Is a Proton Relay Involved in the Mechanism of 3-Oxo-Δ^5-steroid Isomerase?†

David C. Hawkinson§ and Ralph M. Pollack*,§,‡

Laboratory for Chemical Dynamics, Department of Chemistry and Biochemistry, University of Maryland Baltimore County, Baltimore, Maryland 21228-5398, and Center for Advanced Research in Biotechnology, 9600 Gudelsky Drive, Rockville, Maryland 20850

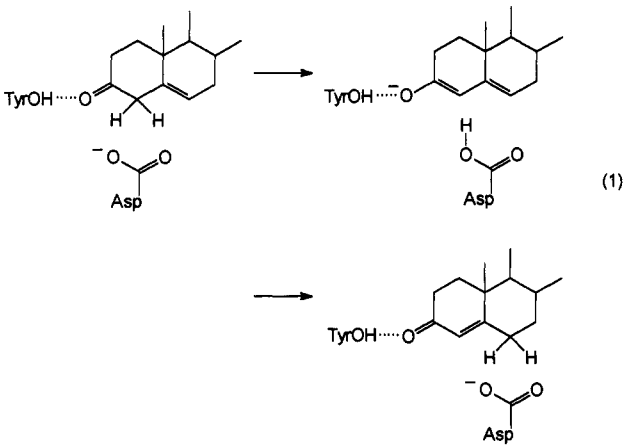
Received July 28, 1992; Revised Manuscript Received September 28, 1992

ABSTRACT: Previous reports of a UV spectral shift upon binding of the competitive inhibitor 19-nortestosterone (**1**) to 3-oxo-Δ^5-steroid isomerase (KSI), coupled with UV resonance Raman results, have led to the conclusion that the enone moiety is polarized to a degree similar to that produced by complete protonation and that a proton relay may be involved in the enzymatic mechanism (Austin et al., 1992). These conclusions were partly based upon interpretations of the corresponding UV spectra of **1** in aqueous acid solutions. These interpretations are shown to be inconsistent with results of deuterium exchange studies and with spectra of model systems. Consequently, there is no evidence either for an extraordinary polarization of **1** produced by binding to the active site of KSI or for a proton relay mechanism.

3-Oxo-Δ^5-steroid isomerase (also called Δ^5-3-ketosteroid isomerase, KSI,[1] EC 5.3.3.1) is an example of a class of enzymes that catalyze proton abstraction adjacent to a carbonyl group. Since these protons have only a moderate intrinsic acidity, the precise mechanism by which they are labilized during catalysis is of considerable interest (Gerlt et al. 1991). Recently, Austin et al. (1992) concluded that the active site of KSI polarizes bound substrate through the combined action of an electrophile (Tyr-14) and the enzymatic base (Asp-38). They further postulated a "proton relay" mechanism, in which the proton of Tyr-14 is donated to the substrate, while another acidic group protonates Tyr-14 during abstraction of the α proton by Asp-38. In this work, we show that simple hydrogen bonding of Tyr-14 to the substrate is consistent with all of the available evidence and that there is no need to invoke a proton relay mechanism.

KSI catalyzes the conjugation of a number of 3-oxo-Δ^5-steroids to their Δ^4-isomers (eq 1) [for reviews, see Pollack et al. (1989) and Schwab and Henderson (1990)]. The enzyme is an extremely efficient catalyst, operating at a rate within a factor of 3 of the diffusion-controlled limit with 5-androstene-3,17-dione as the substrate (Hawkinson et al., 1991b). It is firmly established that the KSI-catalyzed reaction proceeds through formation of an enzyme-bound dienol or dienolate ion intermediate (Eames et al., 1990; Hawkinson et al., 1991a), with Asp-38 acting to transfer a proton from C-4 to C-6 (Kuliopulos et al., 1989). Electrophilic catalysis is provided by Tyr-14 (Kuliopulos et al., 1989), through either hydrogen bonding (Zeng et al., 1992) or complete proton transfer to the C-3 oxygen of the substrate (Malhotra & Ringold, 1965; Kuliopulos et al., 1989, 1990, 1991; Xue et al., 1990).

