonyl compounds, such as in 2-cyclohexenone, has been found to be reduced (Stott *et al.*, 1993; Vaz & Massey, 1994). Like methylene blue and ferricyanide, molecular oxygen is viewed as an adventitious oxidant as evidenced by its slow reactivity with the reduced enzyme in comparison to other oxidases and by the ability of other electron acceptors such as duroquinone and 2-cyclohexenone to shut off the molecular oxygen-dependent NADPH oxidase activity (Schopfer & Massey, 1991; Stott *et al.*, 1993). A well-known property of OYE is its ability to interact with phenolic compounds to yield long wavelength charge transfer bands in the region of 500−800 nm (Matthews & Massey, 1969; Matthews *et al.*, 1975; Abramovitz & Massey, 1976 a,b). Various cyclic enones have been found that yield long wavelength charge transfer bands on equilibration with OYE (Stott *et al.*, 1993; Massey, 1993). Based on the spectral similarity of these charge transfer bands to those formed between OYE and phenolic compounds of comparable carbon skeletal structure, the carbonyl-derived charge transfer bands were attributed

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[1] Abbreviations: OYE, Old Yellow Enzyme isolated from Brewer's Bottom Yeast; OYE 1, Old Yellow Enzyme isoform 1 expressed in *Escherichia coli*; ODE, 3-oxodecalin-4-ene; ODEC, 3-oxodecalin-4-ene-10-carboxaldehyde; HTN, 3-hydroxy-6,7,8,9-tetrahydronaphthalene; GC/MS, coupled gas chromatography/mass spectroscopy. The rings of ODE, ODEC, and HTN have been numbered according to the convention for the A and B rings of steroids as used previously (Vaz *et al.*, 1994) and have been maintained in this manuscript for purpose of comparison.

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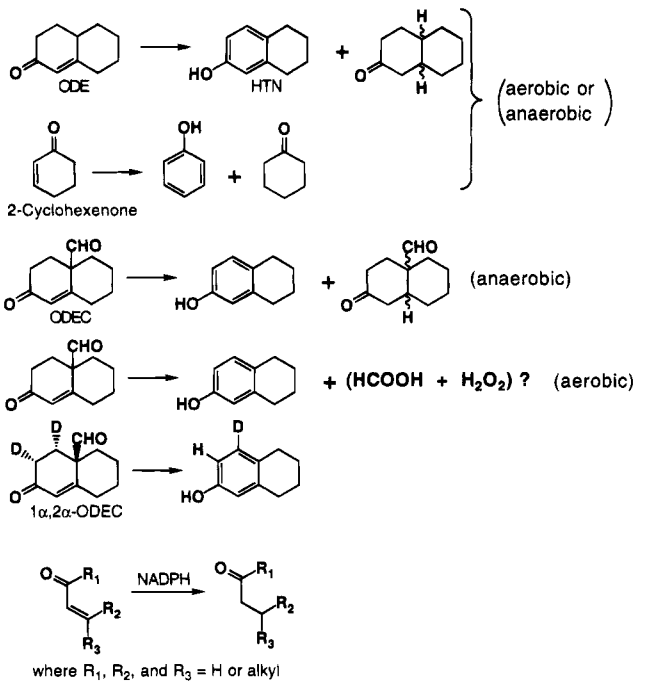


FIGURE 1: Reactions of OYE with cyclic and acyclic enones investigated in this study.

to an OYE−enolate complex (Stott *et al.*, 1993; Massey, 1993).

As outlined in Figure 1, in this study we have examined the reactions of α,β-unsaturated carbonyl compounds with OYE, and we have established that (1) the charge transfer bands which develop under aerobic or anaerobic reaction conditions between OYE and the three α,β-unsaturated carbonyl compounds, 3-oxodecalin-4-ene (ODE), 3-oxodecalin-4-ene-10-carboxaldehyde (ODEC), and 2-cyclohexenone, are due to their oxidative aromatization to 3-hydroxy-6,7,8,9-tetrahydronaphthalene (HTN) and phenol, respectively; (2) the aerobic aromatization of ODEC to HTN occurs by a stereospecific *trans* dehydrogenation; (3) the oxidative aromatization of the cyclic enones as probed by ODE and 2-cyclohexenone has been established to occur by a novel dismutation reaction catalyzed by OYE in which ODE or 2-cyclohexenone is first dehydrogenated to yield HTN or phenol, and subsequently the olefinic bond of a second ODE or 2-cyclohexenone molecule is reduced to 3-oxodecalin or cyclohexanone. The reductive half of the dismutation reaction is analogous to the NADPH-dependent reduction of the olefinic bond of the enones; (4) the NADPH-dependent, OYE-catalyzed reduction of the olefinic bond of α,β-unsaturated carbonyl compounds is limited to α,β-unsaturated ketones and aldehydes. The corresponding esters, acids, amides, and nitriles are not substrates for the reduction reaction. Increased steric bulk at the β-carbon results in a significant decrease in the rate of reduction of the olefinic bond. The reduction involves hydride transfer from the *R*-face of the NADPH to the β-carbon of the substrate and proton uptake from solvent by the α-carbon.

MATERIALS AND METHODS

OYE was isolated from Brewer's Bottom Yeast by the affinity chromatography method previously described (Abramovitz & Massey, 1976a). Unless otherwise specified, all of the results reported here were obtained with this enzyme. OYE 1, OYE 2, and OYE 3 were expressed in *Escherichia coli* and purified as described (Saito *et al.*, 1991; Stott *et al.*, 1993; Niino *et al.*, 1995). Pro-*R* deuterated NADPD was obtained by the enzymatic oxidation of [D,L-$^2H_1$]malate (Lowenstein, 1963) using the enzymatic procedure with malic enzyme described by Ryerson *et al.*, (1982). ODEC and its deuterated isotopomer, [1α,2α-$^2H_2$]ODEC, were synthesized as described (Vaz *et al.*, 1994). ODE was synthesized by the Robinson annelation of cyclohexanone with methyl vinyl ketone essentially as described by Brewster and Eliel (1953). ODE and ODEC were synthesized as racemic mixtures and were used as such in all the studies reported. All other reagents were obtained commercially and purified if found necessary. UV/visible absorbance spectra were recorded with a Hewlett Packard Model 8452A diode array spectrophotometer.

Analytical Methods: HPLC and GC Analysis and Preparative Purification of HTN. A system consisting of a Waters NovaPak C-18 reversed phase column (5.0-μm particle size; Millipore Waters Chromatography Division, Millford, MA) fitted to a Waters HPLC system consisting of a Model 600 multisolvent delivery system, a Model 490 programmable detector, a Model 710 WISP autosampler, and a Model 3390A Hewlett-Packard recording integrator was used for all quantitative analysis by HPLC. HTN was normally quantitated by the method previously described (Vaz *et al.*, 1994). Deuterated HTN from the aerobic reaction of [1α,2α-$^2H_2$]ODEC with OYE was extracted from the reaction mixture and purified as previously described (Vaz *et al.*, 1994). For stoichiometry of the dismutation reaction with ODE, the gas chromatographic method described below was used to quantitate HTN in the presence of 3-oxodecalin. After quenching the reaction mixture with 5 μL of formic acid, *p*-cresol and 2-octanone (100 nmol of each) were added as internal standards and extracted once with a 2-mL volume of methylene chloride. The extract was dried over anhydrous sodium sulfate and concentrated in a vacuum centrifuge to approximately 20 μL. An aliquot (2 μL) was injected onto a fused silica capillary GLC column (Supelco SPB-5 15 m, 0.2 mm i.d.) fitted in a Varian Model 3700 gas chromatograph having a flame ionization detector. The injector and detector temperatures were 180 and 210 °C, respectively. The column temperature was initially maintained at 50 °C for 5 min followed by a temperature gradient of 5 °C/min to a final temperature of 210 °C. The carrier gas was helium at a head pressure of 10 psi. Under these conditions, the retention times of HTN, 3-oxodecalin, *p*-cresol, and 2-octanone were 24.7, 20.3, 13.9, and 10.5 min, respectively. The dectector response was linear between 5 and 40 nmol for all compounds of interest.

