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Orientation, Accessibility, and Mobility of Equilenin Bound to the Active Site of Steroid Isomerase†

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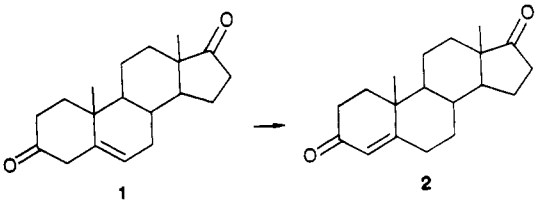
ABSTRACT: The fluorescent aromatic steroid equilenin, which contains a β-naphthol moiety, is bound by 3-oxo-Δ^5-steroid isomerase. The excitation and emission fluorescence spectra of equilenin when bound to the enzyme, as well as the fluorescence decay time, are indicative of ground-state ionization. In view of the high efficiency of tyrosine quenching, which approaches 100%, the β-naphthol moiety of equilenin must be in proximity to all three tyrosines of steroid isomerase to account for the observed efficiency of radiationless energy transfer. From the observed response to an external quencher, it appears that enzyme-bound equilenin is largely shielded from solvent. Fluorescence anisotropy measurements indicate a high degree of immobilization of the bound ligand. These models are consistent with proposed models of the enzyme–substrate complex.

The binding of steroids to proteins is an integral part of the action of steroid-metabolizing enzymes, as well as many hormone receptors and transport proteins (Duax et al., 1983). These steroids mediate a variety of physiological functions, such as sexual differentiation, protein synthesis, calcium uptake, electrolyte balance, and the maintenance of secondary sexual characteristics (Duax et al., 1983). It has also been suggested that steroids may be the natural substrates for hepatatic cytochrome P-450 (Waxman et al., 1983). In many cases, the binding site is relatively tolerant of structural changes in the steroid (Schwarzel et al., 1973; Weintraub et al., 1977; Adams & McDonald, 1981; Rousseau et al., 1981), and several proteins have been found to bind steroids in more than one orientation (Sweet & Samant, 1980; Strickler et al., 1980; Adams & McDonald, 1981; Waxman et al., 1983; Kashino et al., 1987). Ross et al. (1982) have reported that the fluorescent steroid equilenin is bound by human sex steroid binding protein with high affinity.

Probably the most thoroughly characterized protein of this type is the steroid isomerase from *Pseudomonas testosteroni*. This enzyme catalyzes the interconversion of 3-oxo-Δ^5-steroids to their Δ^4-isomers by the transfer of a proton from C-4β to C-6β with the concomitant migration of the double bond from C-5 to C-4 (eq 1). The isomerase is a dimer with identical



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subunits, each having 125 amino acid residues of known sequence (MW 13394), and 1 binding site per monomer (Benson et al., 1972, 1975; Tivol et al., 1975; Batzold et al., 1976). The

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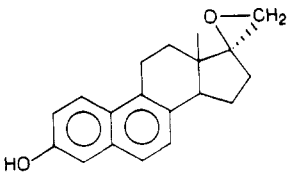
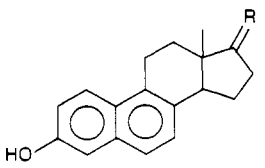
nature of the interactions between the steroid and the binding site has been investigated for this protein by an examination of the binding of competitive inhibitors (Weintraub et al., 1977), by affinity labeling (Benisek et al., 1982; Hearne & Benisek, 1985), by NMR (Benisek & Ogez, 1982; Kuliopulos et al., 1987), ESR (Kuliopulos et al., 1987), ultraviolet (Wang et al., 1963; Bevins et al., 1986; Kuliopulos et al., 1989), and fluorescence spectroscopy (Wang et al., 1963), and by *x*-ray crystallography (Westbrook et al., 1984; Kashino et al., 1987). X-ray crystallographic measurements at 6-Å resolution by Westbrook and co-workers (Westbrook et al., 1984) have revealed that the steroid binding pocket is a hydrophobic pit and that each of the two monomers has its own binding site. Hearne and Benisek (1985) have suggested that the catalytically active residue Asp-38 is located at the base of this pit. Further characterization of the active site comes from the work of Kuliopulos et al. (1987), who examined the binding of a spin-labeled substrate analogue to the isomerase. They were able to combine these measurements with unpublished X-ray results at 2.5 Å to identify hydrophobic groups at the active site that contribute to binding and to locate polar amino acid side chains that might be involved in catalysis. Kuliopulos et al. (1989) have also examined the effect of site-directed mutation of residues 14, 38, and 55 on the spectral properties of bound steroids and the catalytic activity of the enzyme.