Investigation of the nature of the electrophilic catalysis by Tyr-14 of KSI has relied heavily upon spectroscopic examination of the interaction of chromophoric steroids with the enzyme. Changes in the UV absorbance (Wang et al., 1963; Kuliopulos et al., 1989; Zeng et al., 1992), fluorescence (Wang et al., 1963; Kuliopulos et al., 1989; Eames et al., 1989; Zeng et al., 1992) NMR (Kuliopulos et al., 1991), and UV resonance Raman (Austin et al., 1992) spectra of these probe molecules upon binding to KSI and its active-site mutants have been interpreted in terms of the protonation states of enzyme-bound species in the catalytic process. In early studies, Wang et al. (1963) observed that binding of the product analog, and competitive inhibitor, 19-nortestosterone (**1**) to KSI results in a bathochromic shift of the principal absorption band in the UV from 248 nm in water to 258 nm. Later, Kuliopulos et al. (1989) examined the UV spectra of **1** in acidic solutions to provide a nonenzymatic model for this spectral shift. Using this model system, they inferred that the carbonyl group of **1** is protonated upon binding to the active site of KSI.



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More recently, on the basis of NMR data (Kuliopulos et al., 1991) and UV resonance Raman results (Austin et al., 1992), Mildvan and co-workers concluded that 19-nortestosterone is not protonated at the active site of KSI. In order to accout for their data, Austin et al. (1992) postulated that the active site polarizes the bound enone to a greater degree than protonation of the carbonyl does and that a proton relay mechanism is involved in the catalysis. However, it is important to recognize that this inference relies heavily upon the interpretation of the UV spectral changes of 19-nortestosterone in aqueous acid. Clearly, in any attempt to draw conclusions of this nature from spectral data, the choice and evaluation of model systems that mimic the spectral observations is crucial. We describe here evidence that is incon-

† This work was supported by Grant GM38155 from the National Institute of Gereral Medical Sciences, U. S. Public Health Service.

* Address correspondence to this author at the University of Maryland Baltimore County.

§ University of Maryland Baltimore County.

‡ Center for Advanced Research in Biotechnology.

[1] Abbreviations: KSI, 3-oxo-Δ^5-steroid isomerase; $Y14_o$, KSI mutant with only one tyrosine at position (Tyr-14).

0006-2960/93/0432-694$04.00/0

sistent with previous interpretations of the UV spectra of model systems. Thus, there is no reason to invoke any extraordinary ability of KSI to polarize bound steroid beyond that provided by moderate hydrogen bonding to Tyr-14, nor is there evidence for a proton relay mechanism. All of the evidence is consistent with catalysis by hydrogen bonding from Tyr-14.

EXPERIMENTAL PROCEDURES

3-Isobutoxy-2-cyclohexen-1-one was synthesized by Dr. Sang-Ho Lee according to Funk and Vollhardt (1980). Ethyl ether (Aldrich, absolute) was dried by distillation from sodium. Water was purified by passage through an Ion Pure reverse osmosis system coupled with a tank ultrapurification system consisting of two mixed media deionization tanks, a carbon tank, a UV sterilization lamp, and a Pyroguard ultrapurification membrane. 19-Nortestosterone (Sigma, one spot by TLC), lithium chloride (Baker), hydrochoric acid (Baker), sulfuric acid (Baker), and all other reagents were used without further purification.