Mass Spectral Analysis. GC/MS was performed with a Finigan 40-21GC-MSDS mass spectrometer fitted with a 30-m DB-5 fused silica capillary column (S&W Scientific) and operating under electron impact conditions at 70 eV. The splitless injector temperature was maintained at 200 °C, and the carrier gas was helium at a head pressure of 10 psi. In the standard procedure, the sample in 2−5 μL of methylene chloride was injected onto the column, which was held at 50 °C for 4 min, followed by an increase to 275 °C at 20 °C/min. The column was held at the latter temperature for 30 min. The mass spectra shown were obtained by summing the scans within a peak of interest in the total ion current and subtracting a baseline composed of an equal number of scans just prior to the peak of interest.

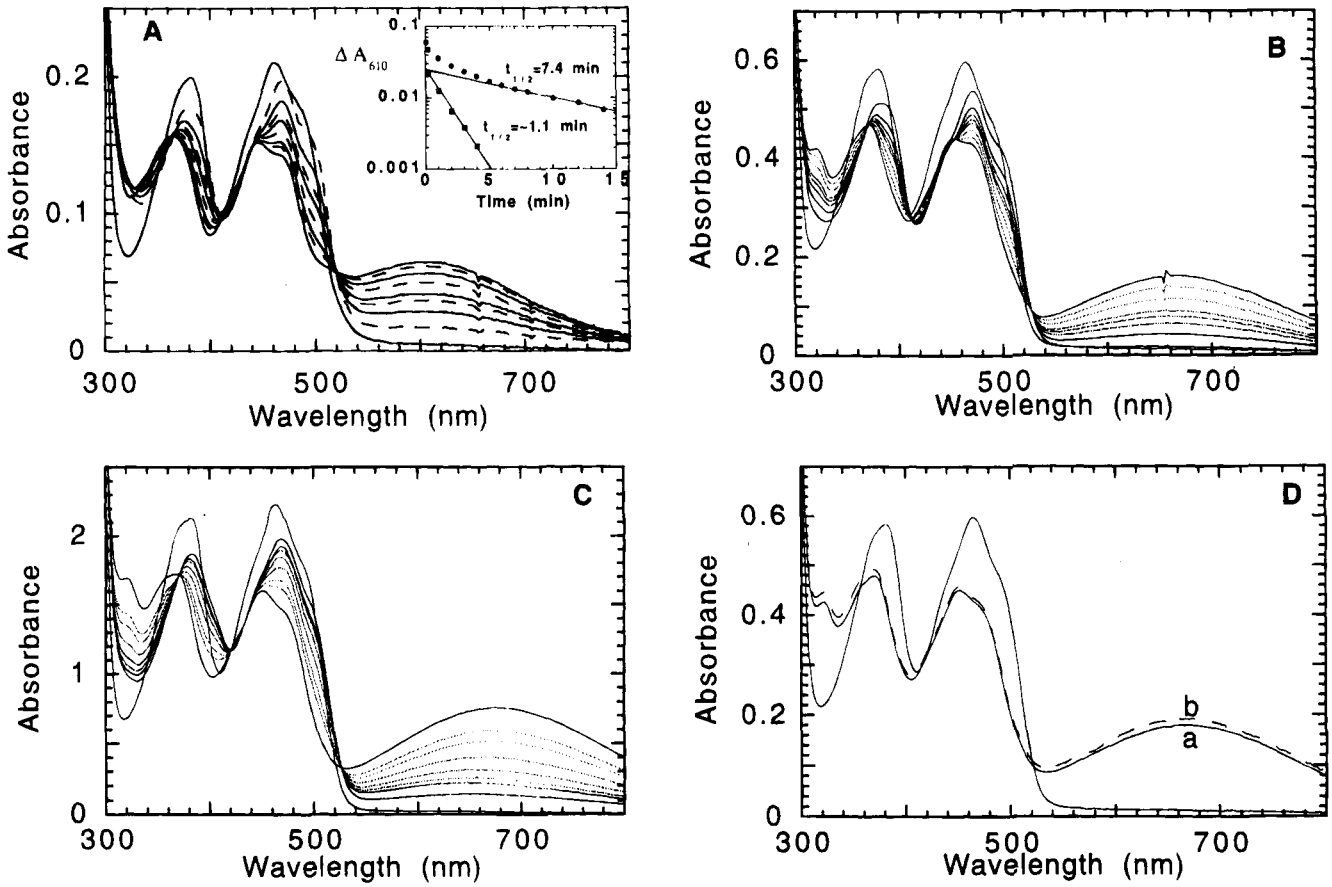


FIGURE 2: Spectral interaction of 2-cyclohexenone (panel A), ODE (panel B), and ODEC (panel C) with OYE under aerobic conditions. The biphasic kinetics for development of the charge transfer complex with 2-cyclohexenone is shown as an inset to panel A. Panel D shows the spectra of OYE with ODE after complete development of the charge transfer complex (trace a) and that of OYE with HTN (trace b). For the experiment of panel A, recombinant OYE 1 was used. For the other studies, OYE isolated from Brewer's Bottom Yeast was used. In panel B, the spectra with increasing absorbance at long wavelengths and decreasing absorbance at 460 nm were recorded before mixing with 1 mM ODE and after 10 s, 1, 2, 3, 4, 8, 20, and 80 min, and 3 day. In panel C, the corresponding spectra were recorded before mixing with 1 mM [1α,2α-$^2H_2$]ODEC and after 15 s and 2, 4, 10, 23, 40, 75, and and 950 min. The spectra of panel A were recorded in 0.1 M phosphate, pH 7.0, 25 °C, those in the remaining panels in 0.1 M pyrophosphate, pH 8.5, 25 °C.

Proton NMR Analysis. Proton NMR spectra were recorded on a GE Omega 500 MHz spectrometer. The ^1H-proton NMR spectrum was recorded for the aromatic region of HTN in deuteriochloroform.

RESULTS

Spectral Interactions of ODE, ODEC, and 2-Cyclohexenone with OYE. The spectral changes that occur on the addition of 2-cyclohexenone, ODE, or ODEC to OYE under aerobic conditions are shown in Figure 2 (Panels A–C). With all three compounds, a rapid pertubation of the flavin spectrum is observed within the 10–15-s mixing time of the carbonyl compound with the enzyme; however, no long wavelength charge transfer band is observed immediately. The charge transfer bands, centered at 670 nm for ODE and ODEC and at 610 nm for 2-cyclohexenone, develop slowly in a biphasic first-order manner (as shown in the inset of panel A for 2-cyclohexenone). Identical charge transfer bands also develop under anaerobic conditions (data not shown). With ODEC, the charge transfer band maximum appears to be slightly blue shifted at early time points (2, 10, and 23 min). However, after complete development of the charge transfer complexes, the spectra obtained from the reactions of OYE with ODE and ODEC are identical. This suggests that under aerobic or anaerobic reaction conditions ODE and ODEC either form identical electronic complexes with OYE or react with OYE to generate a common product that is responsible for the charge transfer complex. Furthermore, as shown in Figure 2, panel D, the spectrum of the complex formed from the reaction of ODE with OYE after complete development of the charge transfer band is identical to the spectrum of the complex of HTN with OYE. As shown below, the reaction mixtures of ODE, ODEC, and 2-cyclohexenone were found to contain the aromatic products HTN and phenol, respectively. Accordingly, we propose that the slow development of the charge transfer bands is due to the oxidation of the enones to the corresponding phenols, which then interact with oxidized OYE to form the well-recognized charge transfer bands. The biphasic development of the charge transfer bands is not due to the two isoforms of OYE in Brewers' Bottom Yeast. Identical patterns are found with the separate isoforms OYE 1, OYE 2, and OYE 3. The results shown in panel A were obtained with the recombinant enzyme OYE 1. It should be noted that the development of the charge transfer complex of oxidized enzyme with phenol is very fast, in the millisecond time range (data not shown). Thus, the slow development of this band from cyclohexenone is due to the slow dismutation reaction.

