Enzymatic and Nonenzymatic Polarizations of α,β-Unsaturated Ketosteroids and Phenolic Steroids. Implications for the Roles of Hydrogen Bonding in the Catalytic Mechanism of Δ^5-3-Ketosteroid Isomerase†

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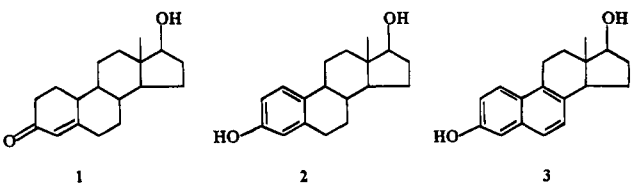
ABSTRACT: Ketosteroids (e.g., 19-nortestosterone) and phenolic steroids (e.g., 17β-estradiol and 17β-dihydroequilenin), which are potent competitive inhibitors of Δ^5-3-ketosteroid isomerase (isomerase, EC 5.3.3.1) of *Pseudomonas testosteroni*, undergo significant polarization upon binding to the active site of the enzyme. The 10 nm red shift of the UV absorption maximum of the enone chromophore of 19-nortestosterone, which occurs in the enzyme−steroid complex, resembles that observed when this steroid is exposed to strong acid. The UV and fluorescence spectral changes of 17β-estradiol and 17β-dihydroequilenin in the enzyme-bound complex resemble the spectra of ionized phenolate species in aqueous basic solutions. Since most enzymes bind their substrates and competitive inhibitors in a solvent-inaccessible hydrophobic environment, and the generation of charges in such nonpolar environments is unfavorable, we investigated the possibility that the spectral perturbations of the steroids might arise from strong hydrogen bonding in nonpolar environments. For this purpose, the spectral properties of model compounds capable of forming intramolecular hydrogen bonds were studied in nonpolar solvents. Thus, 4-hydroxyandrost-4-ene-3,17-dione, in which the 4-hydroxyl group is intramolecularly hydrogen-bonded to the 3-carbonyl group through a five-membered ring, exhibits a λ_{max} of 276.0 nm, while the corresponding 4-methyl ether, 4-methoxyandrost-4-ene-3,17-dione, which cannot form an internal hydrogen bond, shows a λ_{max} of 258.5 nm in aqueous solution. This 17.5 nm difference in λ_{max} increases, as the solvent polarity is lowered, to a difference of 24.0 nm in hexane, presumably because there is less competition for hydrogen bond formation by the less polar solvent molecules. 2-Hydroxybenzoic acid showed progressively increasing red shifts and enhancements of UV absorption as the polarity of solvents was decreased, and these changes resembled those of 17β-estradiol when bound to isomerase. The spectral changes of 17β-dihydroequilenin, when bound to isomerase, are better approximated by those of 1-acetyl-2-naphthol in nonpolar solvents, which strengthen the intramolecular hydrogen bond, than by ionization of 17β-dihydroequilenin in strong aqueous base. Both fluorescence emission and excitation spectra of 17β-dihydroequilenin in aqueous solution can be significantly altered by high concentrations of hydrogen bond acceptors such as malonate, and these changes closely mimic the spectral properties of 17β-dihydroequilenin bound to isomerase. These results indicate that strong, directional hydrogen bond(s) to the functional groups of steroids in the solvent-inaccessible active site can explain the spectral behavior of these steroids when bound to isomerase. Such strong hydrogen-bonding interactions in the enzyme−inhibitor complexes implicate a low-barrier hydrogen bond between Tyr-14 and the enolic intermediate during catalysis.

The Δ^5-3-ketosteroid isomerase of *Pseudomonas testosteroni* (isomerase, EC 5.3.3.1)[1] promotes very rapid conversion of Δ^5- to Δ^4-3-ketosteroids by a conservative and stereospecific intramolecular transfer of the 4β-proton of the substrate to the 6β-position of the product. The reaction mechanism involves concerted enolization followed by reketonization of the enzyme-bound steroid. Tyr-14 is the critical general acid and Asp-38 is the base that participate in the enolization and proton transfer steps (Figure 1). Spectroscopic studies (UV, NMR, fluorescence, resonance Raman) of enzyme-bound steroids have provided critical evidence in support of this mechanism.

However, more than 30 years ago, Wang, Kawahara, and Talalay (1963) described profound changes in the UV spectra of three competitive inhibitors of isomerase—the enone 19-nortestosterone (**1**) and the phenols 17β-estradiol (**2**) and 17β-dihydroequilenin (**3**)—upon binding to the enzyme (Figure 2).



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The red shift of the absorption maximum of 19-nortestoster-

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[1] Abbreviations: DHE, 17β-dihydroequilenin (17β-hydroxy-1,3,5-(10),6,8-estrapentaen-3-ol); isomerase, Δ^5-3-ketosteroid isomerase (EC 5.3.3.1); 19-nortestosterone, 17β-hydroxy-4-estren-3-one.

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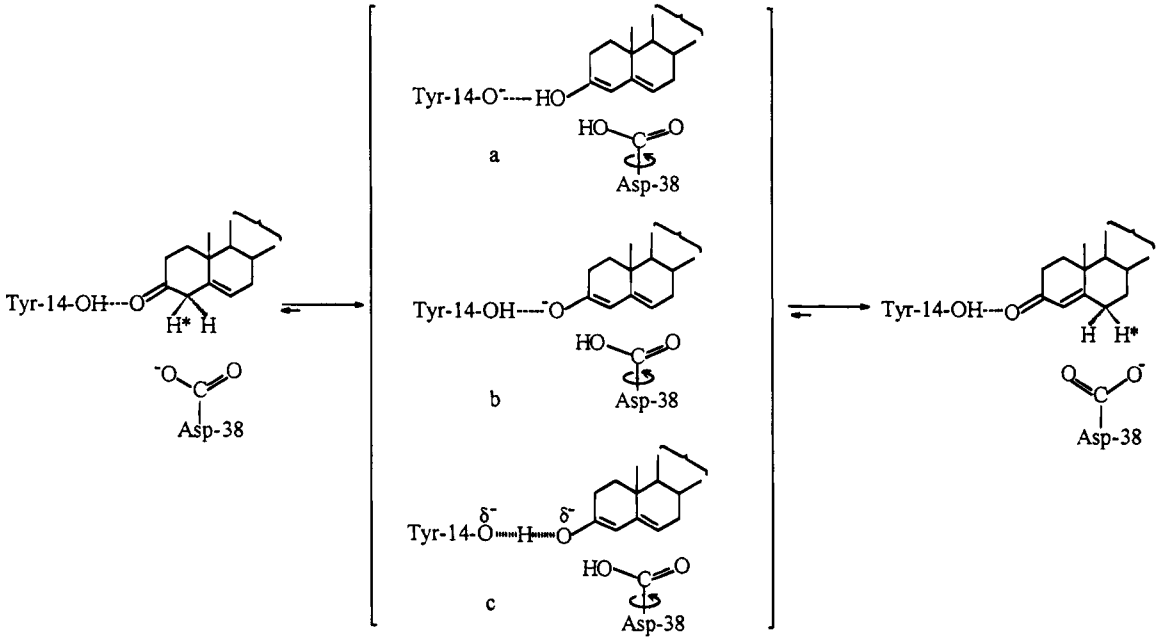


