Nature of the Intermediate in the 3-Oxo-Δ^5-steroid Isomerase Reaction†

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ABSTRACT: The role of Tyr-14 of 3-oxo-Δ^5-steroid isomerase (KSI) was probed by analysis of the spectra of 3-amino-1,3,5(10)-estratrien-17β-ol (**4**) and equilenin (**5**) bound to the active site of KSI. The ultraviolet spectrum of **4** bound to KSI is identical to that for **4** in neutral solution. This observation indicates that Tyr-14 does not protonate the amine group of **4** at the active site. By analogy, it is argued that the 3-oxo group of steroid substrates for KSI is not protonated during the reaction. In contrast, the fluorescence excitation spectra of **5** bound to KSI show characteristics of an ionized phenol, even at pH values as low as 3.8. It is concluded that the pK_a of equilenin is perturbed from its value in solution of 9 to $\leq$3.5 at the active site of KSI. Similarly, the pK_a of the intermediate dienol in the KSI reaction should be lowered to $\leq$4.5 when it is bound to KSI. Thus, the function of Tyr-14 as an electrophilic catalyst is likely the stabilization of the anion of the dienol by hydrogen bonding rather than by proton transfer.

The formation of enol and/or enolate ion intermediates is a critical step in a variety of enzymatic reactions, such as those catalyzed by triosephosphate isomerase (Rieder & Rose, 1959; Albery & Knowles, 1976a; Rose et al., 1990), glyoxylase I (Hall et al., 1978a,b; Kozarich et al., 1981; Creighton & Pourmotabbed, 1988), pyruvate kinase (Kuo et al., 1979; Rose & Kuo, 1989), mandelate racemase (Kenyon & Hegeman, 1979; Kenyon & Whitman, 1986), horseradish peroxidase compound I (Bohne et al., 1986), and 4-oxalocrotonate tautomerase (Whitman & Stolowich, 1991) [for a review of the biochemistry of enols, see Richard (1990)]. One particularly well-studied enzyme that functions through an enol(ate) intermediate is the 3-oxo-Δ^5-steroid isomerase (EC 5.3.3.1) from *Pseudomonas testosteroni*, which catalyzes the isomerization of 3-oxo-Δ^5-steroids (**1**) to their conjugated Δ^4-isomers (**3**) [for reviews, see Pollack et al. (1989a) and Schwab and Henderson (1990)]. This enzyme [also called Δ^5-3-ketosteroid isomerase (KSI)[1]] utilizes the carboxylate of Asp-38 as a base to abstract the 4β-proton to form a dienol(ate) [**2a(b)**], with the hydroxyl group of Tyr-14 acting to polarize the carbonyl, either by proton donation or by hydrogen bonding (Benisek et al., 1980; Viger et al., 1981; Bounds & Pollack, 1987; Kuliopulos et al., 1989). Subsequent protonation by the conjugate acid of Asp-38 at C-6β completes the reaction (Scheme I).

Although the existence of a dienol(ate) intermediate in the isomerization is on firm ground (Eames et al., 1990; Hawkinson et al., 1991a), the timing of the proton transfers in the formation and reketonization of the dienol(ate) is unclear. Three possibilities have been considered (Scheme II): (a) proton transfer to the carbonyl preceding loss of the 4β-proton, with the formation of an oxocarbonium ion intermediate (Malhotra & Ringold, 1965; Bevins et al., 1984; Kuliopulos et al., 1989); (b) concerted proton transfer from Tyr-14 to the carbonyl and from C-4β to Asp-38 (Xue et al., 1990; Kuliopulos et al., 1990, 1991); and (c) deprotonation at C-4 by Asp-38 with only hydrogen bonding from Tyr-14 in the transition state, producing an intermediate carbanion (Pollack et al., 1989a; Zeng & Pollack, 1991).

In order to evaluate the possibilities, we have probed the nature of the intermediate by examining the spectral characteristics of 3-amino-1,3,5(10)-estratrien-17β-ol (**4**) and equilenin (**5**) when bound to KSI. These compounds are analogues of the intermediate dienol (**2**) and can be protonated or deprotonated, respectively, with relative ease. In each case, spectral changes between the ionized and unionized forms permit evaluation of the protonation state of the bound species (Scheme III). No spectral evidence for protonation of the amine was found for the interaction of **4** with KSI, whereas fluorescence measurements of the KSI–equilenin complex indicate that the bound species is anionic, even at pH $\leq$ 3.8. These data suggest an enolate intermediate in the catalytic reaction with **1**, with stabilization by hydrogen-bond formation with the phenolic hydroxyl group of Tyr-14.

Experimental Procedures

Native 3-oxo-Δ^5-steroid isomerase, available from previous work (Bounds & Pollack, 1987), was used for the UV spectral studies with **4**. Recombinant isomerase (Eames et al., 1989) was used for the fluorescence experiments with **5**. Equilenin was obtained from Aldrich and was used without further purification. Thin-layer chromatography on silica gel with ethyl acetate/hexane (1:2) showed only a single spot.

3-Amino-1,3,5(10)-estratrien-17β-ol (**4**) was obtained by base hydrolysis of 3-acetamido-1,3,5(10)-estratrien-17β-ol acetate prepared by the method of Gold and Schwenk (1959). The reactant (190 mg) was refluxed for 48 h with a solution of 100 mL each of 2-propanol and 2 N NaOH. The mixture was cooled to room temperature and extracted with ether (3 × 30 mL); the organic layers were then extracted with 1 N HCl (3 × 30 mL). The aqueous layers were adjusted to pH > 9.5 by the addition of 12 mL of 10 N NaOH and extracted with dichloromethane (3 × 30 mL). The organic layers were washed with water and with saturated sodium chloride solution, dried (Na_2SO_4), and evaporated to any oily residue (131 mg). The product was purified by chromatography on 8 g of silica gel (Woelm Dry Pack) with 5% (v/v) 2-propanol in

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[1] Abbreviation: KSI, 3-oxo-Δ^5-steroid isomerase.