Identification and Characterization of Reaction Products. Figure 3 shows the GC/MS analysis of the methylene chloride-extractable components from the reaction mixtures

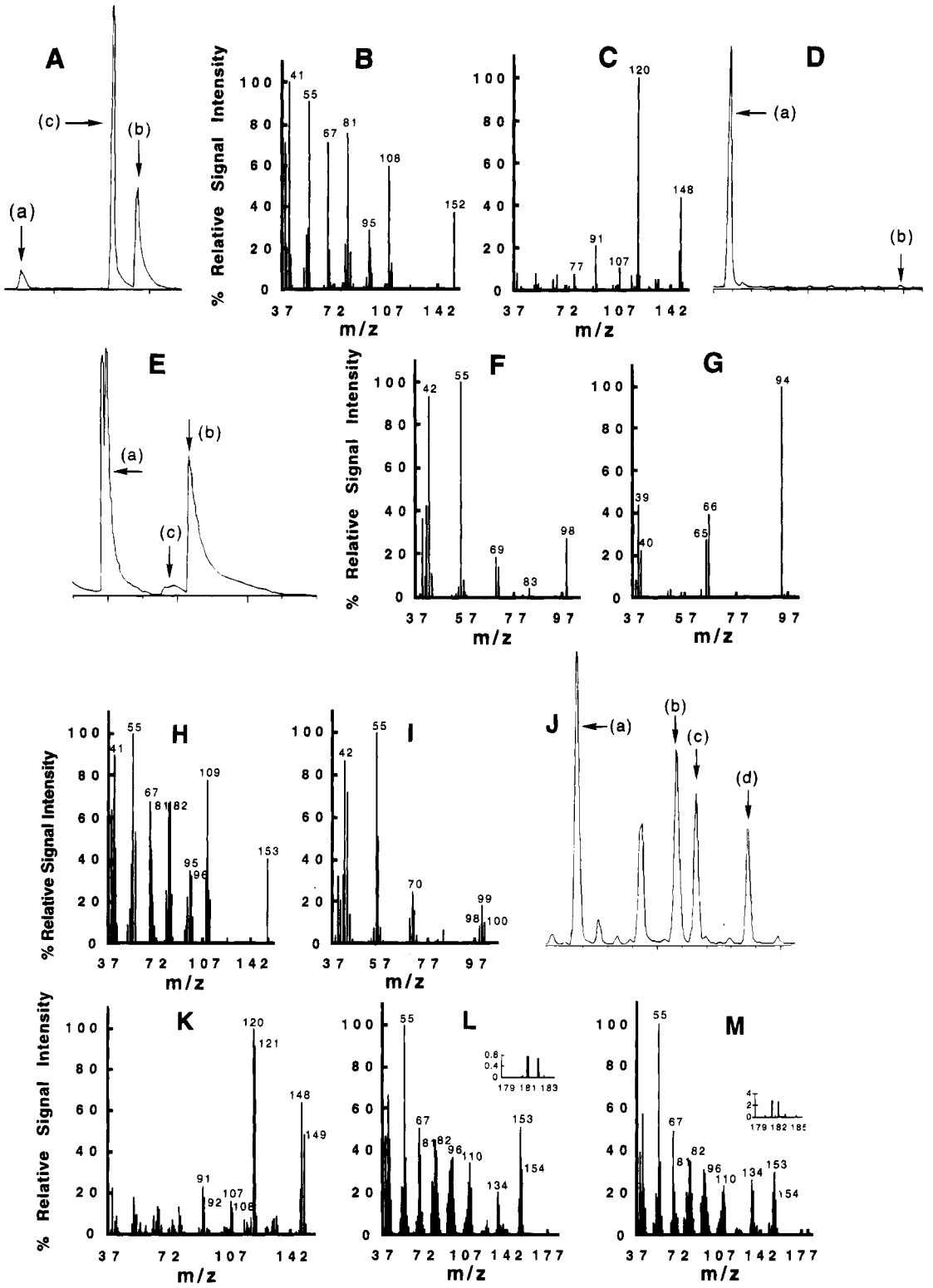


FIGURE 3: Products of the reactions of OYE with ODE, ODEC, and 2-cyclohexenone under aerobic and anaerobic reaction conditions. The reaction mixtures contained 0.1 M potassium phosphate buffer, pH 7, and 20−40 μM OYE, 2−4 mM of ODE, ODEC, or 2-cyclohexenone in a final volume of 1.0 mL. For anaerobic reactions, the enzyme and substrate were first made anaerobic with an oxygen-scrubbed argon atmosphere in the side arms of an anaerobic reaction cell and then mixed prior to monitoring the absorbtion spectra. Reactions were quenched with 5 μL of 88% formic acid and extracted with 2 mL of methylene chloride and analyzed by GC/MS as described in Methods. (Panel A): Ion chromatogram of the aerobic reaction of ODE with OYE showing product peaks decalin-2-one (a) and HTN (b) and unreacted substrate (c); (panels B and C): mass spectra of product peaks (a) and (b), respectively, shown in panel A; (panel D): ion chromatogram of the aerobic reaction of ODEC with OYE showing the HTN product peak (a) and the substrate peak (b); (panel E): ion chromatogram of the aerobic reaction of 2-cyclohexenone with OYE showing product peaks cyclohexanone (a) and phenol (b) and unreacted substrate (c); (panels F and G): mass spectra of peaks (a) and (b) from the ion chromatogram shown in panel E; (panels H and I): mass spectra of 3-oxodecalin and cyclohexanone obtained from ODE and 2-cyclohexenone, respectively, under anaerobic conditions in 90% D_2O; (panel J): Ion chromatogram of the anaerobic reaction of ODEC and OYE with product peaks HTN (a), the isomeric forms of decalin-2-one-10-carboxaldehyde (b and c), and unreacted substrate (d); (panels K−M): mass spectra of product peaks (a−c) shown in panel J.

of ODE, ODEC, and 2-cyclohexenone with OYE under aerobic and anaerobic conditions. Two products were identified in the aerobic reaction of ODE with OYE as shown in the ion chromatogram in panel A. The mass spectrum shown in panel B for peak a corresponds to 3-oxodecalin as determined for the authentic standard obtained from Aldrich Chemicals (data not shown). The mass spectrum shown in panel C for peak b corresponds to HTN (Vaz *et al.*, 1994). Peak c is the unreacted substrate. Under aerobic conditions, 3-oxodecalin is formed in significantly lower amounts than HTN. Under aerobic conditions, ODEC shows a single product peak as shown in panel D, this peak was identified by its mass spectrum as HTN (data not shown). Two product peaks were found in the aerobic reaction of 2-cyclohexenone (panel E). The mass spectra of peaks marked a and b in panel E are shown in panels F and G and correspond to cyclohexanone and phenol, respectively. The cyclohexanone and phenol were formed in comparable amounts. Under anaerobic reaction conditions in 90% D_2O, ODE and 2-cyclohexenone form similar products as under aerobic conditions in H_2O. However, as seen in the mass spectra for the respective reduction products 3-oxodecalin and cyclohexanone (panels H and I, respectively), solvent-derived deuterium atoms are incorporated. A single solvent-derived deuterium atom is incorporated quantitatively into 3-oxodecalin as determine by the intensities of the *m/z* 153 and *m/z* 152 ion masses. Cyclohexanone derived from 2-cyclohexenone is a mixture of 23% un-, 49% mono-, and 27% dideuterated isotopomers as determined by the ion mass intensities at *m/z* 98, 99, and 100, respectively. The unreacted substrates and the aromatization products, HTN and phenol, do not show any solvent deuterium atom uptake (data not shown). Under anaerobic conditions in 90% D_2O, ODEC showed three reaction products (panel J, peaks a–c). Peak a was identified as HTN by its mass spectrum shown in panel K. Peaks b and c correspond to the reduction products, 3-oxodecalin-10-carboxaldehydes, as determined by their similar fragmentation patterns shown in panels L and M. These reduction products are presumably the *cis* and *trans* ring-fused geometric isomers of 3-oxodecalin-10-carboxaldehyde. Additionally, the mass spectrum of HTN shows that partial incorporation of a single deuterium atom from the medium occurred as evidenced by the ion masses at *m/z* 149 and 121 (panel K). The signal intensities of the ion masses *m/z* 121 and 120 indicate that 45% monodeuterio-HTN is formed in the reaction. The reduction products incorporate two deuterium atoms from the medium as seen by the weak molecular ion masses at *m/z* 181 and 182 (insets of panels L and M) and the ($M^+ - CO$) ions masses at *m/z* 153 and 154. Intensities of the ion masses at *m/z* 152, 153, and 154 indicate that the two reduction products are a mixture of 26% un-, 45% mono-, and 28% di-deuterio isotopomers. The mass spectrum of the unreacted substrate (peak d, panel J) indicates that no deuterium exchange with the medium occurred (data not shown). It should be noted that D_2O does not influence the nature of reaction products as the same products were obtained under anaerobic conditions in H_2O from the three substrates (data not shown). The results of product identification and characterization reveal that OYE catalyzes a dismutation reaction under aerobic as well as anaerobic conditions in which one substrate molecule is aromatized and a second substrate molecule is reduced at the olefinic bond. The incorporation of a single deuterium atom by the reduction products of ODE suggests that the reductive half of the dismutation reaction occurs by a hydride transfer (via the enzyme-bound flavin) from the substrate molecule that is oxidized to the substrate molecule that is reduced. The low level of 3-oxodecalin relative to HTN in the aerobic reaction of ODE and the absence of reduction products in the aerobic reaction of ODEC suggest that molecular oxygen may effectively compete with these substrates for reducing equivalents from the enzyme-bound flavin. The incorporation of more than one deuterium atom into the reduction products of 2-cyclohexenone and ODEC under anaerobic conditions suggests that with some substrates the flavin hydride may undergo solvent exchange prior to hydride transfer to the olefinic bond. Quantitative analysis of HTN and 3-oxodecalin from the anaerobic reaction of ODE showed that the oxidation and reduction products were formed in essentially stoichiometric amounts (data not shown).