Investigations from one of our laboratories have shown that steroids can bind to the isomerase in two different orientations, related by a 180° rotation about an axis perpendicular to the plane of the steroid ring system (Bevins et al., 1980, 1986; Kayser et al., 1983; Kashino et al., 1987; Bounds & Pollack, 1987). Furthermore, it appears that for many steroids the binding constants are similar for the two modes of binding (Bevins et al., 1986). These results complicate the interpretation of physical and chemical measurements of steroid bound to the isomerase, since one cannot be sure that the steroid is bound in the catalytically important orientation. Studies with equilenin 17β-oxirane **3a**, however, have shown that the predominant reversible binding mode for this steroid (and presumably for equilenin itself) corresponds to the catalytically active one (Bevins et al., 1986). We report here the results of fluorescence measurements of the interaction of equilenin (**3b**) with steroid isomerase, and the interpretation of these measurements in terms of the characteristics of steroid binding to the active site of steroid isomerase.



3a



3b, R = O
3c, R = α-H, β-OH

Materials and Methods

Materials. The gene for 3-oxo-Δ^5-steroid isomerase of *P. testosteroni* was obtained on plasmid puC 19 from Professor William F. Benisek of the University of California, Davis. This recombinant plasmid (pKC2; Choi & Benisek, 1988) was inserted into DH5α *Escherichia coli*. The isomerase was isolated from cultures of these bacteria and purified by published procedures (Kayser et al., 1983). Preparations obtained in this way appeared homogeneous by the criterion of acrylamide gel electrophoresis. Enzyme prepared in this manner was indistinguishable in its kinetic characteristics toward both 5-androstene-3,17-dione and 5(10)-estrene-3,17-dione from enzyme isolated from *P. testosteroni*. The enzyme used in these experiments had a specific activity of ≥48 000 units/mg.

Equilenin was purchased from Aldrich. Thin-layer chromatography on silica gel using a 1:1 hexane/ethyl acetate solvent showed a single component with an R_f of 0.59. The equilenin was used without further purification.

All other chemicals used were reagent grade or better. Glass-redistilled water was used for the preparation of all solutions.

Methods. Static fluorescence excitation and emission spectra were determined with an SLM 8000 C spectrofluorometer equipped with single photon counting. Emission intensity as a function of wavelength was expressed as a ratio to the intensity of a rhodamine B internal standard, thereby canceling any effect of light source fluctuation. Emission spectra were internally corrected for variation of photomultiplier sensitivity with wavelength. All emission spectra were measured by using vertically polarized exciting light and with the emission polarizer oriented at the "magic angle".

Dynamic measurements of the time decay of fluorescence intensity were made using time domain nanosecond fluorometers located in the Regional Laser and Biotechnology Laboratory of the University of Pennsylvania and in the Laboratory of Dr. J. R. Lakowicz of the Department of Biological Chemistry, School of Medicine, University of Maryland. Both fluorometers are equipped with mode-locked laser sources with frequencies of 2 and 1 MHz, respectively. They utilize rhodamine lasers driven by an Ar^+ and by a Nd-YAG laser, respectively. The excitation wavelength was normally 297 nm and the emission wavelength 400 nm.

Small amounts of methanol (1–2%) were generally present in the aqueous solutions to enhance the solubility of equilenin.

Results

Static Fluorescence Intensity. The fluorescence emission spectrum of equilenin in 0.01 M phosphate is strongly pH dependent (Figure 1A). At pH 7, there is an emission band at 360 nm with a shoulder at 380 nm. With increasing pH, the band at 360 nm is progressively lost, being replaced by a broad emission band at 390–430 nm. The latter is characteristic of the emission of ionized equilenin. By pH 11.5, the band at 360 nm is entirely gone, and only the long-wavelength band remains. The midpoint of the transition is close to pH 9, which corresponds to the ground-state pK of equilenin (Davenport et al., 1986).

The corresponding excitation spectra show, at neutral pH, bands at 290 and 320–340 nm, paralleling the reported absorption spectra (Davenport et al., 1986). The ionization of equilenin at alkaline pH is accompanied by the loss of the latter band and the appearance of a new excitation band at 350 nm (Figure 1B).