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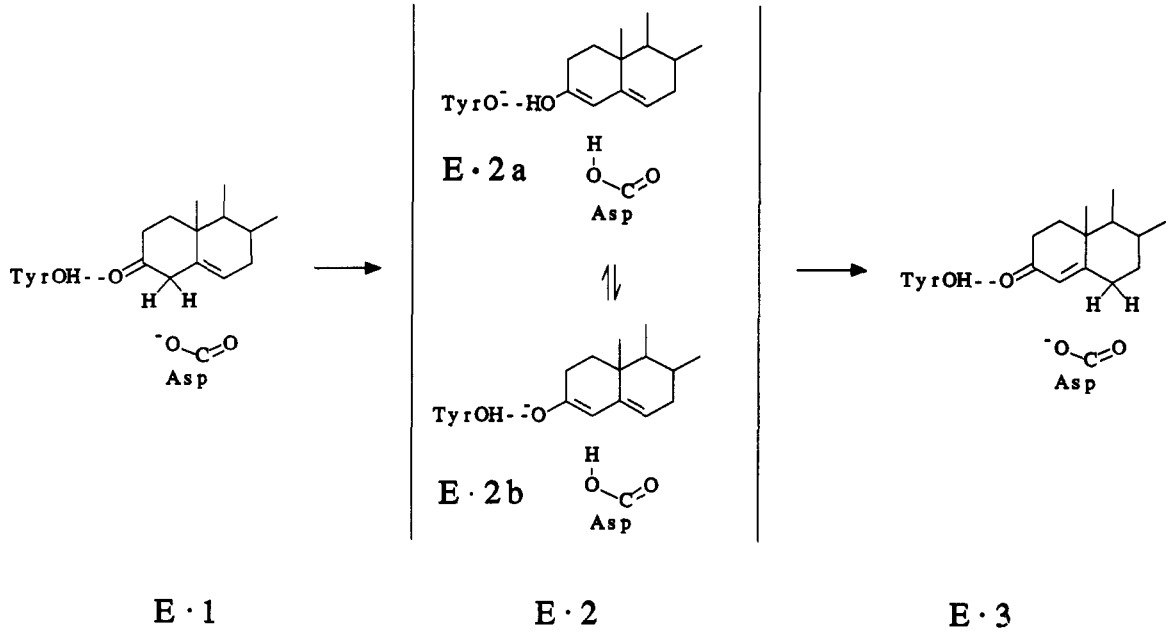
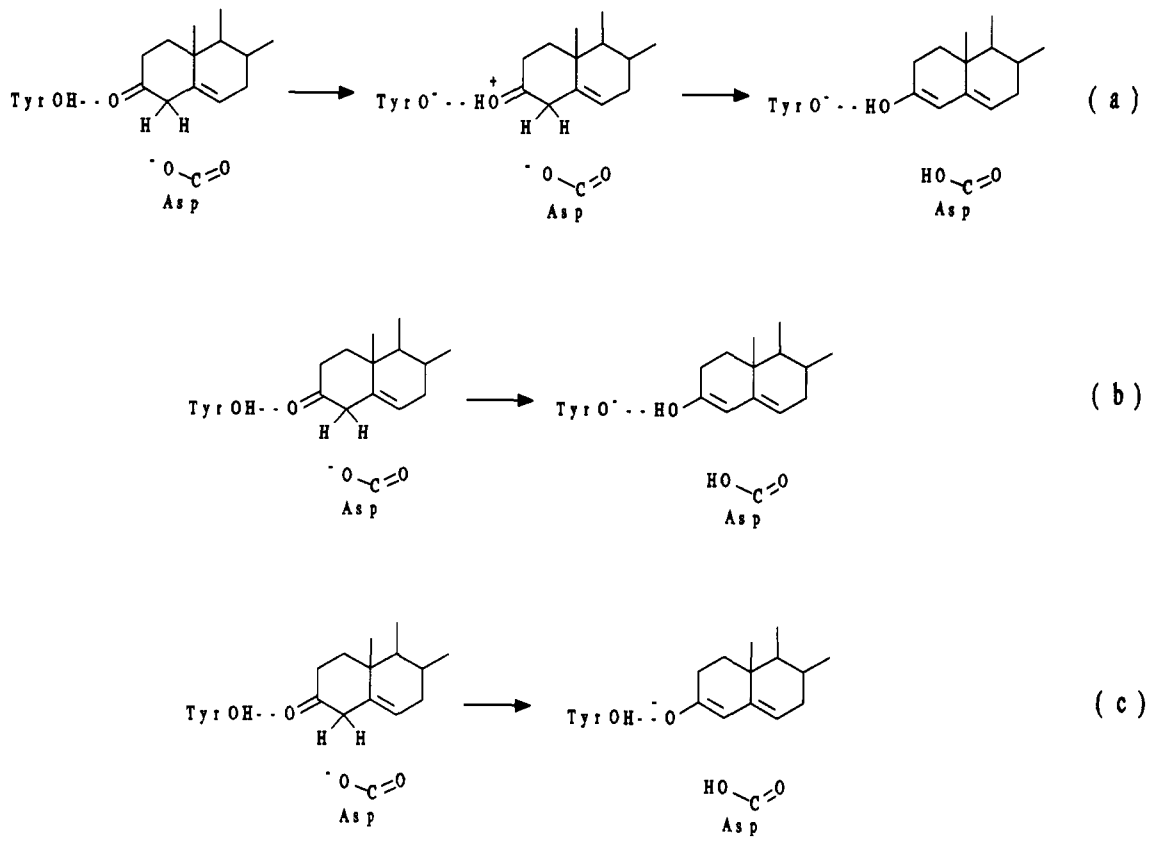
Scheme I



Scheme II



dichloromethane as the mobile phase. The fractions containing product were pooled and evaporated to an oily residue in a stream of nitrogen. The addition of a small amount of methanol resulted in the formation of a fine crystalline precipitate. The methanol was evaporated in a stream of nitrogen to give 88 mg (34%) of solid, which was then recrystallized from methanol: mp 136–138 °C; λ_{max} 235 nm, ϵ 8400 [lit. mp 143 °C, λ_{max} 237, ϵ 7900 (Gold & Schwenk, 1959)].