3-Methyl-2-cyclohexen-1-one (**5**) was prepared by a procedure adapted from that for the synthesis of 3-ethynyl-2-cyclohexen-1-ones (Jeganathan et al., 1984). Methyl lithium (0.012 mol, Aldrich, 1.4 M in ethyl ether) was added with stirring to 2.0 g (0.012 mol) of 3-isobutoxy-2-cyclohexen-1-one in 20 mL of dry ethyl ether at 0 °C. After being stirred for 1 h, the mixture was diluted with 80 mL of ethyl ether and washed with 2 × 50 mL of cold 1 M HCl. The organic layer was concentrated to ca. 15 mL, diluted with 25 mL of THF, and stirred vigorously with 25 mL of 1 M HCl for 30 min. The product was extracted with 2 × 50 mL of ethyl ether, washed with 2 × 50 mL of 10% $NaHCO_3$(aq) and 50 mL of cold water, and dried over anhydrous sodium sulfate. After evaporation of the solvent, the compound was purified by column chromatography (Merck-60 silica; 5:1 hexane/ethyl ether). This procedure yielded 0.682 g (52%) of the desired product: ^{1}H NMR ($CDCl_3$) δ 1.96 (s, 3 H), 1.99 (m, 2 H), 2.28 (t, 2 H, J = 6.0 Hz), 2.34 (t, 2 H, J = 6.6 Hz), 5.88 (s, 1 H); IR (neat) incluses 1665 cm^{-1} (broad, C=O stretch), 1628 cm^{-1} (C=C stretch); the NMR is consistent with literature values (Pletcher & Smith, 1975); mass spectrum, base peak at m/e 82 (M − 56).

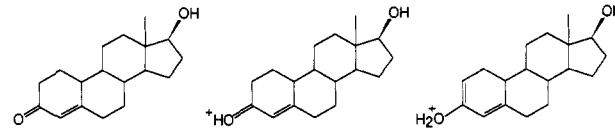
3,6,6-Trimethyl-2-cyclohexen-1-one (**4**) was prepared by acid-catalyzed Robinson annulation (Heathcock et al., 1971) using methyl vinyl ketone (125 mmol, Aldrich) and 3-methyl-2-butanone (100 mmol, Aldrich). Purification by column chromatography (Merck-60 silica; 20:1 hexane/ethyl ether) gave the desired product in 18.8% yield: ^{1}H NMR ($CDCl_3$) δ 1.09 (s, 6 H), 1.80 (t, 2 H, J = 6.7 Hz), 1.92 (s, 3 H), 2.29 (t, 2 H, J = 6.7 Hz), 5.76 (s, 1 H); IR (neat) includes 1658 cm^{-1} (C=O stretch), 1632 cm^{-1} (C=C stretch); NMR and IR peaks are consistent with literature values (Elberson, 1966; Cook & Waring, 1973); mass spectrum, base peak at m/e 82 (M − 56); 2,4-dinitrophenylhydrazone of **4**, mp 206–208 °C (lit. mp 207.5–208 °C; Cook & Waring, 1973).

UV Spectra. UV absorbance spectra were recorded on a Gilford Response spectrophotometer in 10-mm path length cells. In all cases, 1.6% methanol was present as cosolvent.

Mass Spectral Measurements. A 0.5-mL portion of 19-nortestosterone in 12 M H_2SO_4 (5 mg/mL) was added dropwise to 50 mL of 0.48 M sodium acetate/D_2O with rapid stirring. The product was immediately extracted with two 12-mL protions of chloroform. After being dried over anhydrous magnesium sulfate, the sample was evaported to dryness and redissolved in a minimal amount of wet acetonitrile (ca. 3% water) for analysis on a Hewlett Packard HP 5988A mass spectrophotometer (HPLC injection, EI mode). To enhance the sensitivity of the mass spectral measurments, peaks near the m/e of the molecular ion (m/e 272–278) were selectively monitored. An average of 10–15 scans was taken for each sample, and the background was subtracted. The fraction of deuterium incorporated into 19-nortestosterone was calculated as previously described (Hawkinson et al., 1991a).