In the presence of a high concentration (4 M) of acetate ion, which acts as a proton acceptor, the emission spectrum is dominated by the 390–430-nm band characteristic of the ionized form, while the band at 360 nm arising from unionized equilenin is almost abolished, even at a pH as acid as 6.3 (Figure 1C). However, the excitation spectra at pHs below 9 are characteristic of unionized equilenin, and the transition

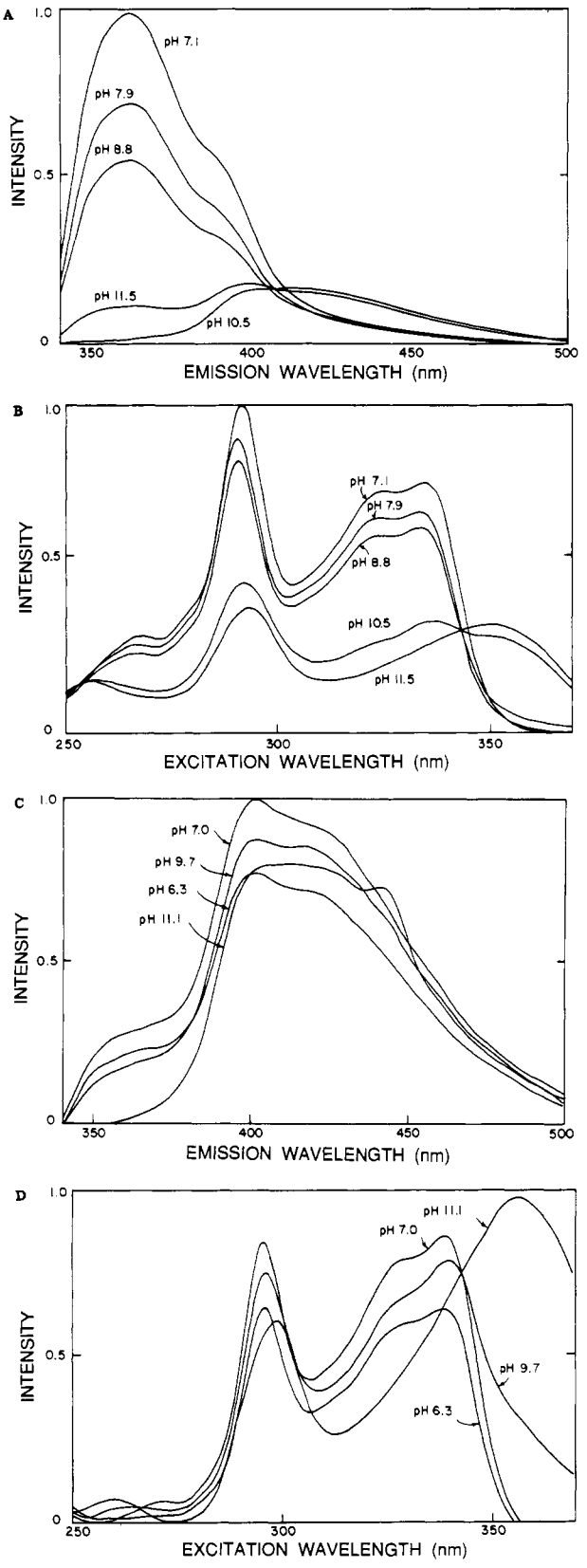


FIGURE 1: (A) Emission spectra for equilenin (10 μM) in 0.01 M phosphate at a series of pHs. The excitation wavelength is 298 nm. (B) Excitation spectra for equilenin at a series of pHs. The emission wavelength is 400 nm. Other conditions are the same as in A. (C) Emission spectra for equilenin (10 μM) in the presence of 4 M sodium acetate, plus 0.01 M phosphate, at a series of pHs. The excitation wavelength is 298 nm. (D) Excitation spectra for equilenin (10 μM) in the presence of 4 M sodium acetate plus 0.01 M phosphate, at a series of pHs. The emission wavelength is 400 nm.