Determination of the pK_a of the Conjugate Acid of 3-Amino-1,3,5(10)-estratrien-17β-ol (**4**). The aniline-like UV chromophore of the steroid was used as an index of its protonation state. The absorbance at 235 nm was determined for solutions of **4** [3.84 × 10^{-5} M, 1% (v/v) methanol] in 0.1 N HCl (pH 1.3), 0.02 M potassium carbonate (pH 10.8), and 0.02 M sodium acetate buffer (pH 4.0–5.8). The pK_a was determined from a weighted least-squares fit of the absorbance data to eq 1, with K_a, A_a, and A_b treated as variables, where A = absorbance of the solution, A_b = absorbance of the free amine, and A_a = absorbance of the protonated amine. All measurements were performed at room temperature (ca. 21 °C).

$$A = (K_a A_b + A_a[H^+])/([H^+] + K_a) \quad (1)$$

Inhibition of the Isomerase by 3-Amino-1,3,5(10)-estratrien-17β-ol (**4**). We have previously described the determination of first-order rate constants (k^{obs}) for the reaction

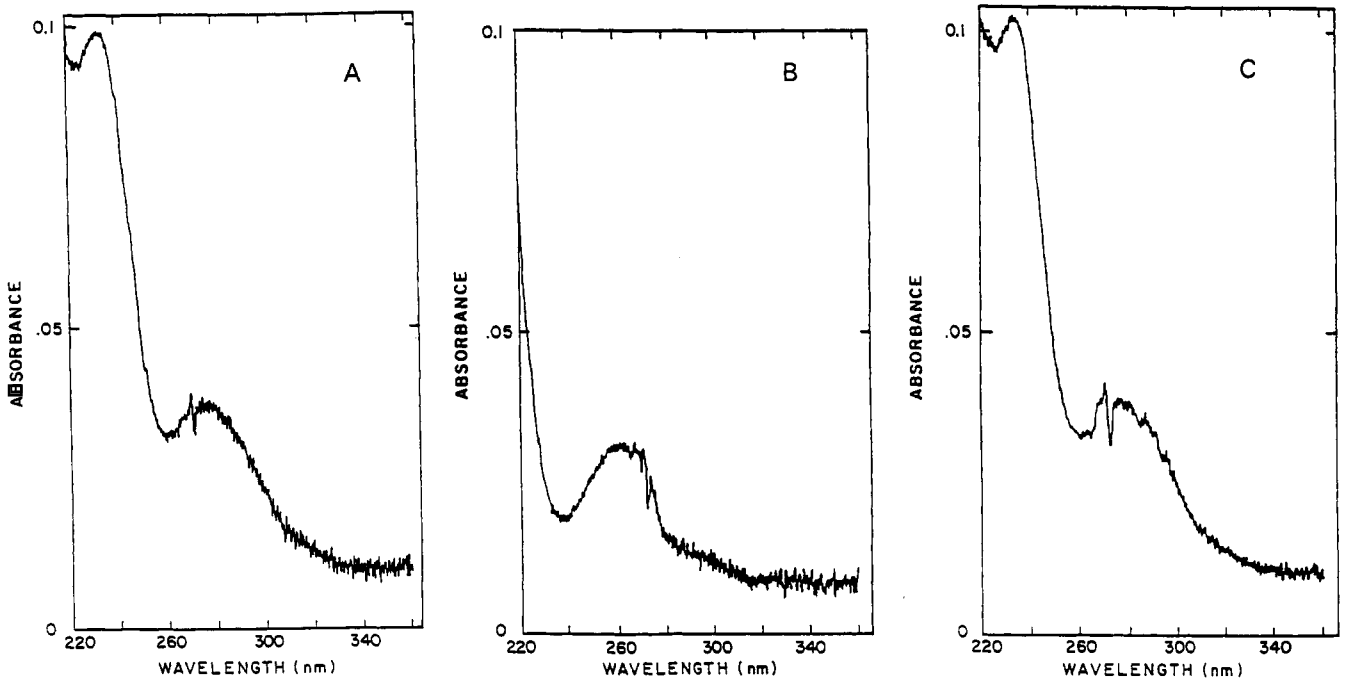
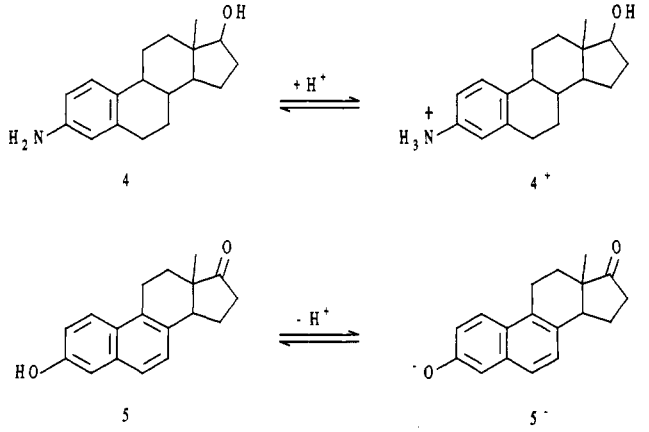


FIGURE 1: Ultraviolet absorbance spectra of 3-amino-1,3,5(10)-estratrien-17β-ol (93.2 μM) in (A) 0.02 M potassium phosphate, pH 7.0, and (B) 0.02 M sodium formate, pH 3.5, and (C) the difference spectrum of 3-amino-1,3,5(10)-estratrien-17β-ol (93.2 μM) with 95.6 μM KSI in 0.01 M potassium phosphate, pH 7.0, against 95.6 μM KSI in the same buffer. All spectra are for a 1-mm path length.

Scheme III



of the isomerase in the presence of a competitive inhibitor and the subsequent calculation of the binding constant (K_i) for the inhibitor (Pollack et al., 1979). The reaction cell contained 3.00 mL of phosphate buffer (0.034 M, pH 7.0), 4% (v/v) methanol, 10 μM 5-androstene-3,17-dione as substrate, and varying amounts of the inhibitor 3-amino-1,3,5(10)-estratrien-17β-ol (0–184 μM). The reaction was initiated by the addition of 20 μL of enzyme (diluted in 0.1% BSA) of sufficiently high concentration to ensure complete isomerization of the substrate in less than 5 min. All measurements were made at room temperature (ca. 21 °C). Isomerization of the substrate was monitored by following the appearance of product at 248 nm. In the presence of a competitive inhibitor (I), the observed first-order rate constant (k^{obs}) may be expressed by eq 2, and the binding constant (K_i) can be calculated from the slope and intercept of a plot of $1/K^{obs}$ at constant enzyme concentration as a function of [I].

$$k^{obs} = \frac{k_{cat}[E]}{(K_m[I])/K_i + K_m} \quad (2)$$

UV Spectral Study of the KSI Complex with **4**. The UV absorbance spectrum of 3-amino-1,3,5(10)-estratrien-17β-ol (93.2 μM) was recorded in neutral (0.02 M potassium phosphate, pH 7.0) and acidic (0.02 M sodium formate, pH 3.5) buffers and in the presence and absence of enzyme (95.6 μM in 0.01 phosphate buffer, pH 7.0). The spectrum of the enzyme was subtracted from that of steroid recorded in the presence of protein; these spectra were measured in 1-mm-path-length cells to minimize background absorbance due to protein.