FIGURE 1: Isomerase-catalyzed conversion of Δ^5-3-ketosteroids to Δ^4-3-ketosteroids via an enolic intermediate. Catalysis has been proposed to proceed via (a) a dienol, (b) a dienolate, or (c) a low-barrier hydrogen-bonded species (Cleland & Kreevoy, 1994; Gerlt & Gassman, 1993; Holman & Benisek, 1994; Li et al., 1993a; Xue et al., 1990, 1991; Zeng et al., 1992).

one was attributed to enolization or protonation (and mimicked the behavior of this steroid in strong acid) (Wang et al., 1963; Kuliopulos et al., 1989). The UV spectra of 17β-estradiol and the UV absorption and fluorescence spectra of 17β-dihydroequilenin, when bound to isomerase, closely resembled those of phenolate anions in aqueous base. Yet, the precise identity of the chemical species responsible for these spectral shifts has remained elusive. Thus, it has been difficult to provide a plausible explanation for the apparent capacity of the enzyme both to act as a proton donor to the 3-carbonyl group of 19-nortestosterone and to promote the abstraction of a proton from the phenolic steroids.

Furthermore, since the active sites of most enzymes are more hydrophobic than the surrounding medium (and this would appear to be especially true for isomerase, which binds hydrophobic steroids), the environment of the bound steroid would be expected to be unfavorable for charge generation. The local dielectric constant near Tyr-14 at the active site of isomerase is similar to that of 2-propanol (Li et al., 1993a; Wu et al., 1994). The environment of the active site of isomerase would be expected to be even more hydrophobic upon binding of a steroid, since most or all of the water molecules at the active site would have been displaced by the more hydrophobic ligand. For instance, X-ray crystallographic studies of cholesterol oxidase have shown that 12 of the 13 water molecules present at the active site were displaced by the steroid, except for the one believed to be catalytically functional (Li et al., 1993b). The presence of Tyr-14 is essential for the aforementioned spectral changes, as shown by mutation of this residue to phenylalanine (Kuliopulos et al., 1989, 1991).

Recent UV resonance Raman studies (Austin et al., 1995) of 19-nortestosterone in different acids, solvents, and in the complex with isomerase showed that the vibrational frequencies of the C=C and C=O groups of 19-nortestosterone in the active site of isomerase are intermediate between those found with full protonation and in 10 M HCl, in which strong hydrogen bonding occurs (Hawkinson & Pollack, 1993), indicating that a very strong hydrogen bond is responsible for the 10 nm red shift in the complex. Here we report the effects of the medium on the UV and fluorescence spectral properties of α,β-unsaturated ketosteroids and phenolic compounds, and we compare these properties to those found with the enzyme-bound steroids. We conclude that neither full protonation of ketosteroids nor full deprotonation of phenolic steroids is required to account for the spectral changes in the isomerase−steroid complexes. Strong, directional hydrogen bonding interactions between Tyr-14 and the steroid ligands in a solvent-inaccessible active site can account for the spectral findings.

EXPERIMENTAL PROCEDURES

Materials. 4-Hydroxyandrost-4-ene-3,17-dione and 4-methoxyandrost-4-ene-3,17-dione were purchased from Sigma (St. Louis, MO). 2-Hydroxybenzoic acid, 4-hydroxybenzoic acid, 1-acetyl-2-naphthol, potassium hydroxide, and malonic acid were obtained from Aldrich (Milwaukee, WI). These compounds were used without further purification. Highest grade solvents were from Aldrich, Sigma, or J. T. Baker. 17β-Dihydroequilenin (DHE) was purchased from Steraloids (Wilton, NH) and was recrystallized from hexane/acetone before use. Recombinant wild-type isomerase was prepared and purified to electrophoretic homogeneity as described previously (Kuliopulos et al., 1987, 1989).

UV Measurements of Δ^4-3-Ketosteroids and Phenolic Compounds. The UV spectra of 4-hydroxyandrost-4-ene-3,17-dione, 4-methoxyandrost-4-ene-3,17-dione, 2-hydroxybenzoic acid, 4-hydroxybenzoic acid, and 1-acetyl-2-naphthol in different solvents were obtained at 25 °C with a Cary 1E UV−visible spectrophotometer in quartz cuvettes with a 1.0 cm light path. The spectra of appropriate blanks (buffers, solvents, or the buffers containing isomerase alone) were obtained before the addition of the compound of interest in methanol or acetonitrile. In most experiments, the concentration of methanol or acetonitrile in the final mixture of 1.0 or 3.0 mL total volume was $\leq$1.0%. Concentrations were adjusted to give absorbances of 0.5 or less and are expressed as molar absorption coefficients (ϵ).