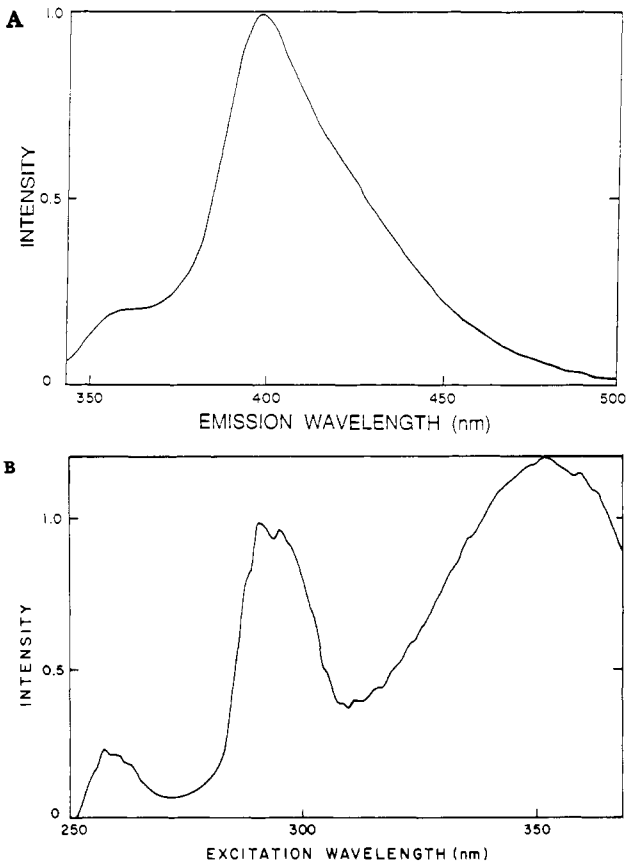


FIGURE 2: (A) Emission spectrum of equilenin (10 μM) plus excess steroid isomerase (50 μM) in 0.01 M phosphate, pH 7.0. The excitation wavelength is 298 nm. (B) Excitation spectrum of equilenin plus excess steroid isomerase for the above conditions. The emission wavelength is 410 nm.

to the ionized spectrum only occurs at pHs alkaline to 9, where ground-state ionization is occurring (Figure 1D). It is clear that excited-state ionization is occurring in the neutral pH range. Despite the low excited-state pK ($\sim$3) of equilenin, the lifetime of its initial excited state in water is too short for significant ionization to take place, unless the rate of ionization is increased by the presence of high levels of a proton acceptor.

In the presence of excess steroid isomerase at a concentration sufficiently high to bind virtually all the equilenin, a drastic change occurs in the emission spectrum, which now resembles that for ionized free equilenin, with a loss of the band at 360 nm and a shift in the emission maximum to 400 nm (Figure 2). However, in contrast to the behavior in 4 M acetate, the excitation spectrum is now also characteristic of ionized equilenin, with the appearance of a new band at 350 nm. From these results, it appears that a ground-state ionization occurs for bound equilenin, which exists on the enzyme predominantly in this form. In the combined state, the pK of equilenin is thus depressed by over 2 pH units.

Measurements were also made of the fluorescence of equilenin (10 μM) in the presence of a large excess (100 μM) of bovine serum albumin at pH 7. Apart from a minor emission tail extending to wavelengths above 400 nm, the emission and excitation spectra are characteristic of unionized equilenin (data not shown).

Dynamic Fluorescence. The time decay of fluorescence for equilenin dissolved in absolute ethanol is dominated by a major component of decay time $\sim$7 ns (Table I). However, there is also a minor component of decay time $\sim$3 ns (Table I). This is unlikely to arise from any contribution of ionized equilenin, as the emission spectrum of equilenin under these conditions

Table I: Time Decay of Fluorescence Intensity for Equilenin[a]

solvent	additive	α_1	τ_1 (ns)	α_2	τ_2 (ns)	α_3	τ_3 (ns)	$\bar{\tau}^b$ (ns)	χ^2
ethanol		0.14	3.56	0.31	6.87			6.24	1.5
0.01 M phosphate, pH 7.0	1% methanol	0.14	1.34	0.10	4.02	0.03	10.34	5.61	2.1
same	5% methanol	0.09	0.61	0.05	2.44	0.02	7.47	4.43	1.5
0.2 M acetate, pH 5.0	1% methanol	−0.40	1.96	0.79	2.62	0.005	9.00		2.5
0.1 M carbonate, pH 12.0	1% methanol	0.19	1.23	0.02	2.34			1.42	3.9
0.01 M phosphate, pH 7.0	steroid isomerase	0.79	0.44	0.32	1.61	0.04	7.05	2.61	1.5

[a] The intensity is assumed to decay according to the relation $I(t)_i = \sum \alpha_i e^{-t/\tau_i}$ where $I(t)$ is the intensity as a function of time, t, and α_i and τ_i are the amplitude and decay time, respectively, of the ith decay mode. [b] The average decay time, defined by $\bar{\tau} = \sum_i \alpha_i \tau_i^2 / \sum_i \alpha_i \tau_i$.

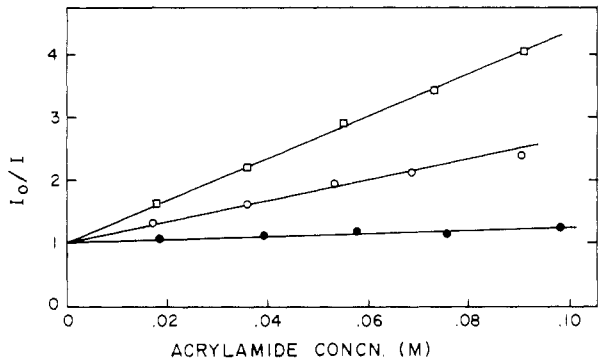


FIGURE 3: Quenching by acrylamide of the fluorescence of equilenin in 0.01 M phosphate at pH 7.0 (□), in 0.01 M carbonate at pH 12 (○), and at pH 7.0 in the presence of excess (50 μM) steroid isomerase (●). The excitation and emission wavelengths are 298 and 400 nm, respectively.

is equivalent to that of the unionized form at pH 5 (data not shown). The minor component may arise from the occurrence of a significant degree of self-association in this relatively polar solvent. In aqueous buffer at pH 7, the time decay becomes more heterogeneous, requiring the assumption of three decay modes to attain an adequate fit (Table I). A plausible explanation for the increase in heterogeneity over that observed in ethanol is an enhanced tendency to self-association arising from the increased polarity of the solvent.