Fluorescence Measurements with Equilenin. Both static and dynamic fluorescence measurements were carried out as previously described (Eames et al., 1989). Background fluorescence excitation spectra due to buffer was always <1%. For spectra of equilenin–KSI complexes, the spectra were first taken of equilenin (ca. 1 μM) in the appropriate buffer solution, followed by addition of enzyme, and the spectrum of the resulting solution was recorded. Duplicate spectra taken after several minutes showed no change, indicating that enzyme denaturation was insignificant on the time scale of the experiment. The time-domain dynamic measurements were carried out at the Center for Fluorescence Spectroscopy, Department of Biological Chemistry, School of Medicine, University of Maryland at Baltimore (J. R. Lakowicz, Director).

RESULTS

3-Amino-1,3,5(10)-estratrien-17β-ol (**4**). The ultraviolet spectrum of 3-amino-1,3,5(10)-estratrien-17β-ol (**4**) was used as a probe for the protonation state of the amine group under various conditions. In neutral buffer, two maxima are seen for the free amine at 235 nm (ϵ_{max} 8600) and 278 nm (ϵ_{max} 2900) (Figure 1A). The spectrum of the protonated amine taken in acidic (pH 3.5) buffer shows a single maximum at 261 nm (ϵ_{max} 2360) and a large end absorption (Figure 1B). A pK_a of 4.92 for the amine group was determined from the UV absorbance at 235 nm for solutions of **4** in acid (pH 1.3), in base (pH 10.8), and at several pH values between 4.0 and 5.8. The spectrum of **4** (93.2 μM) was also recorded in the presence of KSI (95.6 μM) in 0.01 M phosphate buffer at pH 7 (Figure 1C) and is identical to that taken in neutral buffer.

Table I: Time Decay of Fluorescence Intensity for Equilenin (**5**)[a]

pH	KSI	α_1	τ_1 (ns)	α_2	τ_2 (ns)	α_3	τ_3 (ns)	$\bar{\tau}^b$ (ns)	χ^2
3.8[c]	no[f]			0.027	2.3	0.044	5.7	5.0	2.0
7.0[d]	no[g]					0.053	5.9	5.9	1.1
11.0[e]	no[g]			0.052	1.4	0.0016	5.7	1.9	1.3
3.8[c]	yes[h]	0.067	0.36	0.024	1.4	0.0079	4.9	2.5	1.4
7.0[d]	yes[i]			0.095	1.0			1.0	2.2

[a] The intensity is assumed to decay according to the relation $I(t) = \sum_i \alpha_i e^{-t/\tau_i}$. [b] The average decay time, defined by $\sum_i \alpha_i \tau_i^2 / \sum_i \alpha_i \tau_i$. [c] In 10 mM acetate buffer. [d] 10 mM phosphate buffer. [e] In sodium hydroxide solution with 10 mM phosphate. [f] [**5**] = 2.84 μM. [g] [**5**] = 1.36 μM. [h] [**5**] = 2.64 μM; [KSI] = 13 μM. [i] [**5**] = 1.31 μM; [KSI] = 8.1 μM.

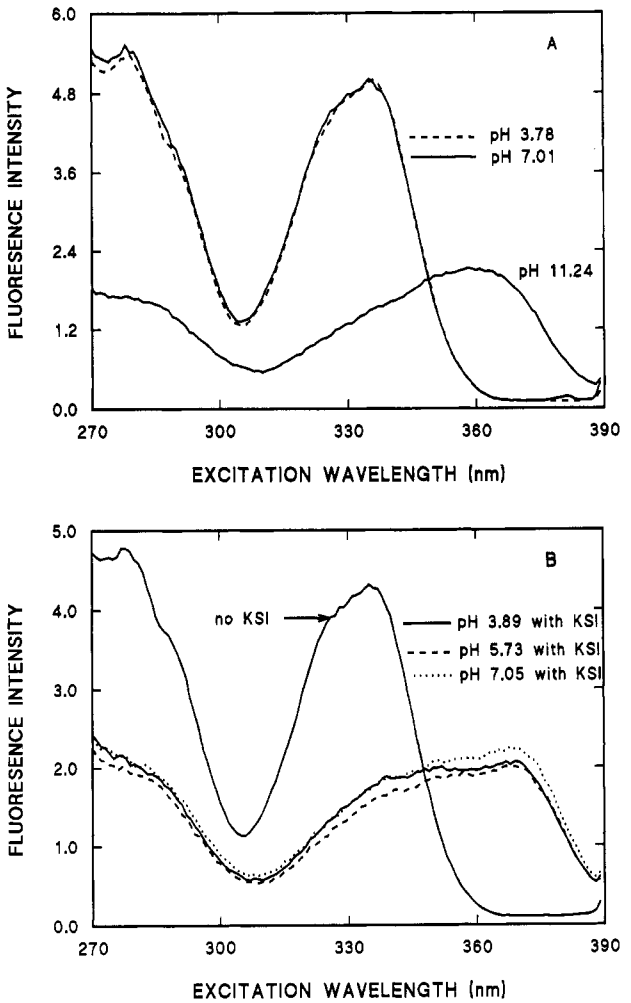


FIGURE 2: (A) Fluorescence excitation spectra of equilenin (1.36 μM) in acidic pH (3.78), neutral pH (7.0), and basic pH (11.24) solutions (1.4% methanol); (B) excitation spectra of equilenin (1.3 μM) in the absence and presence of KSI at various pH values. KSI concentrations were 9.24 μM at pH 3.89 and 7.92 μM at pH 5.73 and pH 7.05. The emission wavelength was 400 nm for both spectra.

A binding constant (K_i) for **4** was determined from its inhibition of the enzymatic isomerization of 5-androstene-3,17-dione. For a competitive inhibitor, K_i can be evaluated by a plot of the reciprocals of first-order rate constants in the presence of various concentrations of inhibitor (0–184 μM) as a function of inhibitor concentration (Pollack et al., 1979). This plot is linear and gives a K_i of 78 μM for **4**.

Equilenin (**5**). The fluorescence excitation spectrum of equilenin was recorded at pH 3.8, pH 7.0, and pH 11.2 (Figure 2A). In addition, spectra were obtained for equilinen in the presence of excess KSI at pH values of 3.9, 5.7, and 7.1 (Figure 2B). At each pH, the spectra of the bound species are essentially identical for modest (25%) changes in the concentration of KSI, suggesting that almost all of the equilenin is bound to KSI under the experimental conditions.