Stereochemistry of Aromatization of ODEC. [1α,2α-$^2H_2$]-ODEC was synthesized as described (Vaz *et al.*, 1994). HTN derived from the aerobic reaction of OYE with [1α,2α-$^2H_2$]-ODEC was isolated by HPLC as previously described (Vaz *et al.*, 1994). Figure 4 shows the 500-MHz ^1H NMR spectra in the aromatic region from 6.48 to 6.9 ppm for deuterio-HTN (panel A) isolated from the reaction of OYE with [1α,2α-$^2H_2$]ODEC and a standard of HTN (panel B). The assignments of the proton signals are C_{1H} 6.83 ppm, C_{2H} 6.56 ppm, and C_{4H} 6.52 ppm as given in the Sadtler reference work (1976). Signal intensities for the C_1 and C_2 protons were integrated relative to the C_4 proton. The intensities of the C_1 and C_2 proton signals of deuterio-HTN are 63% and 100% that of the C_4 proton signal intensity, respectively. Stereospecific loss of the $C_{1\beta}$ hydrogen from [1α,2α-$^2H_2$]-ODEC would result in retention of the 1α deuterium at the C_1 position of HTN. Since the C_1 position of ODEC was labeled in the amount of 44% (Vaz *et al.*, 1994), the NMR signal intensity would be expected to be decreased by a corresponding amount. The observed signal intensity for the C_1 position of deuterio-HTN is 63%. As shown in the inset to Figure 4, panel A, this result is consistent with stereospecific removal of the $C_{1\beta}$ hydrogen during catalysis by OYE. Similarly, stereospecific loss of the $C_{2\alpha}$ deuterium would result in retention of the $C_{2\beta}$ hydrogen and no loss in the signal intensity at the C_2 position of HTN. The observed signal intensity for the C_2 position of deuterio-HTN is 100% of that of the C_4 proton signal intensity, a result consistent with stereospecific loss of the $C_{2\alpha}$ hydrogen of ODEC. Thus, the results show that the aromatization of ODEC by OYE proceeds by an overall *trans*-dehydrogenation at the C_1C_2 positions of ODEC and involves the 1β,2α hydrogens.

Functional Group and Structural Specificity of NADPH-Dependent, OYE-Catalyzed, Reduction of α,β-Unsaturated Carbonyl Compounds. Table 1 shows the rates of the OYE-catalyzed oxidation of NADPH in the presence of various α,β-unsaturated carbonyl compounds. From the cinnamyl class of compounds examined in this study, it appears that α,β-unsaturated acids, esters, or amides do not serve as substrates for OYE, whereas when the carbonyl group is present as an aldehyde or ketone, significant NADPH oxidation activity is maintained. Substitution of the carbonyl by a cyano function also results in complete loss of activity. The ease of protonation of the carbonyl group in aldehydes or ketones relative to that in functional groups such as esters, acids, and amides or cyanides suggests that the aldehyde or ketone function may be protonated or hydrogen bonded to

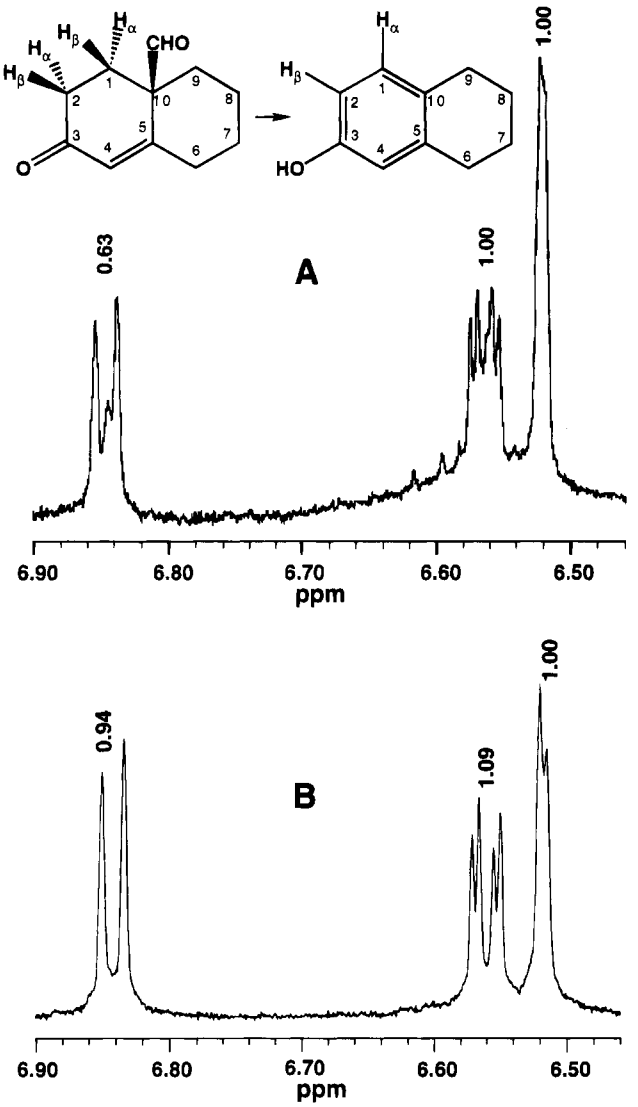


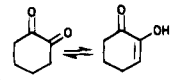
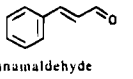
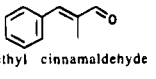
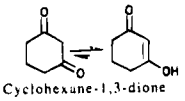
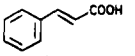
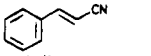
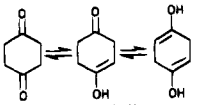
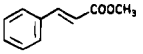
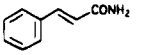
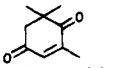
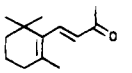
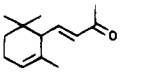
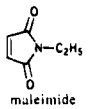
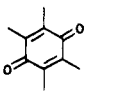
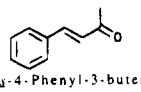
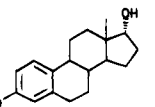
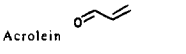
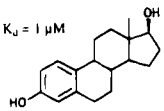
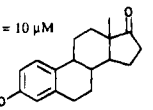
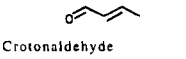
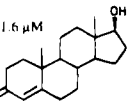
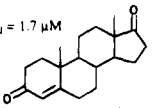
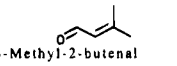
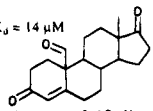
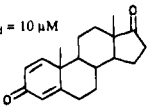
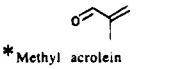
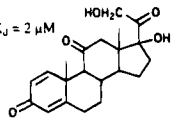
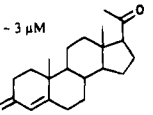
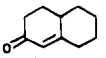
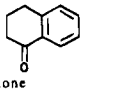
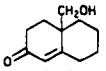
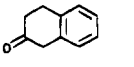
FIGURE 4: 500-Mz proton NMR spectra of HTN in the aromatic region from 6.46 to 6.9 ppm. (Panel A): deuterated HTN isolated from the OYE-catalyzed aromatization of [1α,2α-$^2H_2$]ODEC; (panel B): HTN standard. Numbers above the peaks are the signal intensities unitized to the signal intensity for the C_4 proton at 6.52 ppm. The inset to panel A shows the numbering system used in the substrate and the product and the stereochemistry of the dehydrogenation reaction catalyzed by OYE.