At pH 12, where equilenin exists in the ionized form, there is a major decrease in the average decay time. The time decay is again dominated by a major component, which is now of decay time ~1 ns (Table I). The decrease in quantum yield for the ionized form is thus accompanied by a major decrease in decay time. In the presence of excess steroid isomerase at pH 7, the emission is dominated by components of short decay time, while the average decay time decreases sharply (Table I). This is consistent with the ground-state ionization of bound equilenin.

In the presence of 0.2 M acetate, pH 5.0, the amplitude of the long decay time present at neutral pH in the absence of acetate is substantially reduced (Table I). In addition, there is a component with negative amplitude. This is characteristic of a decay process involving an excited-state reaction leading to a new excited species, which subsequently decays by emission of fluorescence. In the present case, the excited-state reaction is presumably the ionization induced by transfer of a proton to the proton acceptor, acetate.

Accessibility of Bound Equilenin to External Quencher. Uncombined equilenin is quenched by acrylamide with high efficiency (Figure 3) at both pH 7 and pH 12, although some reduction in quenching efficiency occurs for the ionized form at the latter pH, as expected because of the decreased decay time. At both pHs, the data could be fit by a linear Stern–Volmer plot. The computed Stern–Volmer constants for the two pHs are cited in Table II, together with the corresponding bimolecular rate constants. The magnitudes of the latter

Table II: Quenching of Equilenin by Acrylamide in the Absence and Presence of Steroid Isomerase[a]

pH	buffer	enzyme concn (μM)	K_{SV}^b (M^{-1})	k_Q^c (s^{-1} M^{-1})
7.0	0.01 M phosphate	0	33.6	6.0×10^9
12.0	0.01 M carbonate	0	12.0	8.5×10^9
7.0	0.01 M phosphate	50	2.0	0.8×10^9

[a] The concentration of equilenin is 10 μM. All solutions contain 1% methanol. [b] The Stern–Volmer constant. [c] The bimolecular rate constant for quenching; $k_Q = K_{SV}/\bar{\tau}$, where $\bar{\tau}$ is the average decay time (Table I).

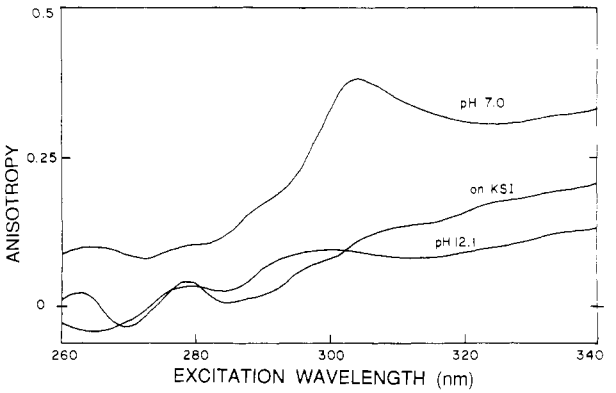


FIGURE 4: Fluorescence anisotropy excitation spectra of equilenin (10 μM) in 99% glycerol at pH 7, at pH 12, and in the presence of 50 μM steroid isomerase (KSI) at pH 7 (no glycerol). The other conditions are as in Figure 1B.

approach the rate constants expected for a diffusion-controlled process (Fersht, 1985).

In the presence of excess steroid isomerase, the efficiency of quenching by acrylamide is greatly reduced (Figure 3). Since bound equilenin exists in the ionized state, it is logical to compare its quenching behavior with that of free equilenin at pH 12. The Stern–Volmer constant for bound equilenin is reduced by an order of magnitude (Table II). The implication of this finding is that the bound equilenin is shielded from the external quencher, presumably through envelopment by the tertiary structure of the enzyme.

Static Anisotropy. The anisotropy excitation spectrum of equilenin in 99% glycerol, pH 12,[1] 10 °C, gives some indication of secondary maxima below 300 nm, suggesting the presence of overlapping transitions. A plateau is approached above 300 nm (Figure 4). At pH 7,[1] the limiting anisotropy is substantially higher and approaches the theoretical limit, 0.4. In view of the high viscosity of the medium and the short decay time of equilenin fluorescence, the fluorophore may probably

[1] The cited pHs refer to the aqueous equilenin stocks at 20 °C in either 0.01 M carbonate, pH 12, or 0.01 M phosphate, pH 7, which were subsequently made 99% in glycerol; the emission spectra were equivalent to those of aqueous solutions at the same nominal pHs.