RESULTS AND DISCUSSION

UV Spectra of Model Systems. In an effort to provide a model for the bathochromic shift in the UV spectrum of 19-nortestosterone (**1**) upon binding to KSI, Mildvan and coworkers (Kuliopulos et al., 1989) determined the spectrum of **1** in 10 M HCl and 10 M H_2SO_4. In 10 M HCl, the spectrum of **1** exhibits a major peak at 258 nm, with a shoulder at about 285 nm, similar to that for **1** bound to the active site of KSI. In 10 M H_2SO_4, the spectrum is dominated by the peak at 285 nm. Kuliopulos et al. (1989) interpreted the shift from 248 nm in water to 258 nm in 10 M HCl in terms of formation of the protonated ketone (**2**) in 10 M HCl. The larger bathochromic shift to 285 nm in 10 M H_2SO_4 was assigned to formation of a protonated homoannular $\Delta^{2,4}$-dienol (**3**). By analogy, they suggested that the bathochromic shift of **1** from 248 nm (water) to 258 nm (bound to KSI) is due to formation of the protonated ketone (**2**) at the active site. Later, on the basis of NMR results, Kuliopulos et al. (1991) postulated that **1** is not protonated at the active site, but rather is subject to hydrogen bonding to the carbonyl from Tyr-14.



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Austin et al. (1992) determined UV resonance Raman spectra for **1** in acidic solutions and for **1** bound to the active site of a KSI mutant having as its only tyrosine Tyr-14 ($Y14_o$). From these results, they concluded that polarization of the enone at the active site of $Y14_o$ KSI "produces a carbonyl frequncy (in the UV resonance Raman) that is nearly as low as that produced by protonation in aqueous solution and a vinyl frequency that is even lower." Clearly, however, this interpretation rests upon the identification of the species absorbing at 258 nm in 10 M HCl as the protonated ketone (**2**).

The unprecedented nature of the structure of **3** prompted us to test for its existence by attempting to trap the proposed protonated dienol (**3**) by quenching in D_2O. A solution of 19-nortestosterone (**1**) in 12 M H_2SO_4 was rapidly quenched into deuterium oxide, with sodium acetate present in the quench solution to regenerate **1**. Mass spectral analysis of the **1** isolated from this quench shows only minor incorporation (ca. 0.8%) of deuterium. If the protonated dienol (**3**) were the predominant species in solution at [H_2SO_4] ≥ 10 M, neutralization by quenching in a large excess (100-fold) of D_2O should result in substantial incorporation of solvent deuterium at the C-2 position. The small quantity of deuterium found in **1** after quenching is clearly inconsistent with the presence of significant quantities of the protonated dienol (**3**) in 12 M H_2SO_4.

In order to further investigate the nature of the structures responsible for the UV spectra in acidic solutions, we examined the spectral behavior of 3,6,6-trimethyl-2-cyclohexen-1-one