functional groups in the active site of OYE. This can result in lowering the reduction potential of the olefinic bond, which is conjugated to the carbonyl group, thus increasing the ease of reduction by the flavin hydride. Increasing the alkyl group substitution at the β carbon of the α,β-unsaturated aldehyde or ketone results in progressive decrease in the rate of NADPH oxidation. For example, 3-penten-2-one and mesityl oxide are progressively less active in NADPH oxidoreductase activity than methyl vinyl ketone. Similarly 2-butenal (crotonaldehyde) and 3-methyl-2-butenal are progressively less active than acrolein. Pertubation of the flavin spectrum by all these compounds at comparable concentrations indicates that alkyl substitutions at the β carbon do not appear to affect binding to OYE; however, the NADPH oxidation activity is significantly affected.

Several steroids having a 3-oxo-Δ^4-ene structure were examined for their ability to form charge transfer complexes or to show NADPH oxidoreductase activity with OYE. At low steroid concentrations (0.1−20 μM), the flavin spectrum of OYE is significantly perturbed indicating tight binding (K_d values ranging from 0.1 to 10 μM, data not shown). However, except for the Δ^1-bond of androst-1,4-diene-3,-17-dione, which was reduced to give androst-4-ene-3,17-dione, the other 3-oxo-Δ^4-ene steroids examined showed no NADPH oxidoreductase activity. With the exception of 19-nortestosterone, none of the other 3-oxo-Δ^4-ene steroid structures examined developed charge transfer bands on incubation with OYE. The charge transfer band obtained with 19-nortestosterone was similar to the charge transfer band of OYE with β-estradiol, and it was confirmed by HPLC analysis that 19-nortestosterone was oxidized by OYE to β-estradiol (data not shown). Furthermore, it was established by HPLC analysis that desaturation at the C_1,C_2 positions of androst-4-ene-3,17-dione did not occur. These results suggest that the inability of OYE to reduce the double bond of the 3-oxo-Δ^4-ene function in steroids must arise from steric factors in the OYE active site that place the Δ^4-double bond in an unfavorable position for hydride transfer from the reduced flavin.

NADPH-Dependent Reduction of Cinnamaldehyde and ODE. Stott *et al.* (1993) have reported the OYE-catalyzed reduction of 2-cyclohexenone to cyclohexanone by NADPH. As shown above, structurally diverse α,β-unsaturated enones and aldehydes are now known to serve as substrates for this reduction reaction; however, the mechanism of this reduction is not known. The protein-bound FMN is rapidly reduced by NADPH, and the reduction is also known to be stereospecific with the pro-*R* hydrogen of the reduced pyridine nucleotide being transferred to the flavin (Massey & Schopfer, 1986). The mechanism of NADPH-dependent reduction of enones has been examined for ODE and cinnamaldehyde. Transfer of hydride from NADPH to the enone via the enzyme-bound flavin can occur with or without prior solvent exchange. To distinguish between these two alternatives, the reduction with NADPH was examined in 90% D_2O. Reduction of the protein-bound flavin by NADPH and exchange of the flavin hydride with D_2O followed by reduction of the α,β-unsaturated carbonyl compound should result in the incorporation of two deuterium atoms into the reduction product. Alternatively, in the absence of exchange of the flavin hydride with solvent, reduction of the substrate in D_2O should result in the incorporation of a single deuterium atom into the reduced product. As shown in Figure 5, panels A and B, the reduction of ODE and cinnamaldehyde to 3-oxodecalin and 3-phenylpropionaldehyde, respectively, results in a single deuterium atom uptake from solvent. The mass fragmentation pattern of [$^2H_1$]-3-oxodecalin is consistent with incorporation of the deuterium atom at the position α to the carbonyl group. The mass fragmentation pattern of [$^2H_1$]-3-phenylpropionaldehyde shows a strong molecular ion at *m/z* 135 corresponding to an 87% incorporation of a single deuterium atom from solvent. The strong fragment ion at *m/z* 91 indicates that the deuterium atom is not incorporated at the benzylic methylene carbon, whereas the fragment ion at *m/z* 106 indicates that incorporation of the solvent deuterium atom occurs at the methylene carbon adjacent to the aldehyde function. When cinnamaldehyde was reduced with [4-pro-*R*-$^2H_1$]NADPD in H_2O, the mass spectrum of the reduction product shows that a single deuterium atom is incorporated (Figure 5, panel C). The fragment ion at *m/z* 92 indicates that the deuterium atom is incorporated at the benzylic methylene carbon. When the [4-pro-*R*-$^2H_1$]NADPD-

Table 1: Reactivity of OYE with Enones and Phenols[a]

Compound (common name)	Turnover Number (min-1)	Compound (common name)	Turnover Number (min-1)	Compound (common name)	Turnover Number (min-1)	Compound (common name)	Turnover Number (min-1)
 *2-Cyclohexenone	150	 *Cyclohexane-1,2-dione	130	 *Cinnamaldehyde	125	 α-Methyl cinnamaldehyde	110
 3-Methyl-2-cyclohexenone	2.5	 Cyclohexane-1,3-dione	0.25	 Cinnamic acid	0.0	 Cinnamanitrile	0.0
 2-Cyclohexenol	0	 Cyclohexane-1,4-dione	0	 Methyl cinnamate	0.0	 Cinnamide	0.0
 Cyclohexanone	0	 2,6,6-Trimethyl cyclohex-2-ene-1,4-dione	35	 βIonone	0.0	 α-Ionone	0.5
 N-Ethyl maleimide	160	 Duroquinone	130	 *trans*-4-Phenyl-3-buten-2-one	33	 α-Estradiol	0.0
 *Methyl vinyl ketone	170	 Acrolein	170	 β-Estradiol	0.0	 Estrone	0.0
 3-Penten-2-one	96	 Crotonaldehyde	150	 Testosterone	0.0	 4-Androstene-3,17-dione	0.0
 Mesityl oxide	0.9	 3-Methyl-2-butenal	30	 4-Androstene-3,17-dione-19-aldehyde	0.1	 Δ1,4-Androstadien-3,17-dione	1.5
 2-Ethyl acrolein	130	 *Methyl acrolein	150	 Prednisone	0.0	 Progesterone	0.0
 3-Oxo-decalin-4-ene (ODE)	2.5	 α-Tetralone	0.3				
 3-Oxo-decalin-4-ene-10-methanol	0.0	 β-Tetralone	0.0	19-Nor-testosterone	0.0		
3-Oxo-decalin-4-ene-10-carboxaldehyde (ODEC)	6.0	Menadione	36				
Coumarin	6.5	4-Hydroxynonenal	~200				

[a] The turnover numbers reported were those obtained under anaerobic conditions in the presence of 100 μM NADPH and 1−2 mM enone in 0.1 M phosphate, pH 7.0, 25 °C. With those compounds marked by asterisks, the values shown are the true k_{cat} values obtained by systematic variation of both the NADPH and acceptor concentrations, giving sets of parallel Lineweaver−Burk plots typical of a ping-pong mechanism (Massey & Schopfer, 1986). The turnover numbers with these compounds under the standard single substrate concentrations were 85−90% those of true k_{cat} values. Separate stopped flow experiments (data not shown) showed biphasic reduction of the enzyme by NADPH, with rate constants 3.9 and 0.9 s^{-1}, the same as those given by the first and third isozyme forms separated by FPLC (Stott *et al.*, 1993). Reoxidation of the reduced enzyme by cyclohexenone under the same conditions occurred at a limiting rate of 65 s^{-1} and with a K_d for binding of cyclohexenone to reduced enzyme of 26 μM (Niino *et al.*, 1995). Hence, the rate-limiting step in NADPH-cyclohexenone reductase activity is the reduction of the enzyme flavin by NADPH. As the two isoforms of OYE in Brewer's Bottom Yeast occur in the ratio of approximately 60:40, this corresponds to an average reduction rate of 160 min^{-1}.

dependent reduction reaction was carried out in 85% D_2O, two deuterium atoms were incorporated into the reduced product (Figure 5, panel D). The mass fragmentation pattern shows a deuterium atom incorporated at each of the methylene groups of the [$^2H_2$]-3-phenylpropionaldehyde product. These deuterium atom incorporations from pyridine nucleotide and solvent are consistent with tranfer of the 4-*pro-R* hydrogen of NADPH to the β carbon of the α,β-unsaturated carbonyl compound and proton uptake from solvent at the α carbon.