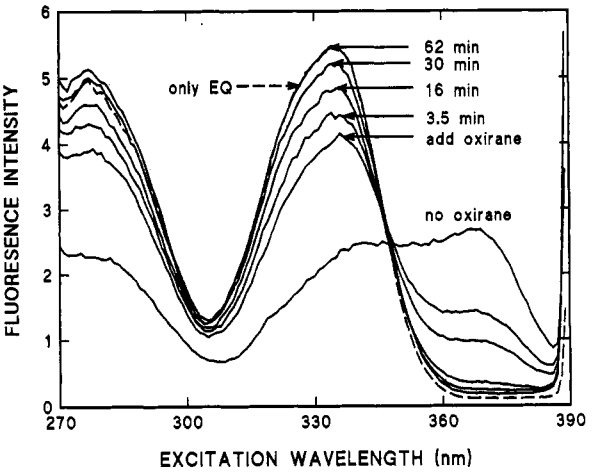


FIGURE 3: Fluorescence excitation spectra of equilenin (2.75 μM) at pH 3.8 (3.1% methanol) in the absence of KSI (dashed line) and in the presence of 9.7 μM KSI with no oxirane [(3*S*)-spiro[5α-androstane-3,2′-oxiran]-17-one)] and after addition of 69 μM oxirane at various time intervals. The emission wavelength was 400 nm.

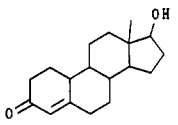
In order to confirm that equilenin binds at the active site of KSI, the active-site-directed irreversible inhibitor (3*S*)-spiro[5α-androstane-3,2′-oxiran]-17-one (Pollack et al., 1986) was added to a solution of equilenin and KSI at both pH 7.1 and pH 3.9. This oxirane is one of a variety of 3β-oxiranes that bind covalently and stoichiometrically at the active site of KSI (Bevins et al., 1984; Pollack et al., 1986; Bounds & Pollack, 1987). Upon addition of the inhibitor to a solution of equilenin and KSI at both pH 7.0 (28 μM oxirane) and at pH 3.8 (69 μM oxirane), the characteristic excitation spectrum of free equilenin slowly increases, as the equilenin is displaced from KSI by the oxirane (Figure 3). After ca. 1 h, the final spectrum is almost identical to that of free equilenin. A control with no oxirane gave no regeneration of the free equilenin spectrum over several hours, showing that the enzyme is stable over the time necessary to perform the experiment.

Dynamic Fluorescence. To further characterize the ionization state of equilenin at the active site at low pH, the time decay of fluorescence was monitored (Table I). The decay of fluorescence of neutral equilenin under these conditions (pH 3.8 and pH 7.0) is dominated by a major decay time of about 6 ns, although there is a significant component of ca. 2.3 ns at pH 3.8. In contrast, ionized equilenin (pH 11) exhibits a major decay time of ca. 1–2 ns. These results are in qualitative agreement with our previous observations (Eames et al., 1989). In the presence of excess KSI equilenin has an emission spectrum that is dominated by short decay time components, with an average decay time of about 1–2 ns at both pH 7.0 and pH 3.8.

Discussion

The spectra of chromophoric steroids bound to KSI were first utilized by Wang et al. (1963) as a tool to investigate the nature of the intermediates in the enzymatic reaction. Later,

Bevins et al. (1986), Kuliopulos et al. (1989), and Eames et al. (1989) expanded on that work. The principal ultraviolet absorption band of the competitive inhibitor 19-nortestosterone (**6**) undergoes a bathochromic shift from 248 to 258 nm upon binding to the enzyme (Wang et al., 1963; Kuliopulos et al., 1989). This spectral shift has been interpreted to indicate that the 3-oxygen of **6** is protonated in the enzyme–inhibitor complex (Kuliopulos et al., 1989). In contrast, characteristic fluorescence and/or absorption spectra of aromatic phenols bound to KSI, such as dihydroequilenin (**7**), equilenin (**5**), and estradiol (**8**), are thought to be due to ionization of the phenolic hydroxyl (Wang et al., 1963; Bevins et al., 1986; Kuliopulos et al., 1989; Eames et al., 1989).[2]



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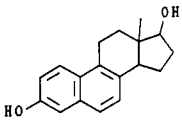

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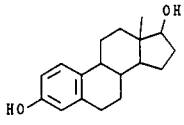

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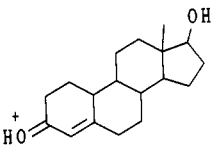
The apparent dual ability of KSI to protonate an α,β-unsaturated carbonyl group (pK_a ca. −3)[3] and to deprotonate phenolic steroids (pK_a's ca. 9; Davenport et al., 1986) has complicated the development of a mechanism of action for KSI. If protonation of 19-nortestosterone occurs when it binds to the enzyme, then it is likely that the mechanism of isomerization involves a cationic intermediate (protonated carbonyl). Alternatively, the formation of a phenolate ion from aromatic steroids suggests that deprotonation of the unconjugated steroid (**1**) leads to a dienolate ion, with no concurrent protonation of the oxygen.

In order to help clarify this ambiguity, 3-amino-1,3,5-(10)-estratrien-17β-ol (**4**) was used as an alternate probe for proton donation by KSI. In addition, we examined the fluorescence changes associated with binding of equilenin (**5**) to KSI as a function of pH. Both of these compounds serve as probes of the ionization state of intermediates in the KSI reaction. A steroid containing an aromatic amine group at the 3-position (**4**) might easily be protonated by an acidic residue at the active site of the isomerase, similar to the postulated protonation of 19-nortestosterone. Similarly, the phenolic group of **5** could ionize in the presence of a basic group of the enzyme, as has been suggested for the dienol intermediate **2**.

3-Amino-1,3,5(10)-estratrien-17β-ol (**4**) *as a Probe for a Protonated Intermediate.* The A-ring of this steroid contains a pH-sensitive chromophore, with a pK_a of 4.92, similar to that of aniline (pK_a 4.6; Kolthoff & Stenger, 1947). The ultraviolet absorption spectrum of **4** in neutral solution (Figure 1A) has wavelength maxima at 235 nm (ϵ_{max} 8400) and 278 nm (ϵ_{max} 2900). In acid, only one absorbance maximum is seen at 261 nm (ϵ_{max} 2360) (Figure 1B). In the presence of KSI, the ultraviolet absorption spectrum of **4** is identical to the spectrum taken in neutral buffer (compare parts A and C of Figure 1). The most straightforward explanation for the lack of a spectral shift upon the addition of KSI is that the amino group of **4** is not protonated by KSI.