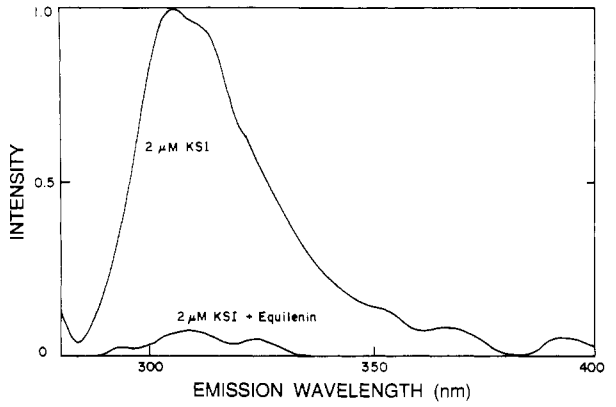


FIGURE 5: Tyrosine fluorescence emission spectra of steroid isomerase (KSI, 2 μM) in 0.01 M phosphate, pH 7.0, in the absence and presence of equilenin (20 μM). The excitation wavelength is 275 nm.

be regarded as effectively completely immobilized under these conditions.

The Perrin equation states that

$$A_0/A = 1 + \tau/\sigma$$

where A_0 and A are the limiting anisotropy for the immobilized fluorophore and the anisotropy under ambient conditions, respectively, τ is the average decay time, and σ is the rotational correlation time.

For a molecule of the size of equilenin, the expected correlation time in water at 20 °C is of the order of 100 ps (Lakowicz, 1983). If it is assumed that σ is proportional to η/T, where η is the solvent viscosity and T the absolute temperature, then, for the conditions of measurement ($\eta = \sim 5 \times 10^3$ cP), σ will be $\sim 5 \times 10^2$ ns. Since the average decay time of fluorescence is 5.6 ns at pH 7 and 1.4 ns at pH 12 (Table I), A should not differ from A_0 by more than ~1% at either pH. The lower limiting anisotropy at pH 12 probably arises from the contribution of multiple overlapping transitions.

In the presence of a sufficient excess of steroid isomerase to ensure complete binding, the anisotropy excitation spectrum resembles that of ionized free equilenin at pH 12, except that the limiting anisotropy at long wavelengths is slightly higher (Figure 4). The anisotropy of combined equilenin is thus close to that of the immobilized ionized form of free equilenin. The implication is that bound equilenin is largely immobilized within the combining site. These data cannot, of course, exclude a minor degree of librational mobility.

Tyrosine Fluorescence. The intrinsic fluorescence of steroid isomerase is characteristic of a class A protein (Steiner, 1983). The fluorescence emission, which arises entirely from tyrosine, has a maximum at about 305 nm (Figure 5). In the presence of excess equilenin, the tyrosine fluorescence of the isomerase is almost entirely quenched (Figure 5). Since the fraction of the fluorescence remaining is comparable to the expected fraction of free enzyme remaining, it is likely that the quenching of tyrosine fluorescence by equilenin binding is virtually quantitative. This parallels an earlier report concerned with the interaction of the enzyme with 19-nortestosterone (Wang et al., 1963), except that the latter ligand only quenches about 75% of the tyrosine fluorescence.

Equilenin contains a single carbonyl group at position 17, which is a potential tyrosine quencher. However, equilenin is unlikely to act as a quencher of all three tyrosines by direct van der Waals contact. Since the absorption spectrum of ionized equilenin overlaps the tyrosine emission band of steroid isomerase, radiationless energy transfer to the β-naphthol portion of equilenin is an obvious possibility as a mechanism for quenching the tyrosine fluorescence.

The efficiency, E, of radiationless energy transfer is related to the separation, R, of donor and acceptor groups by

$$E^{-1} - 1 = (R/R_0)^6 \quad (2)$$

R_0, the separation for 50% transfer efficiency, is given by

$$R_0^6 = (8.79 \times 10^{-5})\kappa^2 n^{-4} Q J \quad (3)$$

where κ^2 is an orientation factor, equal to $^2/_3$ in the case of random orientation of donor and acceptor, n is the refractive index of the medium, Q is the donor quantum yield, and J is the overlap integral, defined by

$$J = \int F(\lambda)\epsilon(\lambda)\lambda^4 \, d\lambda / \int F(\lambda) \, d\lambda \quad (4)$$

where $F(\lambda)$ and $\epsilon(\lambda)$ are the fluorescence intensity of the donor and the extinction coefficient of the acceptor, respectively, at wavelength λ.