The NADPH-dependent reduction of ODE shows biphasic kinetics with turnover numbers of 2.8 min^{-1} for the fast phase and 0.08 min^{-1} for the slow phase with approximately 50% extents of reaction for each phase based on the total ODE

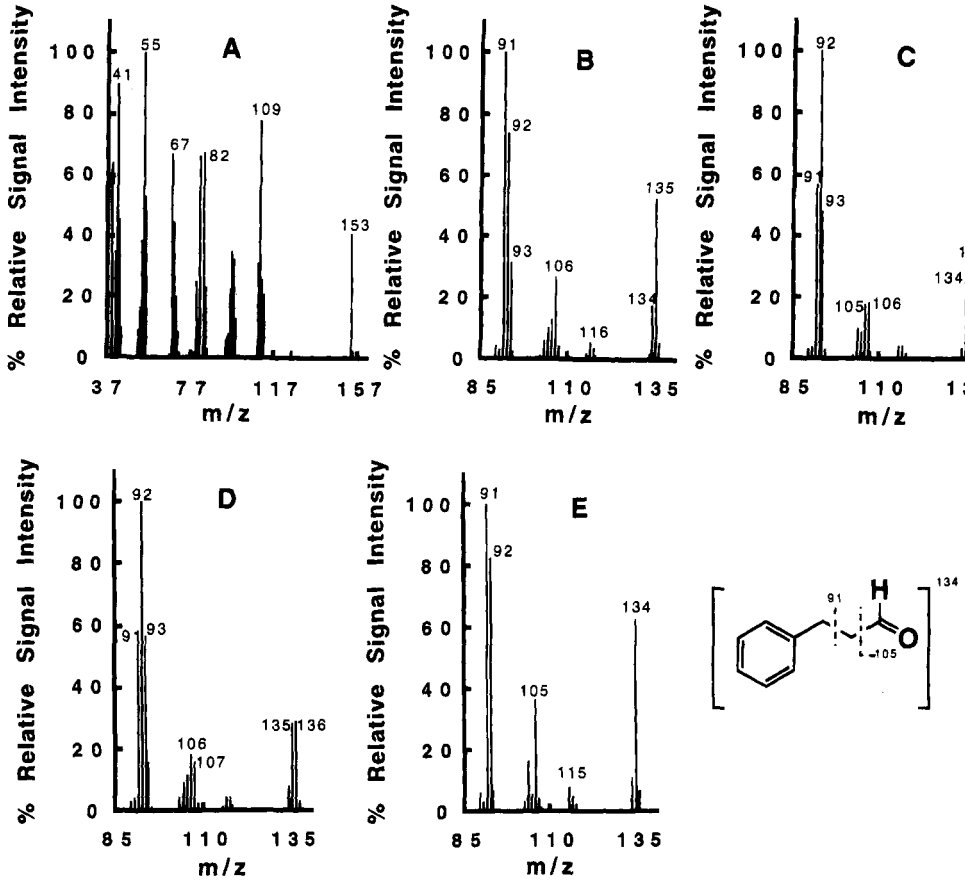


FIGURE 5: Electron impact mass spectra of products from the OYE-catalyzed reduction of ODE and cinnamaldehyde under various conditions. The standard reaction contained 0.1 M potassium phosphate buffer, pH 7.0, 1 mM ODE or cinnamaldehyde, 100−300 μM NADPH or [4-pro-*R*-$^2H_1$]NADPD, and an appropriate concentration of OYE (0.17 μM for cinnamaldehyde and 4 μM for ODE) in a final volume of 1.0 mL of H_2O or D_2O as stated. After completion of the reaction at 25 °C, the reaction mixtures were extracted with methylene chloride and analyzed by GC/MS as described in Methods. (Panel A): 3-oxodecalin from ODE formed in 90% D_2O; (panels B−D): 3-phenylpropionaldehyde from cinnamaldehyde in 85% D_2O with NADPH (panel B); in H_2O with [4-pro-*R*-$^2H_1$]NADPD (panel C); in 85% D_2O with [4-pro-*R*-$^2H_1$]NADPD (panel D); (panel E): 3-phenylpropionaldehyde standard. The fragmentation pattern of 3-phenylpriopionaldehyde is shown.

concentration (Figure 6, panel A). The reaction products were examined by GC/MS at the end of each phase of the reduction reaction. As shown in Figure 6, panel B, at the end of the fast phase primarily one isomer of 3-oxodecalin (presumably the *cis* isomer) is observed along with residual substrate. At the end of the slow phase, analysis of the reaction mixture shows essentially a 1:1 ratio of the *cis* and *trans* isomers of 3-oxodecalin and complete consumption of the substrate (Figure 6, panel C). Since ODE was used as a racemic mixture, the results suggest that the biphasic kinetics of reduction of ODE are a consequence of differential rates of reduction of the enantiomeric forms of ODE. It remains to be established which enantiomeric forms of ODE result in the *cis* and the *trans* forms of 3-oxodecalin.

DISCUSSION

The interaction of phenols with OYE has long been known to result in long wavelength absorbtion bands with wavelength maxima from 500 to 800 nm, the maximum being dependent on the structure of the phenol. These bands were assigned to π-π charge transfer interactions between the phenolate and the oxidized flavin (Abramovitz & Massey, 1976b; Massey *et al.*, 1984). Although an alternate explanation has been proposed for the long wavelength band (Eweg *et al.*, 1982), recent X-ray crystallographic studies of OYE by Fox and Karplus (1994) are consistent with the charge transfer concept. Reduced OYE does not interact appreciably with the phenols. The observation that α,β-unsaturated enones such as 2-cyclohexenone interact with OYE to form charge transfer bands similar to those formed with phenols was interpreted as an enolate complex of the enone and the flavoprotein (Stott *et al.*, 1993; Massey, 1993). Our present study has demonstrated that the enones are oxidized to the corresponding phenols, and the charge transfer band is a consequence of the interaction of the resultant phenol with OYE. As demonstrated with stereospecifically deuterated ODEC, the oxidation involves a stereospecific *trans* dehydrogenation α',β' to the carbonyl group. Furthermore, the β'-hydrogen of the substrate is transferred to the N_5 of the enzyme-bound flavin. For such a transfer to occur, the β' hydrogen must approach the N_5 atom within 1.5−2.5 Å. As shown for the aromatization of ODE in Figure 7A, this may be achieved by placing the substrate on the *si* face of the flavin in a configuration similar to that for phenolate as established for the crystal structure of the OYE−*p*-hydroxybenzaldehyde complex (Fox & Karplus, 1994). In this configuration, the C_1 hydrogen of ODE that faces the *si* face of the flavin is in bonding distance to the flavin N_5 atom. Hydride abstraction from the C_1 position of the substrate and proton loss from the C_2 position then yields the $\alpha'\beta',\alpha\beta$-dienone desaturated product, which is the thermodynamically less stable keto tautomer of the phenol. Enolization results in the phenolate, which can interact with oxidized flavoprotein to form the charge transfer complex. The catalytic