There are two questions that must be addressed in considering the lack of protonation of the amine group of **4**. (1) Does **4** bind at the active site of KSI? (2) If it does, is the binding similar to that of the putative protonated ketone intermediate? A number of steroids have been shown to act as competitive inhibitors of KSI and presumably bind to the active site, among them are several that are structurally similar to **4** (Weintraub et al., 1977). Therefore, it is likely that **4** also binds at the active site. The K_i for **4** of 78 μM allows a calculation that 42% of the steroid is bound to the enzyme under the conditions of the spectral measurements. Certainly, if this fraction of **4** were protonated, it would be obvious from the spectrum. Thus, it appears that the amine group of **4** remains unprotonated at the KSI active site. Alternatively, the lack of protonation of **4** at the active site of KSI might come from an incorrect orientation of the steroid molecule that does not allow interaction between Tyr-14 of KSI and the steroid. Nonproductive modes of binding have been observed for a variety of steroids with KSI (Bevins et al., 1980, 1986; Kayser et al., 1983; Bounds & Pollack, 1987). However, it is likely that at least a portion of the steroid is bound to KSI in an orientation that would allow interaction between Tyr-14 and the amine group of **4** but that proton transfer does not take place.

This result is somewhat surprising in view of the conclusion that the carbonyl group of 19-nortestosterone (**6**) is protonated upon binding to KSI (Kuliopulos et al., 1989), coupled with the greater basicity of aromatic amines than carbonyl oxygens. Kuliopulos et al. (1989) observed that the ultraviolet spectrum of 19-nortestosterone undergoes a red shift from 248 to 258 nm upon binding to KSI. By comparing these spectra to corresponding spectra in 10 M HCl (λ_{max} 258 nm, with a pronounced shoulder at ca. 285 nm) and 10 M H_2SO_4 (λ_{max} 285 nm with a shoulder at 258 nm), they concluded that the species absorbing at 258 nm is the protonated ketone (**9**) and that the one absorbing at 285 nm is a protonated dienol (**10**).



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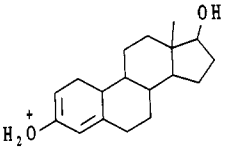

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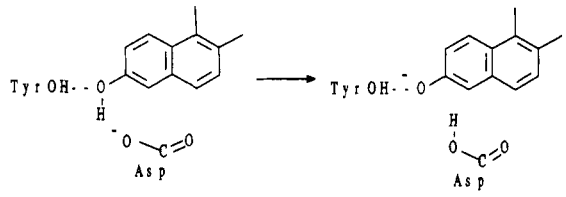
A consideration of previous results on the basicity of α,β-unsaturated carbonyl compounds and spectral shifts associated with changes in solvent polarity suggests that this interpretation is incorrect. The spectra of α,β-unsaturated ketones typically undergo wavelength shifts due to medium effects in acid solutions in addition to the effects produced by protonation (Zalewski & Dunn, 1969; Smoczkiewicz & Zalewski, 1968; Geribaldi et al., 1982). As the acidity of the medium increases, the λ_{max} for the unprotonated ketone increases by as much as 10–15 nm. This bathochromic shift is likely due to the greater ability of more acidic solutions to hydrogen bond to the carbonyl group.

Since the pK_a of 19-nortestosterone is ca. −3[3] and 10 M HCl has an H_A value of ca. −3.2 (Yates & Riordan, 1965), approximately equal amounts of protonated and unprotonated 19-nortestosterone should exist in this solution. Thus, the λ_{max} of 258 nm for 19-nortestosterone in 10 M HCl is almost certainly due to the *unprotonated* ketone, whereas the peak at 285 nm in 10 M sulfuric acid (and the shoulder at 285 nm in 10 M HCl) is characteristic of the protonated ketone (**9**) (Smoczkiewicz & Zalewski, 1968). Consequently, the peak at 258 nm for 19-nortestosterone bound to KSI is most rea-

[2] The possibility that these fluorescence changes may be due to a medium effect caused by a hydrophobic active site is unlikely, since similar spectral changes are not seen in the excitation spectra of equilenin in methanol, dioxane, or hexane.

[3] The pK_a of 19-nortestosterone was estimated as −4.3 in sulfuric acid based upon the H_0 scale (Smoczkiewicz & Zalewski, 1968). Later, Geribaldi et al. (1982) determined that the H_A acidity function is more appropriate for measuring the acidities of protonated α,β-unsaturated ketones. Conversion of the pK_a to the H_A scale using the data of Yates et al. (1964) gives a pK_a of ca. −3.1 for 19-nortestosterone.

Scheme IV



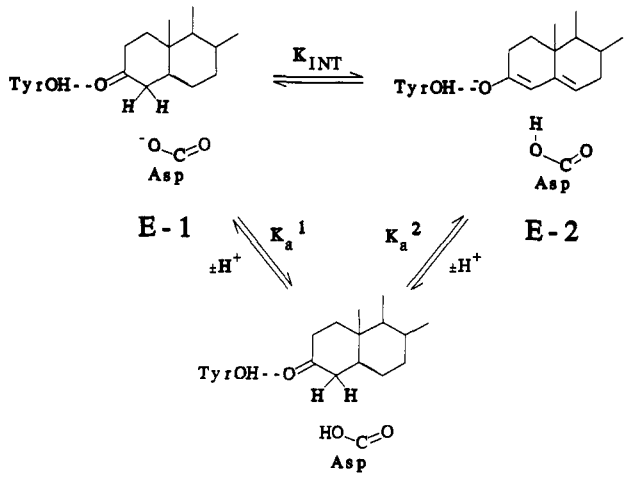
sonably assigned to the unprotonated ketone. This interpretation is consistent with hydrogen bonding from the hydroxyl group of Tyr-14 to the carbonyl being stronger than hydrogen bonding from water in aqueous solution.