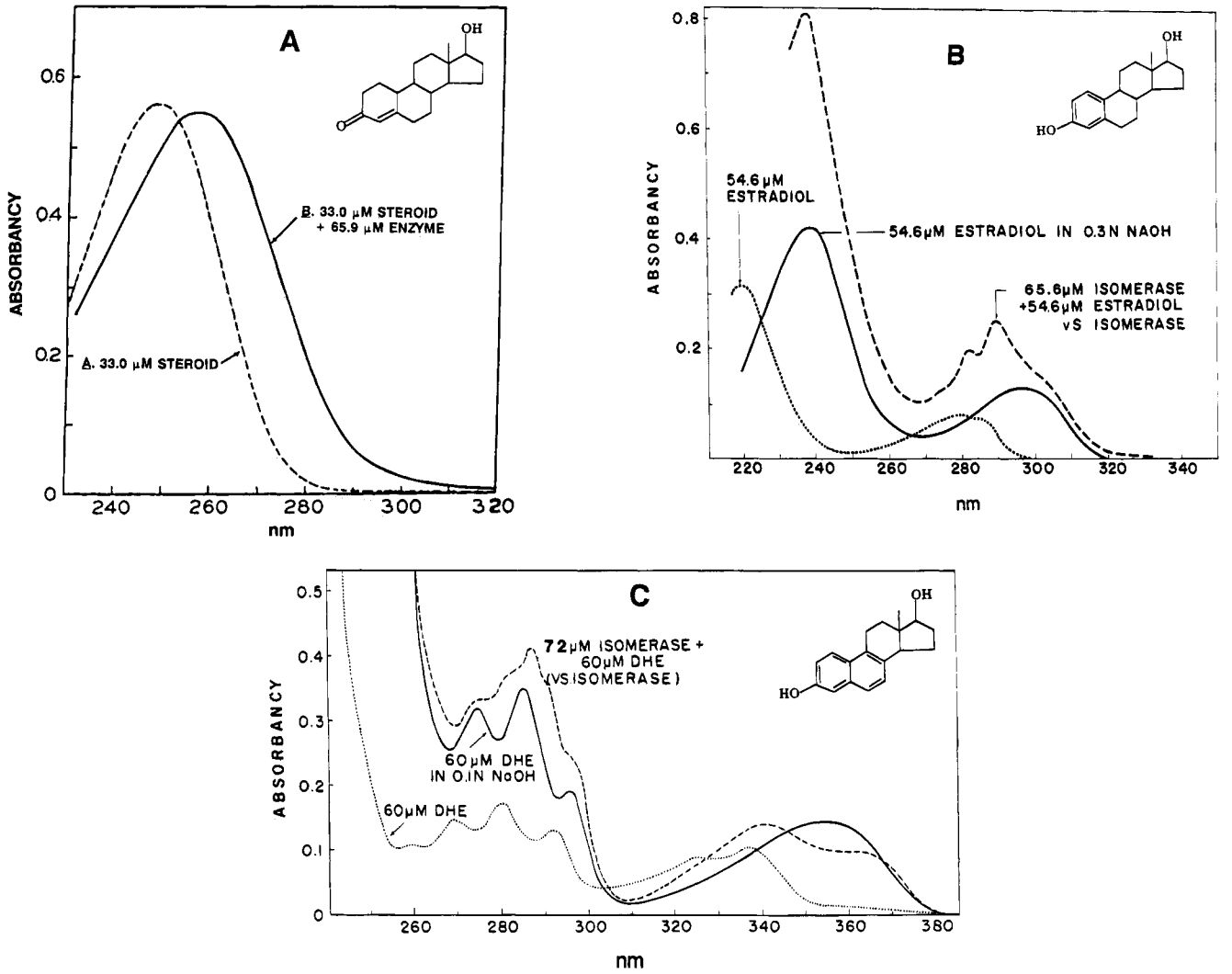


FIGURE 2: Spectral changes of competitive inhibitors of isomerase upon binding to the enzyme: (A) 19-nortestosterone (**1**); (B) 17β-estradiol (**2**); and (C) 17β-dihydroequilenin (**3**). The spectra of the phenolic steroids are compared to those obtained upon ionization in 0.1−0.3 N NaOH. The spectra are reproduced from Wang et al. (1963) and have been adjusted for the correct enzyme concentration based on revision of the molecular weight and the molar absorbance of isomerase.

Protein Concentration Determinations and Activity Assay. Concentration measurements of wild-type isomerase and activity assays were carried out at 25 °C with a Cary 1E UV−visible spectrophotometer according to published procedures (Kuliopulos et al., 1989; Li et al., 1993a). The concentrations of isomerase for all experiments are expressed in terms of subunits of the dimeric enzyme.

Excitation and Emission Spectra of 17β-Dihydroequilenin−Isomerase Complexes and 17β-Dihydroequilenin in Different Solutions. All fluorescence spectra of DHE were obtained at 25 °C with a Perkin-Elmer Model LS50 luminescence spectrometer. A slit width of 5.0 nm was used for both the excitation and emission beams. The spectra of blank buffers, solvents, or buffers containing wild-type isomerase were obtained before DHE was added and were subtracted from the spectra of the final mixtures. Spectra of DHE bound to isomerase were obtained with 1.0 μM DHE in the presence of 10.8 μM wild-type isomerase in 3 mM sodium phosphate buffer (pH 7.0).

Measurements of the Dissociation Constants of Complexes between 17β-Dihydroequilenin and Wild-Type Isomerase or between 17β-Dihydroequilenin and Malonate. Since the fluorescence emission of DHE undergoes dramatic changes upon binding to isomerase or malonate, fluorescence intensity measurements can be used to monitor the formation of the bound species. In the experiments with isomerase, the fluorescence intensity was measured with excitation at 350 nm and emission at 400 nm, which are the respective maxima of bound DHE. Similarly, for the titration with malonate ion, the fluorescence intensity was measured with excitation at 340 nm and emission at 420 nm, which are the approximate maxima of the DHE−malonate complex. The slit width was set at 5.0 nm for excitation and 10.0 nm for emission. The background fluorescence was subtracted from the total fluorescence of the mixture containing DHE.

The titration of DHE with isomerase or malonate ion was carried out in a total volume of 2.0 mL containing 3 mM sodium phosphate buffer (pH 7.0). For the experiments with isomerase, the final concentration of DHE was 0.5 μM and the isomerase concentrations ranged from 1.4 to 15.8 μM. This unconventional design, with constant inhibitor concentration and increasing concentrations of the enzyme, was selected because DHE is the fluorogenic species, and the K_D value was unaffected by the way in which the binding was measured. Titrations of DHE with malonate ion (50−1000 mM) were carried out in 3 mM sodium phosphate buffer (pH 7.0), at a final concentration of DHE of 1.0 μM. All solutions were adjusted to pH 7.0.

Table 1: Effects of Hydrogen Bonding on the UV Absorption Spectra of α,β-Unsaturated Ketosteroids[a]

		compound **4a**		compound **4b**		difference between **4a** and **4b**	
solvent	acceptor number[b]	λ_{max} (nm)	ϵ (M^{-1} cm^{-1})	λ_{max} (nm)	ϵ (M^{-1} cm^{-1})	$\Delta\lambda_{max}$ (nm)[c]	$\Delta\Delta\lambda_{max}$ (nm)[d]
water[e]	54.8	276.0	12 000	258.5	13 100	17.5	0.0
methanol	41.3	277.5	11 000	255.0	11 900	22.5	5.0
ethanol	37.1	278.0	11 300	255.0	12 500	23.0	5.5
2-propanol	33.5	278.0	11 800	255.0	12 100	23.0	5.5
acetonitrile	18.9	275.5	13 000	252.0	13 400	23.5	6.0
n-hexane	0	274.0	9 950	250.0	9 870	24.0	6.5

[a] The absorption maxima of 4-hydroxyandrost-4-ene-3,17-dione (**4a**) and 4-methoxyandrost-4-ene-3,17-dione (**4b**) were measured in different solvents. All measurements were made at 25.0 °C in the presence of 1.0% CH_3CN. The steroid concentrations were 60−75 μM. [b] Acceptor numbers (Gutmann, 1976) are measures of the capacity of the solvent to accept electron pairs and are a better reflection of the capacity for hydrogen bond formation than are dielectric constants or dipole moments. [c] Difference between the UV absorption maxima of **4a** and **4b** reflects the red shifts caused, at least partially, by the intramolecular hydrogen-bonding contribution to the UV absorption of the enone moiety in a given medium. [d] All of the $\Delta\Delta\lambda_{max}$ values refer to the $\Delta\lambda_{max}$ in water. [e] In 50 mM Tris-HCl (pH 7.50) containing 100 mM NaCl.

Table 2: Effects of Hydrogen Bonding and Solvents on the UV Absorption Spectra of 2-Hydroxybenzoic Acid (**5a**), 4-Hydroxybenzoic Acid (**5b**), and 1-Acetyl-2-naphthol (**6**)

		compound **5a**[a]		compound **5b**[a]		compound **6b**[b]	
solvent	acceptor number[c]	λ_{max} nm	ϵ (M^{-1} cm^{-1}	λ_{max} nm	ϵ (M^{-1} cm^{-1})	λ_{max} (nm)	ϵ (M^{-1} cm^{-1})
water[d]	54.8	297.0	3790	250.0	12 000	331.0	3040
		230.0	7660			306.0	3080
						288.0	3160
methanol	41.3	298.0	4030	252.5	14 600		
2-propanol	33.5	303.0	4700	256.0	15 600		
acetonitrile	18.9	303.0	4280			355.0	3640
						312.0	5090
n-hexane	0	311.0	6080	254.0	14 100	357.5	5250
		240.0	12900			312.0	6870
						302.0	6090
1.0 N NaOH		301.0	3590	281.0	17 500		
0.1 N NaOH						369.0	4300

[a] All spectra were obtained in presence of 1.0% methanol except the spectra in hexane, for which neat hexane was used. [b] All measurements were made at 25.0 °C in the presence of 1.0% CH_3CN. The concentrations of the phenolic compounds in the cuvettes were 50−150 μM. [c] Acceptor numbers were from Gutmann (1976). [d] In 50 mM Tris-HCl (pH 7.50) containing 100 mM NaCl.