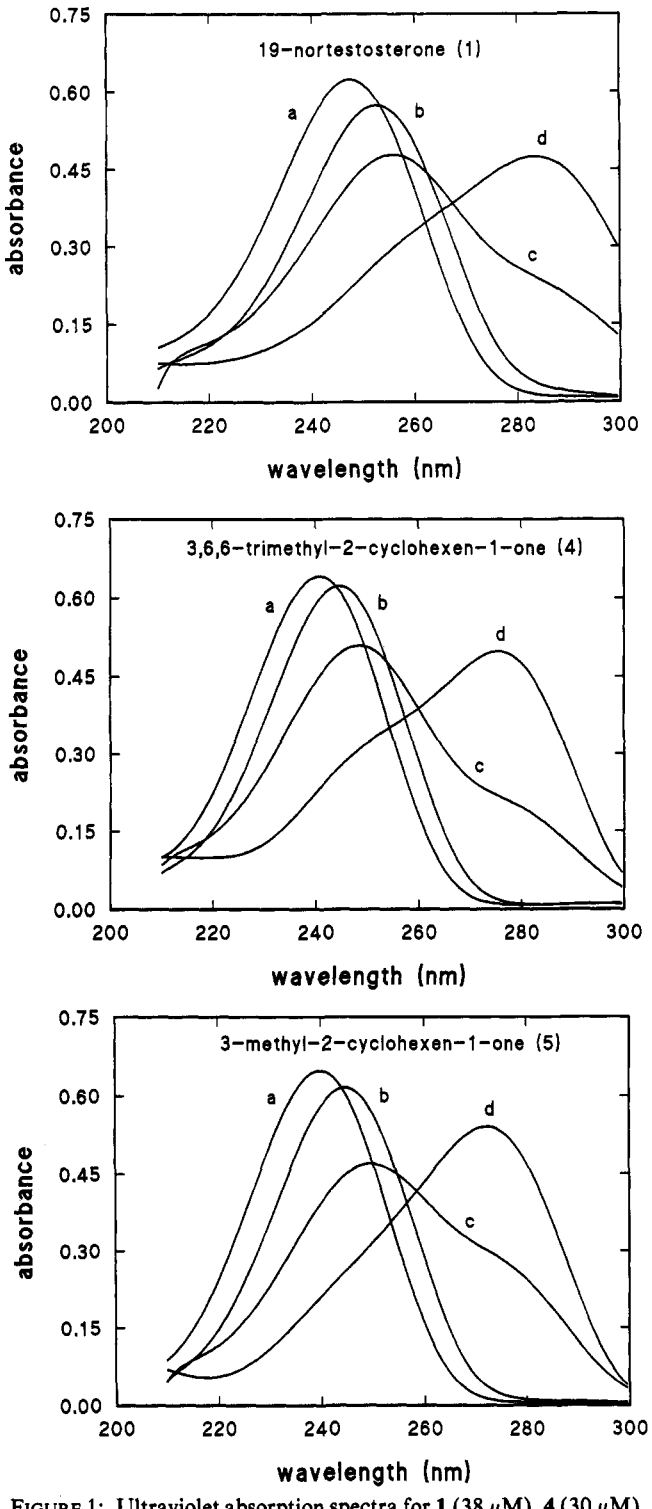

FIGURE 1: Ultraviolet absorption spectra for **1** (38 μM), **4** (30 μM), and **5** (30 μM) in water (a), 10 M LiCl (b), 10 M HCl (c), and 10 M H_2SO_4 (d).

(**4**). The geminal methyl groups of **4** preclude formation of a protonated dienol, analogous to **3**. The UV absorption spectra of **4** in water, aqueous lithium chloride (10 M), aqueous hydrochloric acid (10 M), and aqueous sulfuric acid (10 M) are compared with those for 3-methyl-2-cyclohexen-1-one (**5**) and for **1** in Figure 1 and in Table I. As expected, the λ_{max} for **1** appears at a substantially longer wavelength than those of **4** and **5** (ca. 7 nm in water), due to the presence of an exocyclic carbon–carbon double bond in **1** (Silverstein et al., 1981). The UV spectra of **1** in water, 10 M HCl, and 10 M H_2SO_4 are similar to those described by Kuliopulos et al.

Table I: Wavelength Maxima (λ_{max}) and Bathochromic Shifts ($\Delta\lambda_{max}$) of **1**, **4**, and **5** Relative to Water in Nanometers

compound	H_2O	10 M LiCl	10 M HCl	10 M H_2SO_4
1	248	252.5 (4.5)	256 (8)	283.5 (35.5)
4	241.5	244.5 (3)	249 (7.5)	275.5 (34)
5	240.5	244.5 (4)	250 (9.5)	273 (32.5)

(1989), although Austin et al. (1992) report a somewhat different acidity dependence of the absorption maxima.[2] We also observe a bathochromic shift of 4.5 nm (relative to water) in the λ_{max} of **1** in 10 M LiCl. The values of the shifts in λ_{max} ($\Delta\lambda_{max}$) for **4** and **5** are similar to those for **1**.