The values of J and R_0 computed on this basis are 2.06×10^{13} and 19.8 Å, respectively. Since transfer efficiency approaches 100%, it follows from eq 2 that the tyrosines that contribute significantly to the fluorescence emission must lie within ~15 Å of the acceptor, which is presumably the β-naphthol portion of equilenin.

Discussion

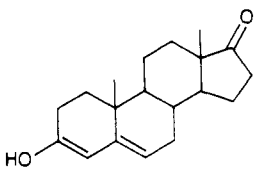
Before discussing the implications of the present measurements to the structure of the binding site of the isomerase and its catalytic mechanism, it is important to establish that equilenin is an appropriate probe. Two questions must be addressed: first, whether equilenin binds in the same orientation as the catalytically important orientation of the substrate at the active site and, second, whether equilenin is sufficiently similar structurally to the substrate to allow extrapolation of the results to binding and reaction with substrate.

Several lines of evidence suggest that steroids may bind to the isomerase in more than one orientation. We have shown that both 3β- and 17β-oxiranes inactive the isomerase in an active-site-directed process (Pollack et al., 1979; Bevins et al., 1980). Each forms a covalent bond to Asp-38 (Kayser et al., 1983; Bounds & Pollack, 1987), a group known to be at the active site (Martyr & Benisek, 1975; Ogez et al., 1977), showing that both ends of a steroid molecule can have access to the same part of the enzyme active site. The fact that only the β-isomers inactivate the enzyme (Pollack et al., 1979; Bevins et al., 1980) is evidence for these two binding modes being related by a 180° rotation about an axis perpendicular to the plane of the steroid nucleus. Furthermore, the second-order rate constants for inactivation by a variety of oxiranes (both 3β and 17β) are similar, consistent with a similar binding affinity for both modes (Bevins et al., 1986).

Studies with the 17β-oxirane of equilenin suggest that, although this oxirane can bind in two orientations, the two binding modes are not of equal energy. Bevins et al. (1986) have observed that the 17β-oxirane of equilenin is initially bound reversibly in a form that shows enhanced fluorescence, but subsequently undergoes a slow rearrangement to form a new species in which the steroid is irreversibly bound by reaction of the oxirane with Asp-38; the equilenin in the latter complex shows no enhanced fluorescence. This finding was interpreted in terms of two alternative binding modes for the steroid. The irreversibly bound form places the D ring of the steroid near Asp-38, whereas the initial reversibly bound complex has the A ring near Asp-38. The orientation with the aromatic A ring near the base of the hydrophobic binding pit (the same orientation proposed for the substrate in catalysis) is the predominant one (Bevins et al., 1980, 1986). This

orientation shows UV spectral properties of an ionized phenol, as previously observed for dihydroequilenin (Wang et al., 1963) and estradiol (Wang et al., 1963; Kuliopulos et al., 1989). Thus, the environment of the A and B rings of equilenin when it is bound to the isomerase should be similar to that of the A ring of the substrate during catalysis.

Although the A and B rings of equilenin bear little resemblance to the A and B rings of either the substrate or the product of the catalytic reaction, equilenin may be viewed as an analogue of the intermediate dienol (**4**) postulated for the



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reaction (Batzold et al., 1976; Pollack et al., 1989). We have obtained evidence for the intermediacy of an enol intermediate in this reaction by showing that the putative intermediate trienol in the enzyme-catalyzed isomerization of 5,7-estradiene-3,17-dione to 4,7-estradiene-3,17-dione can serve as a substrate for the isomerase (Bantia & Pollack, 1986). In addition, **4** itself is isomerized by the enzyme (R. M. Pollack, T. C. M. Eames, and D. C. Hawkinson, unpublished results). The combination of the structural similarity of equilenin to the dienol intermediate, the fact that it binds perferentially in the correct orientation, and its excellent binding constant[2] suggest that equilenin is a good probe for the active site of the isomerase.

The results of the present study are in agreement with the model of the active site proposed by Westbrook et al. (1984), which identifies the steroid binding site as a deep hydrophobic activity. Recent work (Kuliopulos et al., 1987) suggests that all three tyrosines of the enzyme (14, 55, and 88) are located at the base of this pit. The near-total quenching of the tyrosine fluorescence by bound equilenin, which makes it likely that the ligand is in the proximity of all three tyrosines, is consistent with this view. Interestingly, Wang et al. (1963) previously reported that only 75% of the tyrosine fluorescence of the isomerase is quenched upon binding of the competitive inhibitor 19-nortestosterone. The shielding from external quencher of the bound equilenin is consistent with the A ring of the steroid being bound at the base of the hydrophobic pocket. This result agrees with the buried location of Asp-38 (Hearne & Benisek, 1985) and the results of Bevins et al. (1986), who concluded that the preferred orientation of bound equilenin has the A ring near Asp-38.