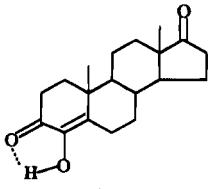
If we assume that both free and bound DHEs contribute to the fluorescence, the following hyperbolic function is used to fit the fluorescence intensity with respect to the concentration of hydrogen bond acceptor, i.e., isomerase or malonate (Li et al., 1993a):

$$F = (F_f/2[\mathrm{DHE}]_t)\{[\mathrm{DHE}]_t - K_D - A_t + [([\mathrm{DHE}]_t + K_D + A_t)^2 - (4A_t[\mathrm{DHE}]_t)]^{1/2}\} + (F_b/2[\mathrm{DHE}]_t)\{[\mathrm{DHE}]_t + K_D + A_t - [([\mathrm{DHE}]_t + K_D + A_t)^2 - (4A_t[\mathrm{DHE}]_t)]^{1/2}\}$$

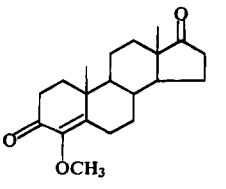
wherein F is the fluorescence intensity, K_D is the dissociation constant, $[\mathrm{DHE}]_t$ is the concentration of total DHE, A (acceptor) is the concentration of isomerase or malonate ion, and the subscripts f, b, and t refer to the free, bound, and total species, respectively. The nonlinear least-squares fit was carried out using GraFit (Leatherbarrow, 1992).

RESULTS

Effects of Intramolecular Hydrogen Bonding on the UV Spectra of α,β-Unsaturated Ketosteroids. The binding of 19-nortestosterone (**1**) to isomerase in neutral aqueous solution results in a 10 nm red shift of the λ_{max} of the UV spectrum of the enone chromophore without a significant change in intensity (Wang et al., 1963; see Figure 2A). In an attempt to model this spectral shift, we compared the effects of different solvents on the spectra of 4-hydroxyandrost-4-ene-3,17-dione (**4a**) and 4-methoxyandrost-4-ene-3,-17-dione (**4b**). In aqueous solution (Table 1), the intramolecular hydrogen bond of the enone moiety to the 3-carbonyl group of **4a** resulted in a 17.5 nm red shift of the absorption maximum from 258.5 to 276 nm. This difference was substantially enhanced when steroids **4a** and **4b** were dissolved in progressively less polar solvents. As shown in Table 1, the difference in λ_{max} ($\Delta\lambda_{max}$) between intramolecular hydrogen-bonded **4a** and its methyl ether **4b** increased from 17.5 nm in water to 24.0 nm in hexane. This 6.5 nm increase is most probably due to the strengthening of the intramolecular hydrogen bond in the less polar solvents, in which competing hydrogen bond formation from bulk solvent molecules is much less efficient. This interpretation is supported by the correlation of $\Delta\lambda_{max}$ with decreasing solvent acceptor number (Gutmann, 1976), which reflects the decreasing Lewis acidity of the solvents (see Figure 3). There were only minimal differences (11% or less) in the molar absorbance coefficients of the two steroids in the various solvents, and these differences were not correlated with solvent polarities. No change in absorbance intensity was observed when 19-nortestosterone bound to isomerase (Wang et al., 1963).



4a



4b

Spectral Properties of Intramolecularly Hydrogen-Bonded Phenolic Compounds in Solvents of Different Polarities. The

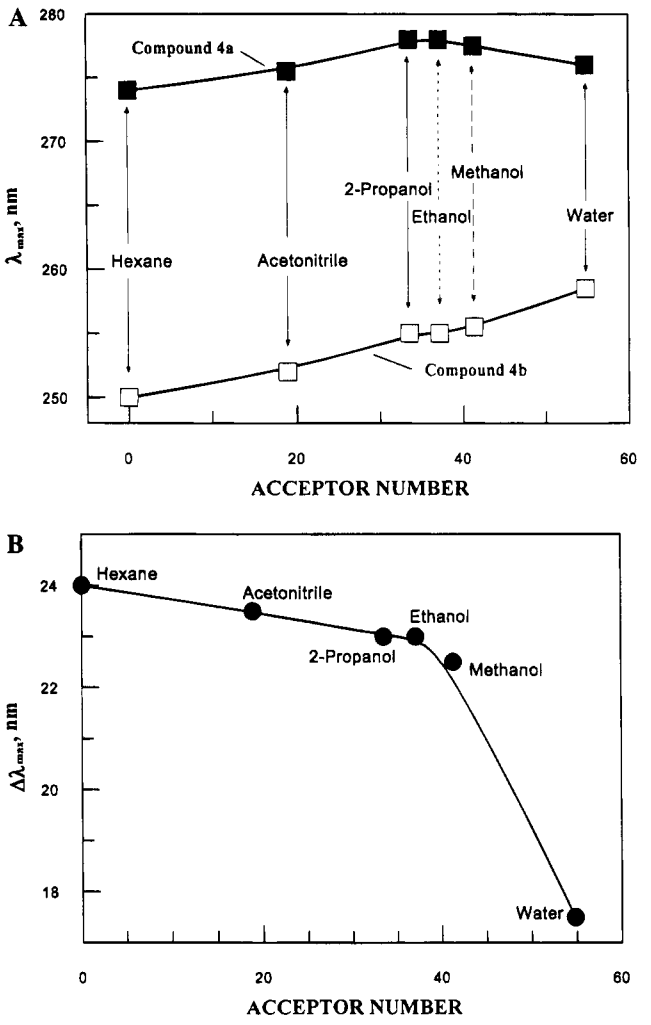


FIGURE 3: Comparison of the red shifts of UV spectra of 4-hydroxyandrost-4-ene-3,17-dione (**4a**) with those of 4-methoxyandrost-4-ene-3,17-dione (**4b**) in different solvents. (A) The absorption maxima of **4a** and **4b** are plotted against the solvent acceptor number. (B) The differences between the absorption maxima of **4a** and **4b** are plotted with respect to the solvent acceptor number.

binding of 17β-estradiol (**2**) or DHE (**3**) to isomerase in neutral aqueous solutions results in changes in both the positions and magnitudes of the UV absorption maxima (Figure 2B,C). The high-intensity absorption band at 220 nm is increased by 2.5-fold and shifted to 238 nm, and the lower intensity absorption is shifted to 290 nm and also increased about 2.7-fold. Upon binding to isomerase, similar but more complex changes in the absorption spectrum of DHE, in which both the A and B rings of the steroid are aromatic, were observed (Wang et al., 1963).

In order to determine the chemical identity of the isomerase-bound forms of 17β-estradiol, we compared the UV spectra of two model compounds—2-hydroxybenzoic acid (**5a**) and 4-hydroxybenzoic acid (**5b**)—in water and solvents of different polarities. With **5a**, where intramolecular hydrogen bond formation is favored, absorption intensity was enhanced and significant red shifts were observed in more hydrophobic media (Table 2). In contrast, 4-hydroxybenzoic acid (**5b**), in which intramolecular hydrogen bonding between the carboxyl and hydroxyl groups cannot take place, showed far smaller spectral changes as the hydrophobicity of the solvents was increased, whereas the ionization of the phenolic group in base still produced significant effects on the UV spectra (Table 2).