Since the pK_a of conjugated cyclohexenones depends largely on the substitution of the vinyl group (Zalewski & Dunn, 1969), the basicities of **1**, **4**, and **5** are expected to be similar (pK_a ca. −2.8 to −3).[3] While tautomerization of a protonated enone (e.g., **2**) to a protonated dienol (e.g., **3**) could conceivably occur with **1** and **5**, this possibility is not an option for **4**, which possesses a quaternary carbon atom at the α'-position. The similar spectral changes observed for **1**, **4**, and **5**, coupled with the fact that **4** can display only simple acid–base behavior (i.e., reversible protonation of the carbonyl oxygen), provide convincing evidence that the protonated dienol of **1** (**3**) is not formed in any appreciable amount in acidic solution. A more reasonable interpretation is that the bathochromic shift of **1** in 10 M H_2SO_4 ($\Delta\lambda$ 35.5 nm) is caused by formation of the protonated ketone (**2**).[4] In 10 M HCl ($\Delta\lambda$ 8 nm), the wavelength shift is likely due to the increased polarity of the solvent, as exemplified by the significant red shift observed in 10 M LiCl (4.5 nm). Alternatively, hydrogen bonding from aqueous 10 M HCl may be more efficient than from water.

This conclusion is consistent with previous explanations of the UV absorption spectra of carbonyl compounds in acidic media in terms of a combination of protonation at the carbonyl oxygen and medium effects (Yates et al., 1964; Cox & Yates, 1981). As Hammett (1970) has noted, "...the absorption spectrum of a solute often changes with changing medium even in the absence of a recognizable chemical reaction." This medium effect, which generally produces much smaller wavelength shifts than protonation,[5] precludes the observation

[2] Austin et al. (1992) report UV spectra of **1** that are quite different than those of Kuliopulos et al. (1989) or those that we observe. Austin et al. (1992) report that in 3.5 M H_2SO_4 λ_{max} appears at 260 nm, while in 6 M H_2SO_4 it is shifted to 290 nm. These wavelength shifts are considerably longer than those obtained by us in these solutions.

[3] The pK_a of **1** [ca. −3.1 (Smoczkiewicz & Zalewski, 1968; Zeng et al., 1992)] and **5** [−2.82 (Zalewski & Dunn, 1969)] have been experimentally determined. The three enones examined in this study are predicted to have the same pK_a (−2.82) using the additivity method of Zalewski and Dunn (1969). Alkyl groups at the α'-position of unsaturated ketones have little effect on the pK_a (Campbell & Edward, 1960).

[4] It is interesting to compare the acidities of 10 M HCl and 10 M H_2SO_4 in terms of their ability to protonate α,β-unsaturated ketones. The extent of protonation of these ketones correlates with the H_A acidity function for both sulfuric acid and perchloric acid solutions, although there does not appear to be any systematic examination of the protonation behavior with respect to hydrochloric acid solutions (Zalewski, 1989). Both 10 M HCl and 10 M_2SO_4 have similar H_A values (−3.2 for HCl and −3.3 for H_2SO_4, calculated from the data of Yates and Riordan (1965) and Yates et al. (1964). This result would suggest that the extent of protonation of these enones should be similar in the two acid solutions. However, H_o values for these solutions differ significantly [−3.6 for HCl and −4.9 for H_2SO_4 (Rochester, 1970)], suggesting that sulfuric acid might be better able to protonate these enones.

Table II: Raman Frequencies and UV Spectra of 19-Nortestosterone (1)[a]

system	λ_{max} (nm)	$\nu_{C=C}$ (cm^{-1})	$\nu_{C=O}$ (cm^{-1})
H_2O	248	1613.5	1641
3.5 M H_2SO_4[b]	260[b]	1608	c
KSI[d]	258	1596	1614
6 M H_2SO_4[b]	290[b]	1573	c

[a] Data taken from Austin et al. (1992). [b] The λ_{max} for these solutions are not in agreement with those previously reported. See footnote 2. [c] Only one peak is seen in the Raman. The C=O peak may be underneath the C=C peak. [d] The $Y14_o$ mutant of KSI, containing only one tyrosine (Tyr-14), was used.

of an isosbestic point for a series of spectra in solutions of differing acidity.