Equilenin (5) as a Probe for an Anionic Intermediate. Both the emission and excitation spectra of equilenin and of dihydroequilenin at pH 7 previously have been interpreted to indicate that the phenolic hydroxyl is ionized at the active site of KSI (Wang et al., 1963; Eames et al., 1989). We have now extended this work to moderately acidic solutions. Figure 2 shows that the excitation spectrum of equilenin in the presence of KSI continues to resemble that of ionized equilenin even at pH values as low as 3.8. Dynamic fluorescence measurements provide further evidence that ionization of equilenin at the active site persists in moderately acidic solutions. The dominant decay times of equilenin in the presence of KSI at pH 3.8 and at pH 7.0 are similar, and both resemble the decay times observed for ionized equilenin at pH 11, rather than for neutral equilenin. The modest contribution of a component of relatively long decay time (4.9 ns) in the presence of KSI at pH 3.8 suggests either that there is a small concentration of free equilenin in solution or that a minor portion of the equilenin at the active site is not ionized. Since there is no spectral evidence that a *substantial* amount of the bound equilenin is present as the unionized phenol, even at pH 3.8, it can be estimated that the pK_a of equilenin bound to KSI at the active site is $\leq$ ca. 3.5. Since the pK_a of equilenin in aqueous solution is about 9 (Davenport et al., 1986), the acidity of the phenolic hydroxyl group has been increased by $>10^5$-fold at the active site relative to that in solution.

Ionization of equilenin bound to the active site of KSI may be rationalized by a proton transfer from the phenolic hydroxyl group of an initially formed complex to a basic residue on the enzyme, possibly Asp-38 (Scheme IV).[4] A proton transfer of this type is analogous to the proton transfer from C-4β of **1** in the normal catalytic reaction to produce the dienol(ate) intermediate. Since both the proton donor and acceptor in Scheme IV are electronegative atoms, this reaction should be quite facile (Eigen, 1964). We previously have argued for a similar proton transfer from the dienol (**2**) to explain its catalytic competence with KSI (Hawkinson et al., 1991a).

Mechanistic Implications. The difference in pK_a of $\geq$5.5 units for free equilenin and bound equilenin suggests that the structurally similar anion of the dienol **2** might also be stabilized upon binding to KSI. If this rationale is correct, the pK_a of the free dienol would be lowered from its value of 10 in solution (Zeng & Pollack, 1991) to $\leq$4.5 when bound to KSI, corresponding to a stabilization of the intermediate dienolate of $\geq$7.5 kcal/mol by binding to the active site. Since

Scheme V[a]



[a] K_{INT} = [E − 2]/[E − 1]; K_a^1 = [E − 1][H$^+$]/[EH − 1]; K_a^2 = [E − 2][H$^+$]/[EH − 1].

the same dienolate ion is formed from deprotonation of **1** and **2**, the pK_a of **1** at the active site may likewise be lowered $\geq$5.5 units from its value of 12.7 in solution (Pollack et al., 1987, 1989b) to $\leq$ ca. 7. This stabilization of $\geq$7.5 kcal/mol is consistent with our recent conclusion that the intermediate dienolate ion is stabilized by up to 11 kcal/mol, relative to the Δ^5-ketone by binding to KSI (Hawkinson et al., 1991b).

A thermodynamic cycle illustrating the effect of the lowering of the pK_a of bound **1** on the equilibrium between **E-1** and **E-2** is shown in Scheme V. An efficient enzyme for this process would likely have a internal equilibrium constant (K_{INT}) near unity (Albery & Knowles, 1976b; Knowles, 1987; Burbaum et al., 1989; Hawkinson et al., 1991b). This equilibrium constant is related to the pK_a's of Asp-38 and the dienol at the active site by $K_{INT} = K_a^2/K_a^1$. In solution, proton transfer from **1** (pK_a 12.7) to acetate ion (pK_a 4.7) is extremely unfavorable ($K_{INT} = 10^{-8}$). If, however, the pK_a of **1** on the enzyme surface (pK_a^2) is lowered to ca. 7, the equilibrium constant for proton transfer to Asp-38 (p$K_a^1 \approx$ 4.7; Pollack et al., 1986a) would become much more favorable ($K_{INT} \approx 10^{-2}$). A p$K_a^2 < 7$ would, of course, raise K_{INT} even more, further stabilizing the intermediate relative to reactants.

Although the above discussion assumes that the enzyme–dienol complex (**E-2**) consists of neutral Tyr-14 and ionized **2**, the energetics of the reaction would be identical if the perturbed pK_a were that of Tyr-14 rather than that of the dienol. That is, the proton might be covalently bound either to Tyr-14 or to the oxygen of **2**, with a hydrogen bond to the other oxygen. It is the pK_a of the *system* that must be perturbed to ca. 5 in order for K_{INT} to be near unity. We have chosen to depict ionized **2** and neutral Tyr because of the observation that equilenin is ionized at the active site. However, equilenin in solution (pK_a 9; Davenport et al., 1986) is somewhat more acidic than **2** (pK_a 10; Zeng & Pollack, 1991). Since the pK_a of **2** may be similar to that of Tyr-14 in the intermediate complex (pK_a of Tyr-14 in free KSI > 10.9; Kuliopulos et al., 1991), the position of the proton in the hydrogen bond cannot readily determined. Xue et al. (1991) have recently inferred that dienol bound to the D38N mutant of KSI is present as a mixture of free dienol and dienolate ion.

Although it is difficult to predict the position of the proton in the intermediate KSI–dienol complex, it is likely that transfer of the proton from Tyr-14 to **2** is not part of the

[4] Although the simplest explanation involves Asp-38 as the base, Kuliopulos et al. (1989) have observed spectral changes upon binding of estradiol to the D38N mutant that mimic those with the wild type. This observation suggests that estradiol is bound to the D38N mutant as the phenolate ion and would argue that another basic residue of the isomerase is able to abstract the phenolic proton. Two other basic residues (Asp-32 and Glu-37) have been found at the active site (Kuliopulos et al., 1987); studies with additional mutants will be necessary to clarify the roles of the three carboxylic residues.

reaction coordinate. We have previously argued that the pK_a of a protonated carbonyl oxygen *in the transition state* is almost certainly much lower than the pK_a of the tyrosine hydroxyl (Zeng & Pollack, 1991). Since there would be no driving force for transfer of the proton from Tyr-14 to the carbonyl oxygen in the transition state, the stabilization of the transition state is probably due to hydrogen bonding of the incipient negative charge.[5] If proton transfer does occur, it is probably *after* the transition state.

Summary. The ability of KSI to stabilize the anion of equilenin at pH values as low as 3.8 suggests that KSI acts by an anionic mechanism, with Tyr-14 acting to stabilize the transition state by hydrogen bonding and not by the transfer of a proton to the incipient negatively charged oxygen of the dienolate ion. In contrast, there is no evidence for a cationic mechanism, involving prior proton transfer from Tyr-14 to the carbonyl oxygen of the β,γ-unsaturated ketone substrate before abstraction of the 4β-hydrogen by Asp-38. The third possibility, concerted proton transfer from Tyr-14 to the carbonyl of the substrate with abstraction of the 4β-hydrogen by Asp-38, is unlikely, as transfer of the proton of Tyr-14 to the incipient dienolate ion is almost certainly energetically unfavorable.