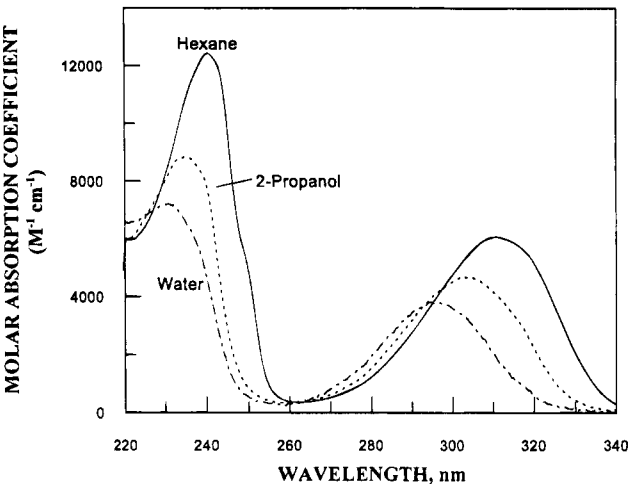


FIGURE 4: Effects of solvent polarity on the UV spectra of 2-hydroxybenzoic acid (**5a**). Spectra in 2-propanol and in 50 mM Tris-HCl buffer (pH 7.50) were obtained in the presence of 1.0% methanol. The spectrum of 2-hydroxybenzoic acid in hexane was obtained in the absence of methanol, since even 1% methanol (ca. 200 mM) in hexane was found to alter the spectrum of 2-hydroxybenzoic acid significantly because of the hydrogen-bonding capacity of methanol.

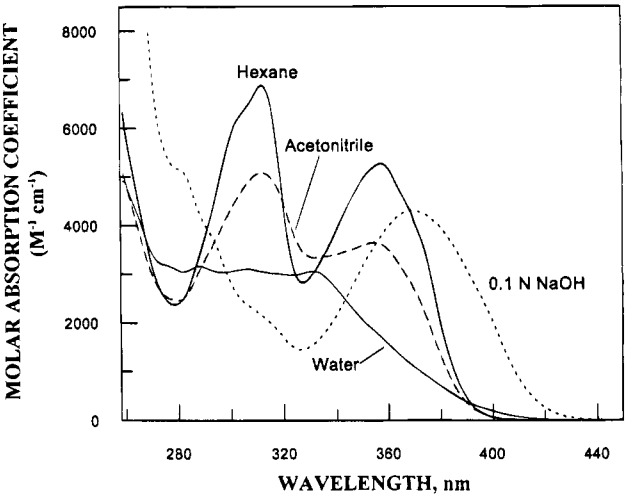
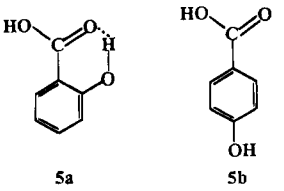


FIGURE 5: Effects of solvent polarity on the UV spectra of 1-acetyl-2-naphthol (**6**) in different solutions. The spectra were obtained with 150 μM 1-acetyl-2-naphthol and a final concentration of 1.0% acetonitrile. The background of the blank solution was subtracted. The following solvents are shown: 3 mM sodium phosphate buffer (pH 7.0) ("water"), 0.1 N NaOH, acetonitrile, and hexane.



5a **5b**

Strengthening the intramolecular hydrogen bond in a nonpolar solvent produces more profound effects on the UV absorption spectra than the ionization of the phenolic group in aqueous solution. In aqueous solution, the UV spectra of 2-hydroxybenzoic acid (**5a**) remain essentially identical from pH 1.0 to 14.0. Presumably, even when the ionization of the phenolic hydroxyl group (pK_a = 13.4) has occurred, the COO^- and O^- might still interact strongly by hydrogen bonding in a six-membered ring involving a proton from a neighboring water molecule. This experimental model demonstrates that a strong hydrogen bond or full ionization

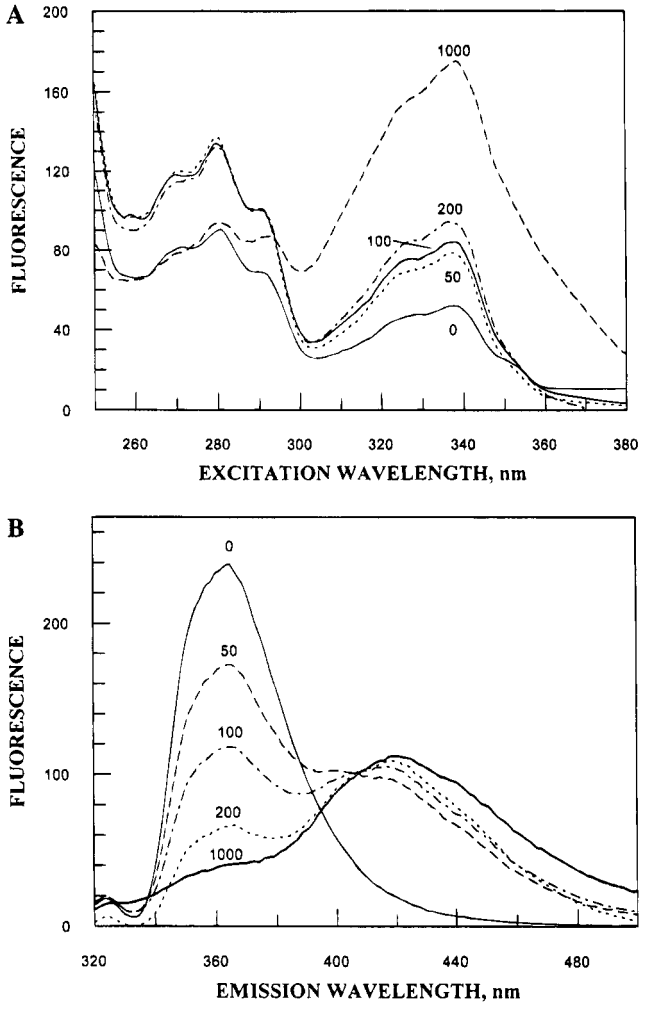


FIGURE 6: Excitation and emission spectra of 17β-dihydroequilenin (DHE) in the presence of increasing concentrations of potassium malonate in 3 mM sodium phosphate buffer (pH 7.0). (A) Excitation spectra of DHE in the presence of 50, 100, 200, and 1000 mM malonate. Emission was measured at 400 nm, which is near the isosbestic point of the emission spectra. (B) Emission spectra of DHE excited at 292 nm. The excitation wavelength was selected to be close to the isosbestic point of the excitation spectra in different solvents. Note, however, that at low concentrations of malonate, there is no isosbestic point. This suggests that the binding interaction is complex and that a high-affinity binding mode may occur at low concentrations of malonate.