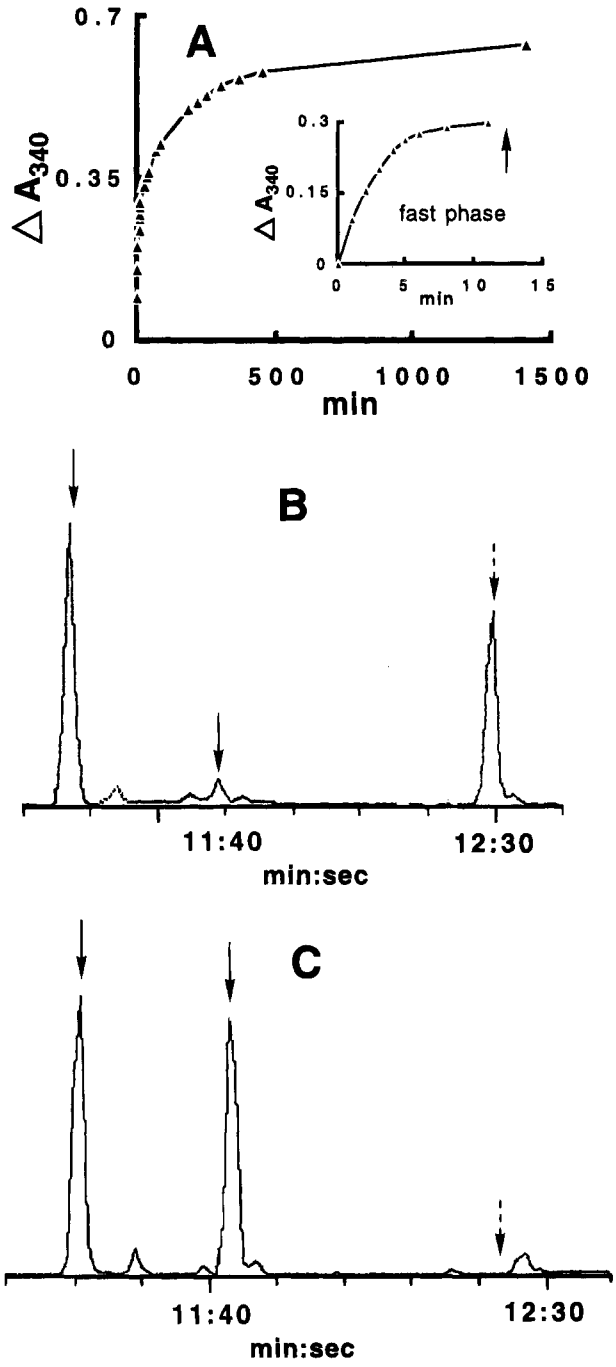


FIGURE 6: Kinetics and product analysis by GC/MS of the OYE-catalyzed anaerobic reduction of ODE by NADPH. The reaction mixture contained 0.1 M Tris-HCl buffer, pH 8.0, 100 μM ODE, 200 μM NADPH, and 5.1 μM OYE in a final volume of 1.0 mL. The kinetics were followed by monitoring the absorbance at 340 nm. The reactions were maintained at 25 °C. (Panel A): Kinetic plot showing the fast and slow phases of the NADPH oxidation by ODE; (panels B and C): relative ion chromatograms of the methylene chloride extracts of the reaction mixtures 14 min (panel B) and 25 h (panel C) after initiation of the reaction. The solid arrows show product peaks whose mass spectra correspond to the geometric isomers of 3-oxodecalin (data not shown). The dashed arrows show the substrate after the fast phase (panel B) and its complete consumption after the slow phase (panel C) as determined by no peak at the expected elution position.

oxidation of the enones to the phenols by OYE implies the existence of an ultimate electron acceptor in the reaction system. Reoxidation of the flavin can occur by molecular oxygen as in the aerobic reaction of ODEC or, as demonstrated, by a second molecule of the substrate that is reduced at the olefinic bond. As determined by solvent deuterium

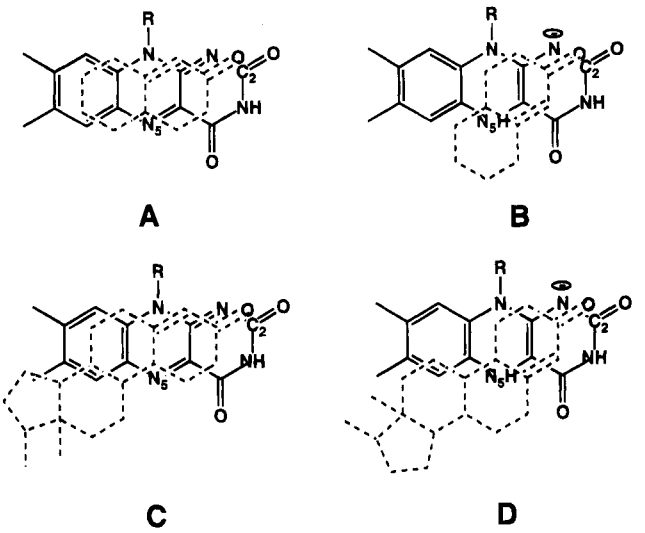


FIGURE 7: (Panels A and B): Schematic diagrams showing the proposed orientation for ODE with respect to the N_5 atom of flavin for the oxidative and reductive halves, respectively, of the dismutation reaction. The carbonyl oxygen has been placed in a position similar to the phenolate oxygen of *p*-hydroxybenzaldehyde as determined from the crystal structure of Fox and Karplus (1994) and with the rings coplanar with the flavin; (panels C and D): schematic representation of the 3-oxo-Δ^4-steroid nucleus coplanar with the flavin, consistent with the ability of OYE to effect oxidation and reduction at the C_1,C_2 carbon center (C) but not at the C_4,C_5 center (D). The orientation of the steroid in panel C is the same as that found by Fox and Karplus (1994) for β-estradiol.

uptake studies, the reductive half of the dismutation reaction occurs with hydride transfer from the oxidized substrate to the β-carbon of the enone via the enzyme-bound flavin. If binding of the substrate to the reduced flavoprotein is assumed to be similar to that of phenolate to the oxidized protein, then the substrate must be oriented such that the β-carbon of the enone is in bonding distance to the N_5 hydride. As shown in Figure 7A,B for ODE, this requires that the binding mode be distinct for the oxidative and reductive halves of the dismutation reaction. Thus, it is entirely probable that some cyclic enones may bind favorably for either the oxidative or reductive half of the dismutation reaction and have unfavorable steric effects with the protein structure at the active site for the other half of the dismutation reaction. Also from solvent deuterium atom uptake studies, the reductive half of the dismutation reaction appears to be similar to the NADPH-dependent reduction of enones as discussed below.

Some cyclic enones, such as 4,4-dimethyl-2-cyclohexenone, bind to OYE as determined by pertubation of the flavin spectrum and reduction of the olefinic bond by NADPH; however, such enones do not form charge transfer complexes with OYE and are not oxidized to the corresponding α,α'-dienones. The inability of OYE to oxidize such enones to the α,α'-dienones may reflect an unfavorable redox potential of such enones from that of the aromatizable enones. However, ODEC, which resembles such a structure, has been clearly demonstrated to undergo the dismutation reaction with OYE. The formyl group at the C_{10} position of ODEC is extremely labile in the α,α'-dienone structure and is readily hydrolyzed in water to yield formate and the aromatic product (Vaz, unpublished observations). This may account for its ability to be aromatized by the enzyme and for the slight shift in the spectral maximum observed at early time points during the development of the charge transfer complex. However, no evidence for the α,α'-dienone from

ODEC was obtained in mass spectral analysis of reaction products. The incorporation of a solvent-derived proton into HTN from the aromatization of ODEC suggests a somewhat more complex mechanism that is as yet unclear.

The stereospecific dehydrogenation observed with ODEC implicates specifc roles for active site functional groups in catalysis. The crystal structure of OYE provides a framework for speculation of potential residues and their role in the dismutation cycle. As previously assumed, if the binding of enones is comparable to that of phenols, then His 191, which hydrogen bonds to the phenolate, may be regarded as a general acid in the active site. In the oxidative half of the reaction, such an acid may function to protonate or hydrogen bond to the carbonyl group of the enones. Protonation would serve to increase the acidity of the C_2 carbon acid by stabilizing the enol form. The stereospecific loss of the $C_{1\beta}$ hydride and $C_{2\alpha}$ proton from ODEC indicates that the β-face of ODEC is proximal to the *si* face of the flavin and that a general base on the α-face of ODEC must serve to abstract the $C_{2\alpha}$ proton. An examination of the crystal structure shows that the phenolic oxygen of Tyr 196 may possibly function in such a capacity. Since unreacted substrate does not appear to accumulate deuterium from solvent when the reaction is done in 90% D_2O, an enolization equilibrium between enzyme-bound and free substrate clearly does not precede hydride abstraction by the flavin. It is tempting to speculate that hydride abstraction by N_5 of the flavin precedes or is in concert with proton abstraction. We favor the concerted process on the basis that hydride abstraction prior to proton loss would result in a carbonium ion at the C_1 carbon, which would then favor nonspecific proton loss from the C_2 carbon. This is contrary to the observed reaction stereochemistry. For similar reasons, the reductive half of the dismutation reaction is also expected to be stereospecific.