The high degree of immobilization of bound equilenin has implications for the mechanism of interconversion of bound species in the active site. The existence of two modes of binding of steroids to the active site of the isomerase suggests a significant degree of adaptability in the vicinity of the active site. However, in view of the absence of evidence for localized mobility of bound equilenin, there does not appear to be a major degree of flexibility of the enzyme. The 180° shift in orientation of bound steroid must therefore correspond to a transition between two well-defined energy minima, presumably requiring dissociation–association from the enzyme.

We have previously demonstrated that Asp-38 is located at the α side of bound steroid during inactivation by 3β- and 17β-oxiranes (Bevins et al., 1984; Bounds & Pollack, 1987). It has also been postulated that Asp-38 is the base involved in the proton transfer during the catalytic reaction (Kuliopulos et al., 1987, 1989). This interpretation would require that Asp-38 be able to approach the β side of the steroid ring system as well as the α side, since proton transfer is predominantly 4β to 6β (Malhotra & Ringhold, 1965; Viger & Marquet, 1977; Viger et al., 1981). A possible solution to this apparent inconsistency would be the existence of free rotation of the steroid about an axis parallel to the plane of the steroid ring system at the active site, thus allowing Asp-38 to have access to both faces of bound steroid. The lack of localized mobility of bound equilenin, however, implies that, if both of these binding modes exist, there is no direct interconversion between them on the enzyme surface but rather the steroid must first dissociate from the enzyme and then bind in its new orientation.

A perturbation of the fluorescence emission spectrum similar to that observed here for equilenin upon binding to the isomerase has previously been observed for dihydroequilenin (Wang et al., 1963). Similarly, spectral shifts in the ultraviolet spectra of both 17β-estradiol and 17β-dihydroequilenin when bound to the isomerase have been reported (Wang et al., 1963; Kuliopulos et al., 1989). Wang et al. (1963) concluded that the enzyme "induces alterations in electron density that resemble the ionization of the phenols in basic solution". However, observation of a perturbation in the emission spectrum alone is not sufficient evidence for ionization of equilenin in the ground state. An alternative explanation would be binding of unionized equilenin to the enzyme, followed by excitation, and *then* ionization. Emission could then occur from the ionized excited state, even though equilenin might be bound in the unionized ground state. Our results confirm the interpretation of Wang et al. (1963) by demonstrating that the *excitation* spectrum of equilenin for bound steroid is also similar to that for ionized equilenin.

The ionic state of bound equilenin may be relevant to the mechanism of action of the isomerase. Although Wang et al. (1963) postulated an anionic ring A phenolate in the binding of dihydroequilenin 25 years ago, the implications of this suggestion have gone largely unappreciated. Virtually all the proposed mechanisms for the isomerase involve either a cationic mechanism or a concerted acid–base reaction to produce a neutral dienol intermediate (Pollack et al., 1989; Kuliopulos et al., 1989). Recently, however, we observed that the isomerase substrate 5-androstene-3,17-dione has a pK_a of 12.7 in aqueous solution (Pollack et al., 1987), and we suggested that the isomerase may act through the formation of a dienolate intermediate rather than a neutral dienol. On the basis of results with other dienols (Duhaime & Weedon, 1987; Keeffe et al., 1988), it is reasonable to assume that the pK_a of the intermediate dienol in aqueous solution is about 9–10, similar to that of equilenin (pK_a ca. 9; Davenport et al., 1986), implying that the dienol may also be bound to the isomerase in the ionized form.

It is also of interest to compare the fluorescence properties of equilenin bound to steroid isomerase with those of the complexes formed with equilenin and other steroid binding proteins. The sex steroid binding protein of human plasma forms a complex with equilenin for which excitation and emission spectra are slightly red-shifted with respect to the free ligand in water (Ross et al., 1982). However, the major quenching and shift to wavelengths above 400 nm of the emission maximum, which are characteristic of ionized equilenin, are not observed. Thus, complex formation in this

[2] The binding constant for equilenin is 8 μM in 10% methanol (Weintraub et al., 1977). Binding should be substantially tighter in the 1–2% methanol solutions used in this work (Falcoz-Kelly et al., 1968).

case is not accompanied by the spectral properties associated with ground-state ionization. In the case of binding by serum albumin, there is some indication of a contribution from excited-state ionization, but ground-state ionization again appears to be absent. The ionization of equilenin bound to steroid isomerase is thus in contrast to what is observed when equilenin is combined with other steroid binding proteins.

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