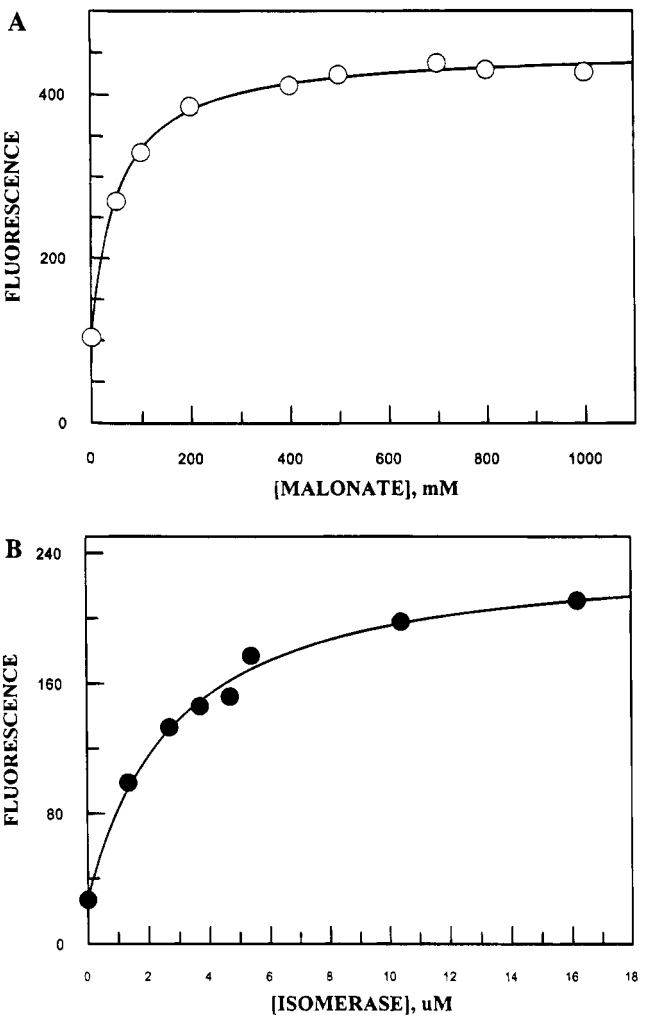
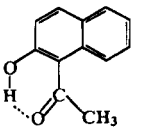


FIGURE 7: Fluorescence titration curves of 17β-dihydroequilenin (DHE) in the presence of increasing concentrations of potassium malonate and wild-type isomerase in 3 mM sodium phosphate buffer (pH 7.0). The fluorescence of the buffer containing malonate ion or isomerase was measured prior to the introduction of DHE and was subtracted from the total fluorescence of the mixture. (A) Fluorescence titration of DHE as excited at 340 nm and observed at 420 nm at increasing malonate ion concentrations. The concentration of DHE is 1.0 μM. An apparent dissociation constant, K_D = 51 ± 5 mM, was obtained by curve fitting described in the Experimental Procedures. (B) Binding curve of DHE to the active site of isomerase with the enzyme present in excess. The DHE concentration was 0.5 μM, and the final methanol concentration was 0.2%. The fluorescence intensity was monitored with an excitation wavelength of 350 nm and an emission wavelength of 400 nm. A K_D = 2.6 ± 0.4 μM (0.2% methanol) was obtained by the data fitting described in the Experimental Procedures and can be compared to inhibition constants K_i = 6.3 μM in 1.5% methanol (Wang et al., 1963) and K_i = 16 μM in 10% methanol (Weintraub et al., 1977) previously reported for DHE.

of the phenolic moiety can produce similar effects on UV spectra. This may be an extreme example in which one cannot identify whether or not the phenol is ionized from its UV spectrum, since 1 N NaOH exceeds the reported pK_a value by 0.6 pH unit. In contrast, 4-hydroxybenzoic acid (**5b**), where intramolecular hydrogen bonding is not possible, exhibits normal characteristics of phenolic compounds—significant spectral changes upon ionization and insensitivity to the hydrogen-bonding capacity of the medium. Hence, caution in the interpretation of UV and fluorescence measurements in terms of the ionization status of phenolic compounds is necessary since the excited state, as well as the ground state, contributes significantly to the spectra.

The complex red shifts and intensifications of the spectrum of DHE upon binding to isomerase were simulated by the behavior of 1-acetyl-2-naphthol (**6**). Figure 5 shows the dramatic effects of solvent polarity on the UV spectrum of 1-acetyl-2-naphthol (**6**). The red shifts and absorption enhancements, as the intramolecular hydrogen bond is



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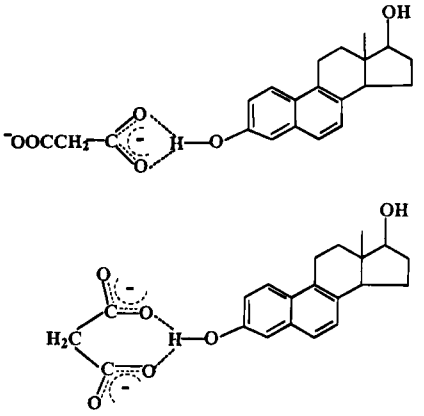
strengthened in nonpolar solvents (i.e., acetonitrile and hexane), mimic the UV spectral changes of the isomerase-bound DHE and 17β-estradiol. Surprisingly, no fluorescence of 1-acetyl-2-naphthol (**6**) was observed in water or acetonitrile at concentrations ranging from 10 to 325 μM, whereas 10 μM 2-naphthol is strongly fluorescent (data not shown).

Perturbation of Fluorescence Spectra of 17β-Dihydroequilenin in the Presence of Isomerase or Hydrogen Bond Acceptors. At neutral pH in aqueous solution, the fluorescence of DHE is sensitive to certain anions, which can act as hydrogen bond acceptors and thereby evoke significant shifts in the fluorescence spectra. The excitation and emission spectra of DHE at increasing concentrations of malonate ion are shown in Figure 6. Malonate ion was chosen as the hydrogen bond acceptor to study the spectral changes of DHE because it can interact with protons either through a four-membered ring, involving one COO^- group, or a six-membered ring, involving the two COO^- groups.



The spectra observed in high concentrations of hydrogen bond acceptor more closely mimic the spectra of the enzyme-bound DHE than do the ionization spectra obtained above pH 11, since the fluorescence intensity is much lower and the emission band is much broader for the ionized DHE (Eames et al., 1989). Similar effects on the spectra of DHE can also be produced by suitable concentrations of ammonium sulfate, sodium phosphate, and sodium acetate in neutral aqueous solutions. However, sodium chloride at concentrations as high as 4.0 M did not affect either the excitation or the emission spectra of DHE in aqueous solution (data not shown), possibly due to the inability of chloride ion to act as a strong hydrogen bond acceptor.

Interestingly, the fluorescence of DHE is titratable both by isomerase and by a hydrogen bond acceptor. Figure 7 shows the binding curves of DHE to isomerase and malonate ion. Reasonable correlations were obtained by fitting the measurements to the equation given in the Experimental Procedures, assuming 1:1 binding stoichiometry. K_D values of 2.6 ± 0.4 μM for isomerase and 51 ± 5 mM for malonate were obtained. The absence of consistent isosbestic points in Figure 6 indicates the occurrence of more than one complex of malonate with DHE. Curve fitting of fluorescence titrations at several wavelengths (not shown) reveals a second and weaker malonate binding site on DHE. A larger red shift from the fluorescence emission maximum of DHE in water (363 nm) was observed with malonate ion (or with other anions such as sulfate or phosphate) (λ_{max} ~ 420 nm) in comparison to the isomerase-bound ligand (λ_{max} ~ 400 nm).