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[5] It has been argued that proton transfer from Tyr-14 to the carbonyl is cocerted with abstraction of the 4β-proton by Asp-38 (Xue et al., 1990; Kuliopulos et al., 1990). This conclusion is based upon additivity in the primary and solvent deuterium isotope effects (Xue et al., 1990) and in the combined effects of D38N and Y14F mutations on the activity of KSI (Kuliopulos et al., 1990). Although these results are good evidence that both residues are acting in the same step, stabilization by hydrogen bonding from Tyr-14 to the carbonyl, rather than proton transfer, is equally consistent with the evidence.

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Role of Glu318 at the Putative Distal Site in the Catalytic Function of Cytochrome $P450_d$†

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ABSTRACT: Most microsomal P450s have a conserved "threonine cluster" composed of three Thrs (Thr319, Thr321, Thr322 for $P450_d$) at a putative distal site. An ionic amino acid at 318 is also well conserved as Glu or Asp for most P450s. To understand the role of these conserved polar amino acids at the putative distal site in the catalytic function of microsomal P450, we studied how mutations at this site of $P450_d$ influence the activation of molecular oxygen in the reconstituted system. Catalytic activity (0.02 min^{-1}) toward 7-ethoxycoumarin of the Glu318Ala mutant of $P450_d$ was just 6% of that (0.33 min^{-1}) of the wild type, while those of Glu318Asp, Thr319Ala, and Thr322Ala were comparable to or even higher than that of the wild type. Consumption rates of O_2 and formation rates of H_2O_2 of those mutants varied in accord with the catalytic activities. Especially, the efficiency (0.5%) of incorporated oxygen atom to the substrate *versus* produced H_2O_2 for the Glu318Ala mutant was much lower than that (3.7%) of the wild type, while that (58.8%) for the mutant Glu318Asp was 16-fold higher than that of the wild type. In addition, the autoxidation [Fe(II) → Fe(III)] rate (0.074 s^{-1}) of the Glu318Ala mutant was much lower than those (0.374–0.803 s^{-1}) of the wild type and other mutants. Thus, we strongly suggest that Glu318 plays an important role in the catalytic function toward 7-ethoxycoumarin of microsomal $P450_d$.

For monooxidation reactions catalyzed by cytochrome P450, molecular oxygen must be activated on the heme plane for transferring activated oxygen atom to the organic substrate (Guengerich, 1991; Ortiz de Montellano, 1986; Porter & Coon, 1991). From the crystal structure of water-soluble $P450_{cam}$,[1] it was suggested that highly conserved Thr252 (numbered for $P450_{cam}$) (Figure 1) in the distal site must be very important for the activation of the molecular oxygen (Poulos et al., 1985, 1987). This suggestion was proven by site-directed mutagenesis of the distal Thr252 of $P450_{cam}$ (Imai et al., 1989; Martinis et al., 1989). Namely, the Thr252Ala mutant of $P450_{cam}$ had a very low catalytic activity as compared with that of the wild type, and consumed O_2 in the enzyme solution was recovered as H_2O_2 (Imai et al., 1989; Martinis et al., 1989) and H_2O (Martinis et al., 1989). In addition, the autoxidation rate of the O_2 complex of the Thr252Ala mutant was much faster than that of the wild type (Imai et al., 1989; Martinis et al., 1989).

Although microsomal membrane-bound P450s monooxygenate organic substrates as does $P450_{cam}$, the detailed mechanism of the monooxidation reaction involved with microsomal P450 may be different from that of $P450_{cam}$ in some respects. For example, electrons necessary for the reaction are supplied from a flavoprotein, cytochrome P450 reductase, for microsomal P450s, while those are supplied from a nonheme iron protein, putidaredoxin, for $P450_{cam}$ (Guengerich, 1991; Ortiz de Montellano, 1986; Porter & Coon, 1991). O_2 in the enzyme solution is not efficiently used, and the production of H_2O_2 is accompanied with the hydroxylation reaction for microsomal P450s (Ingelman-Sundberg & Johansson, 1984; Gorsky et al., 1984), whereas O_2 in the enzyme solution is efficiently used for the reaction of $P450_{cam}$ (Atkins & Sligar, 1988; Imai et al., 1989; Martinis et al., 1989). The autoxidation rate of the O_2 complex of microsomal P450s is fast ($1–10^{-1}$ s^{-1}) (Bonfils et al., 1979; Estabrook et al., 1971; Guengerich et al., 1976; Oprian et al., 1983), while that of $P450_{cam}$ is slow ($10^{-2}–10^{-3}$ s^{-1}), and thus the absorption spectrum of the O_2-bound complex is obtained with a conventional spectrometer (Gettings et al., 1990; Ishimura et al., 1971; Lipscomb et al., 1976; Peterson et al., 1972).

Microsomal P450s have a conserved "threonine cluster" at the putative distal site (Nelson & Strobel, 1988) (Figure 1). Thr319, Thr321, and Thr322 apparently compose the Thr cluster for $P450_d$. Prior to the conserved Thr (Thr252 for $P450_{cam}$, Thr319 for $P450_d$; note that the cluster is absent from $P450_{cam}$), the polar amino acid such as Glu318 for $P450_d$ or Asp251 for $P450_{cam}$ is fairly conserved for P450s (Figure 1). To understand the role of these polar amino acids at the putative distal site of membrane-bound $P450_d$, we changed the

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[1] Abbreviations: P450, cytochrome P450; $P450_d$, rat liver microsomal cytochrome $P450_d$ (which corresponds to P4501A2 or CYP1A2; Nebert et al., 1991); $P450_{cam}$, cytochrome P450 purified from *Pseudomonas putida* grown in the presence of camphor (which corresponds to P450101 or CYP101; Nebert et al., 1991); EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; 7-ethoxycoumarin, 7-ethoxy-2*H*-1-benzopyran-2-one; Emulgen 913, poly(oxyethylene)-*p*-nonylphenyl ether containing 13.1 oxyethylene units on average; DLPC, dilauroyl-L-α-phosphatidylcholine; methyl viologen, 1,1-dimethyl-4,4′-bipyridinium dichloride hydrate; metyrapone, 2-methyl-1,2-di-3-pyridyl-1-propanone.

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