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Interpretation of UV and UV Resonance Raman Spectra. Table II shows both the UV absorption maxima and the Raman frequencies for **1** under various conditions, as reported by Austin et al. (1992). These authors attributed the 1573 cm^{-1} peak for the 285 nm (290 nm) species to the *cis*-diene stretch of **3**. However, it is clear that the 285 nm species is the protonated ketone, so that this band might better be assigned to the C=C stretch of **2**. The data in Table II also show that the peaks in both the UV spectrum and the resonance Raman spectrum of **1** bound to KSI are significantly shifted from the corresponding spectra in water. However, neither of these spectral probes reveals a polarization of the enone that resembles that of the protonated species (290 nm). Rather, both spectra are in accord with those for the 260 nm species. Thus, the simplest explanation of the peak at 258 nm for the KSI–**1** complex is that hydrogen bonding from the hydroxyl group of Tyr-14 to the carbonyl oxygen is somewhat stronger than hydrogen bonding from water in aqueous solution. An effect due to active site polarization produced by the combination of the catalytic residues Tyr-14 and Asp-38 is unlikely since **1** bound to the D38N mutant of KSI (Asp → Asn) shows the same UV spectral properties as **1** bound to the wild-type KSI (Kuliopulos et al., 1989).

Our interpretation also offers a satisfactory explanation for the results used to postulate a proton relay mechanism. The tyrosine ν_{8b} band in the UV resonance Raman spectrum of $Y14_o$ KSI is similar to that for $Y14_o$ KSI with **1** bound at the active site, suggesting that only a weak hydrogen bond is formed between Tyr-14 and the carbonyl group of **1**. However, this conclusion conflicts with the interpretation of the UV spectrum that a strong hydrogen bond is formed between Tyr-14 and the steroid carbonyl in the complex. To explain this discrepancy, Austin et al. (1992) proposed that there is an additional residue at the active site (DH) that donates a hydrogen bond to Tyr-14. This hypothesis led them to postulate a proton relay mechanism for the isomerization reaction catalyzed by KSI, in which DH donates a proton to Tyr-14 as the Tyr-14 proton is transferred to the carbonyl oxygen of the steroid. In light of the present results, which are consistent with a *weak* hydrogen bond between Tyr-14 and the carbonyl, there is no basis to propose either an additional proton donor at the active site or the complication of a proton relay.

We have recently presented other evidence which argues against the possibility of protonation of 3-oxo-steroids, such as **1**, at the enzyme active site (Zeng et al., 1992). Catalysis of the isomerization reaction (eq 1) is probably facilitated by a strengthening of the hydrogen bond from Tyr-14 to the carbonyl oxygen upon proton abstraction by Asp-38, due to the heightened basicity of the C-3 oxygen in the dienolate ion. The electrophilic catalytic function of Tyr-14 then is likely due to better hydrogen bonding to the intermediate (and the transition states) than to the reactant.

A lack of complete proton tranfer from Tyr-14 during catalysis is consistent with the pK_a of Tyr-14 (>10.9; Kulipopulos et al., 1991). Since the initial carbonyl group is only weakly basic and the dienol has an aqueous pK_a of 10.0 (Zeng et al., 1991), the transition state will almost certainly have a pK_a lower than 10, and proton transfer from Tyr-14 would be energetically unfavorable. Thus, at least in the case of KSI, the electrophilic component of the reaction may be simply hydrogen bonding to the incipient dienolate ion and not proton transfer from the electrophile concerted with abstraction of the carbon bound proton.

[5] In some instances, however, the shift due to medium effects may be comparable to that of protonation (Cox & Yates, 1981).

ACKNOWLEDGMENT

We thank Professors Dale Whalen and Donald Creighton and Dr. Sang-Ho Lee for helpful discussions.

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