While organic solvents, such as acetonitrile and hexane, did not cause significant alterations in the fluorescence spectra of DHE (data not shown), dimethyl sulfoxide significantly perturbed the excitation spectrum of DHE (Figure 8), probably due to its hydrogen bond-accepting properties, since dimethyl sulfoxide exists largely in its

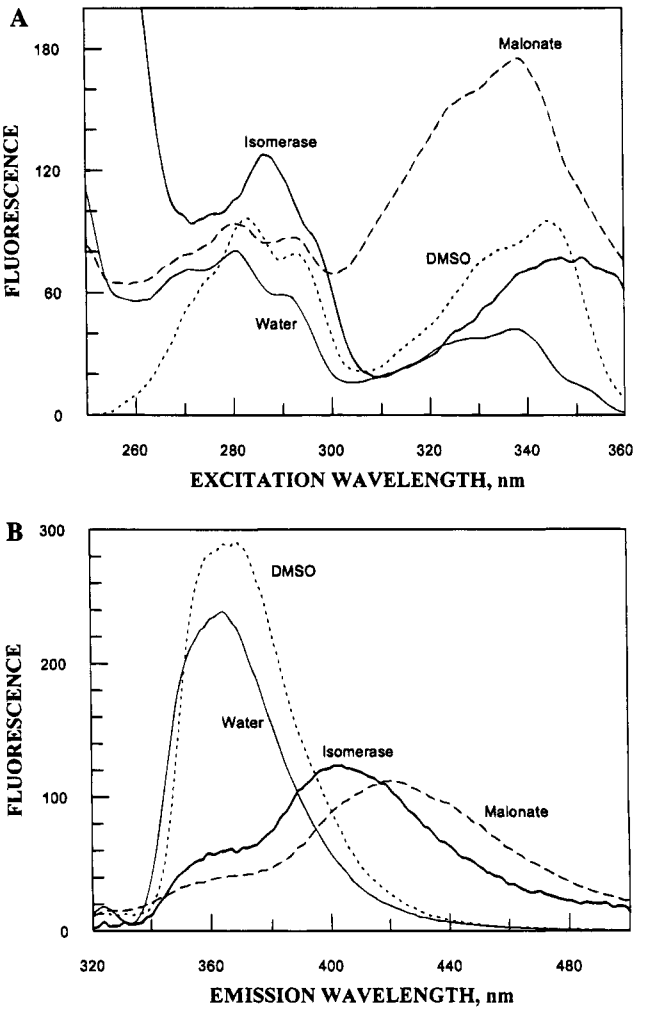
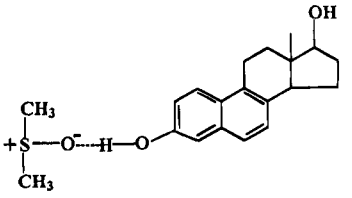


FIGURE 8: Excitation and emission spectra of 1 μM 17β-dihydroequilenin (DHE) in 3 mM potassium phosphate buffer, pH 7.0, alone ("water"), and in the presence of 10.8 μM isomerase, 1.0 M malonate ion, or dimethyl sulfoxide (DMSO). (A) Excitation spectra of DHE with the emission observed at 400 nm. (B) Emission spectra of DHE when the solutions were excited at 292 nm. The slit widths of both the excitation and emission were set at 5.0 nm. All the solutions except dimethyl sulfoxide contained the phosphate buffer.

charged resonance form (Martin, 1975):



The fluorescence spectra of DHE in water, in 1.0 M potassium malonate, in dimethyl sulfoxide, and bound to isomerase are compared in Figure 8. Although the emission spectra of DHE are not significantly changed in dimethyl sulfoxide, the changes in the excitation spectra are similar to the spectral changes that DHE undergoes upon binding to isomerase. Hydrogen bond formation in a lower dielectric environment could therefore be a model for the enzyme-bound species.

DISCUSSION

These studies were designed to provide understanding of the mechanisms underlying the spectral changes that 19-

nortestosterone (an enone) and phenolic steroids (17β-estradiol and DHE) undergo when bound to isomerase and of what insight such information might provide into the catalytic mechanism of the isomerase reaction. We have observed, by UV absorption and fluorescence spectroscopy of model enones and phenols, that intramolecular hydrogen bonding in nonpolar environments can simulate the spectroscopic changes that these compounds undergo upon protonation and deprotonation in aqueous (polar) media. However, since the development of charges in the hydrophobic binding site of the enzyme is unfavorable, and it is unlikely that Tyr-14 is capable of acting both as a proton donor to an enone and as a proton acceptor from a phenol, the formation of hydrogen bonds in a hydrophobic environment provides a more plausible and consistent interpretation of the aforementioned spectral changes.

Comparison of the Spectroscopic Red Shifts of the Enone Moiety of Steroids Induced by Strong Hydrogen Bonding in Nonpolar Environments and by Isomerase. The 10 nm apparent red shift of 19-nortestosterone upon binding to isomerase underestimates the actual polarization because the active site of isomerase has a dielectric constant close to that of 2-propanol (Li et al., 1993a). The absorption maximum of 19-nortestosterone is 239 nm in 2-propanol and 248 nm in water [cf. Fieser and Fieser (1959)]. Thus, the actual polarization of the enone moiety of 19-nortestosterone by isomerase could be approximately 18−20 nm. This polarization is well-simulated by an intramolecularly hydrogen-bonded ketosteroid (**4a**). The UV absorption spectrum of the related steroid **4b**, which cannot undergo intramolecular hydrogen bonding, shows a normal medium effect, i.e., λ_{max} becomes blue-shifted as the polarity (or acceptor number in Table 1 and Figure 3) decreases. In **4a**, however, in which intramolecular hydrogen bonding occurs through a five-membered ring, λ_{max} increases in less polar solvents. Presumably, less competition for hydrogen bond formation from solvent molecules strengthens the intramolecular hydrogen bond. The differences in λ_{max} between **4a** and **4b**, which represent the contribution of intramolecular hydrogen bonding, rose progressively from 17.0 nm in water to 24.5 nm in hexane as the acceptor number decreased. The especially sharp increase in $\Delta\lambda_{max}$ from water to ethanol is likely to be the result of a sharp decrease in hydrogen-bonding ability of the solvents.

Nonpolar Environments Are Unfavorable for the Ionization of Phenols. The fluorescence emission maximum of 2-naphthol in benzene (350 nm) was shifted dramatically to 410 nm in the presence of triethylamine. These effects have been interpreted as resulting from strong charge transfer from the filled nonbonding nitrogen orbital to the vacant hydroxyl antibonding orbital in the excited Franck−Condon state, followed by essentially complete proton transfer from oxygen to nitrogen in the excited state (Mataga & Kaifu, 1963). In the ground state, however, charge transfer or electron delocalization, rather than full proton transfer, is the major stabilizing factor for hydrogen-bonded donor−acceptor complexes in inert solvents. For instance, UV spectra of solutions of acetic acid and triethylamine in carbon tetrachloride, cyclohexane, or carbon disulfide have been ascribed to hydrogen bonding without ionization (De Tar & Novak, 1970). These model studies make it unlikely that phenolic steroids could undergo ionization in the hydrophobic binding site of isomerase.