Of a number of α,β-unsaturated carbonyl compounds examined, only α,β-unsaturated enones were found to be substrates that promoted NADPH oxidation by OYE and were reduced at the olefinic bond. α,β-Unsaturated acids, esters, amides, and nitriles were not substrates for the reaction. Aldehyde and ketone carbonyl groups are far more basic than ester and amide carbonyl and cyano groups (Palm *et al.*, 1966; Grundres & Klaboe, 1970). If His 191 functions as a general acid at the active site to protonate the carbonyl oxygen of aldehydes and ketones that undergo redox chemistry, its estimated pK_a of 15 (Fox & Karplus, 1994) would appear inadequate to protonate the carbonyl group of esters and amides. Furthermore, such a protonation would increase the electrophilicity of the β carbon of the enone toward the flavin hydride. This may account for the reason why carbonyl compounds other than aldehydes and ketones are not substrates for the NADPH-dependent reduction of the olefinic bond. Within the aldehyde and ketone class of α,β-unsaturated enones, structural constraints have been observed. Increasing the alkyl substituents at the β carbon of the substrate affects the rate of hydride transfer to the olefinic bond, consistent with a steric hindrance to hydride transfer from the N_5 position of the flavin. The reduction by NADPH of racemic ODE proceeds in a biphasic manner. Each phase contributed approximately 50% to the overall reaction with the fast phase associated with essentially a single geometric isomer, the chromatographic characteristics of which on the phenyl methylsilane capillary column suggest it to be the *cis* ring-fused product. The slow phase of the reaction results in the other isomer, presumably the *trans* ring-fused product. The identity of these isomers has not been rigorously established; however, the results clearly demonstrate that the biphasic kinetics are a consequence of enantiomeric selectivity by OYE. In this connection, it is worth noting that the dismutation reaction with racemic ODE also results in enantioselectivity as determined by the ratio of isomeric products observed on partial and complete consumption of ODE. At this point the entioselectivity in the oxidative half of the dismutation reaction has not been clearly established.

In view of the ability of OYE to catalyze the reduction of the olefinic bond in α,β-unsaturated carbonyl compounds, it is likely that reduction of quinones by OYE also involves reduction of the olefinic bond, resulting in an unstable product which on enolization gives the hydroquinone. Thus, the mechanism of reduction of quinones by OYE may be different from that with other flavoproteins where reduction of the carbonyl oxygen is commonly assumed. *p*-Benzoquinone and duroquinone are good substrates of OYE-catalyzed NADPH oxidoreductase activity.

A number of Δ^4-3-keto steroids such as progesterone, testosterone, and androst-4-ene-3,17-dione bind tightly to OYE but fail to be reduced at the Δ^4-double bond. The $\Delta^{1,4}$-diene steroid, androst-1,4-diene-3-one, is reduced by NADPH and OYE to androst-4-ene-3,17-dione; also 19-nortestosterone undergoes oxidation by OYE to yield β-estradiol. Both these substrates involve the C_1,C_2 positions of the steroid nucleus. From the studies presented above, these results can be rationalized on the basis of orientation of the steroid in only one configuration with respect to the *si* face of the flavin, as seen in Figure 7C, such that the C_1 carbon of the steroid is proximal to the flavin N_5 atom, and any configuration which places the C_5 atom of the steroid proximal to the N_5 atom of the flavin encounters unfavorable steric effects with the protein structure at the active site.

A structural/mechanistic question raised by significant retention of the hydride in the NADPH-dependent reduction reaction as well as the dismutation reaction is the access of solvent water to the reduced flavin N_5 position. The crystal structure reveals that the flavin *si* face is exposed to solvent in the oxidized and reduced states, and it is this face to which the phenolates bind (Fox & Karplus, 1994). It has long been known that flavoproteins, such as pig heart diaphorase (lipoamide dehydrogenase), that catalyze transhydrogenase reactions do so with solvent proton exchange (Weber *et al.*, 1957). Furthermore, NMR studies of reduced flavin in free solution demonstrate that the N_5 hydride exchanges with solvent protons in a pH-dependent manner with rates of $\sim$200 s^{-1} at pH 7.0 and $\sim$10 s^{-1} at pH 9.5 (Ghisla *et al.*, 1990). These exchange rates are not greatly in excess of the oxidation rate of reduced OYE by cyclohexenone (see footnote to Table 1), which is among the faster reacting substrates with OYE. Rates for the oxidation of reduced enzyme with cinnamaldehyde and ODE are unavailable. That the reductions of cinnamaldehyde and ODE and the dismutation of ODE under anaerobic conditions proceed apparently without solvent proton exchange of the flavin N_5 hydride as determined by a single proton uptake from solvent by the reduced products suggest solvent expulsion from the environment of the flavin N_5 hydride with these substrates. However, the dismutations of ODEC and 2-cyclohexenone proceed by an excess of one solvent proton uptake, indicating limited access of solvent to the environment of the flavin N_5 hydride with substrates such as 2-cyclohexeneone and

ODEC. Thus, it appears that accessibility of solvent in the flavin environment is controlled dynamically by the substrate at the active site.

A recent report by French and Bruce (1994) on the metabolism of morphine by *Pseudomonas putida* M10 has demonstrated another FMN-containing dimeric flavoprotein reductase that specifically reduces the olefinic bond of morphinone. These investigators also found the enzyme to be active with 2-cyclohexenone but not with the α,β-unsaturated acids. Δ^4-Steroids were also found to bind to the enzyme but were not reduced at the olefininc bond. The enzyme appears to share similar substrate specificity but utilizes NADH instead of NADPH. Whether it catalyzes the aromatization of compounds such as 2-cyclohexenone is not known. The N-terminal amino acid sequence has no homology to that of known OYE isoforms. However, it should be noted that primary amino acid sequence homology may have no connection to the functional aspects of the enzymes from *Pseudomonas putida* and Brewer's Bottom Yeast.

ODEC was designed as a substrate analog of the 19-oxo steroid intermediate in the aromatase cytochrome P450 reaction. The P450-catalyzed aromatization of ODEC is molecular oxygen-dependent and occurs by a stereospecific *cis* oxidative deformylation of the $C_{1\beta}$ hydrogen and the formyl group, with nonspecific enolization at the C_2 position (Vaz *et al.*, 1994). In contrast, the OYE-catalyzed reaction is not molecular oxygen-dependent and involves a stereospecific *trans* dehydrogenation of the $C_{1\beta},C_{2\alpha}$ hydrogens, with the formyl group presumably hydrolytically removed as formate. The ability of OYE to effect aromatization of 19-nortestosterone to β-estradiol poses an intriguing question as to the role of this enyzme in yeast. It is worthy to note that estrogen receptors have recently been identified and cloned from yeast, although no distinct role for such receptors or steroid hormones in yeast has been identified (Madani *et al.*, 1994). Furthermore, 19-norandrogens have been identified in mamalian steroid hormone-synthesizing tissue (Short, 1962; Dehennin *et al.*, 1984; Silberzahn *et al.*, 1985), but no clear role for such anabolic steroids has been identified. Although it is known that 19-norandrogens can be aromatized to estrogens by the aromatase cytochrome P450 system (Kellis & Vickery, 1987; Gaillard & Silberzahn, 1987; Raeside *et al.*, 1989), it is tempting to speculate the presence of an as yet unrecognized "aromatase flavoprotein" in mammalian tissue that is distinct from the well-recognized aromatase cytochrome P450 hemoprotein. In this connection, the observations of Raeside *et al.* (1989) on the production of estrogens from norandrogens by boar Leydig cells in culture and of Dintinger *et al.* (1989) on the partial inhibition of the aromatization of norandrogens by testosterone in equine testicular homogenates may be particularly relevant.

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