Strong Hydrogen Bonding in Nonpolar Medium Simulates the Polarization of Phenolic Steroids by Isomerase. Comprehensive studies on the effect of intramolecular hydrogen bonding on the electronic spectra of *o*-substituted phenols (and phenylamines) have shown (Dearden & Forbes, 1960) significant red shifts, together with increases in intensity, for both the B-band (~220−240 nm) and C-band (~270−290 nm) of the *o*-substituted phenols when compared to the corresponding *m*-isomers. Some of these changes were attributable to steric effects, because an increase in intensity is also observed for compounds that cannot form intramolecular hydrogen bonds. Steric effects could also contribute to the changes in the UV spectra of isomerase-bound 17β-estradiol since intimate atomic contacts are expected in the enzyme−steroid complex in a solvent-inaccessible active site.

The spectra of 2-hydroxybenzoic acid (**5a**) in nonpolar solvents closely resemble those of 17β-estradiol upon binding to isomerase (Figures 2 and 4). The UV spectra of 1-acetyl-2-naphthol (**6**) in nonpolar solvents (Figure 5) resemble those of DHE bound to isomerase, and they "suggest that the enzyme-steroid mixture contains forms of dihydroequilenins that resemble both the un-ionized and ionized forms of steroids" (Wang et al., 1963). Nevertheless, ionization of the phenolic steroids in aqueous solution failed to simulate completely the absorption enhancement observed when DHE is bound to isomerase. The strong, directional intramolecular hydrogen bonding shown by 1-acetyl-2-naphthol (**6**) in nonpolar solvents (see Figure 5 and Table 2) provides a better model for the UV spectra of enzyme-bound phenolic steroids.

Comparison of the Polarization of 17β-Dihydroequilenin by Malonate and by Isomerase in Aqueous Solutions. The red shift in the maximal fluorescence emission wavelength of DHE, when associated with malonate ion ($\Delta\lambda \sim 57$ nm), is considerably larger than that observed when this steroid binds to isomerase ($\Delta\lambda \sim 37$ nm) (see Figure 8), suggesting that the hydrogen bond in the DHE−isomerase complex at the active site is weaker than that in the complex of DHE with a negatively charged hydrogen bond acceptor (i.e., malonate, sulfate, or phosphate). Alternatively, this finding may indicate that proton transfer in the excited state in the nonpolar active site is not as efficient as in aqueous solution. In the excited state, the hydrophobic active site of isomerase could generate an extraordinarily strong (possibly low-barrier) hydrogen bond to the 3-hydroxyl group of the liganded steroid. Full proton transfer with charge generation would be highly unfavorable since ion pair stabilization in nonpolar environments is difficult. An analogous interpretation of the perturbation of the fluorescence of 2-naphthol by triethylamine in cyclohexane was proposed by Mataga and Kaifu (1963). The difference in the emission maxima of free and triethylamine-bound 2-naphthol was ~ 45 nm (from 345 to 390 nm) in cyclohexane, whereas this difference was ~60 nm (from 350 to 410 nm) in benzene. As discussed earlier, the larger shift in benzene was attributed to complete proton transfer from oxygen to nitrogen in the excited state, resulting in an ion pair. Such a complete proton transfer is more difficult in cyclohexane solution because of the instability of the ion pair in this less polar solvent. Thus, for cyclohexane, "an extraordinarily strong, almost symmetrical" (or low-barrier) hydrogen bond in the excited state was proposed (Mataga & Kaifu, 1963).

Implications for a Low-Barrier Hydrogen Bond in the Catalytic Mechanism of Isomerase. On the basis of the present results and the preceding discussion, it is clear that

strong, directional hydrogen bonding in the solvent-inaccessible active site, rather than full protonation of ketosteroids or ionization of phenolic steroids, accounts for the observed spectral changes (Figure 2). Unfavorable charge development in the hydrophobic active site argues against the previously proposed dienol (with a negative charge developed on Tyr-14) or dienolate (with a negative charge localized at the 3-carbonyl oxygen atom) intermediates in the catalysis (Figure 1, path a or b). A low-barrier hydrogen-bonded intermediate (Figure 1, path c) delocalizes the negative charge originally on Asp-38 along the vector of C(3)−O· ·H· ·O− Tyr-14 after the 4β-proton is removed by Asp-38 and provides an attractive alternative. Further charge delocalization may be achieved by another compensating hydrogen bond donor to Tyr-14, such as a backbone amide NH or a bound water molecule, as suggested by UV resonance Raman studies of the 19-nortestosterone−isomerase complex (Austin et al., 1992, 1995) and UV spectra of the Y55F/Y88F mutant bound to 3-ketosteroids.[2] Such a hydrogen bond relay network also allows Tyr-14 to function as either a hydrogen bond donor to the carbonyl group of ketosteroids or in its deprotonated form as a hydrogen bond acceptor that stabilizes the enzyme complex with phenolic steroids (see Figure 1).

Closely matched pK_a values of Tyr-14 and the dienolic intermediate and exclusion of solvent from the active site meet the prerequisites for the formation of a low-barrier hydrogen bond (Cleland, 1992; Gerlt & Gassman, 1993; Cleland & Kreevoy, 1994). The pK_a of the dienol (~10 in water; Zeng et al., 1991) could be higher in the nonpolar active site, while the pK_a of Tyr-14 (11.6 in the free enzyme; Li et al., 1993a) could be slightly decreased by repositioning Tyr-14 in response to steroid binding (Kuliopulos et al., 1991). The proposed low-barrier hydrogen bond in the intermediate is also consistent with the solvent kinetic isotope effect of 1.6 on k_{cat} (Xue et al., 1990), extensive polarization of Δ^4-3-ketosteroids detected by UV resonance Raman spectroscopy (Austin et al., 1992, 1995), and Brønsted analysis of general base and general acid groups in catalysis by isomerase (Holman & Benisek, 1994; Brooks & Benisek, 1994). Mutation of Tyr-14 to phenylalanine results in the loss of at least 7.6 kcal/mol in the free energy of binding of the dienolic intermediate (Xue et al., 1991). A low-barrier hydrogen bond could account for up to 20 kcal/mol stabilization free energy of the intermediate, and this could offer an explanation for this extremely efficient catalytic process, which approaches the diffusion-controlled rate limit. Participation of a strong (low-barrier) hydrogen bond has also been proposed in the catalytic mechanisms of mandelate racemase, triosephosphate isomerase, and other enzymes that involve enolic intermediates (Gerlt & Gassman, 1993; Cleland & Kreevoy, 1994). Recently, the presence of a low-barrier hydrogen bond in the mechanism of serine proteases has been demonstrated by studies of appropriate model compounds by Frey et al. (1994).

On the basis of the similarities in the polarization of ketosteroids and phenolic steroids by strong hydrogen bonding in nonpolar environments and by isomerase, we conclude that the spectral changes first observed by Wang et al. (1963), which occur when α,β-unsaturated ketosteroids and phenolic steroids are bound to isomerase, are best explained by the propensity of Tyr-14 at the active site of the enzyme to generate a strong, low-barrier hydrogen bond to the 3-oxygen groups of the steroids during the process of catalysis. This suggestion provides a satisfactory resolution of the vexing question of the nature of these spectral changes, which were first observed more than 30 years ago.

[2] Q. Zhao, unpublished results (1994).

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