Environments and Mechanistic Roles of the Tyrosine Residues of Δ^5-3-Ketosteroid Isomerase†

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ABSTRACT: Δ^5-3-Ketosteroid isomerase (EC 5.3.3.1) from *Pseudomonas testosteroni* converts Δ^5-3-ketosteroids to Δ^4-3-ketosteroids by a stereoselective and conservative transfer of the 4β-proton to the 6β-position. The $10^{9.5}$-fold enzymatic rate acceleration can be attributed to a concerted rate-limiting enolization in which Tyr-14 and Asp-38, positioned orthogonally, act as general acid and base, respectively. The pK_a value of the phenolic hydroxyl group of Tyr-14 of the Y55F/Y88F double mutant is 11.6 ± 0.2 by UV titration. However, the fluorescence titration of Tyr-14 shows biphasic sigmoidal behavior with apparent pK_a values of 9.5 and 11.5. This suggests the assistance of a basic residue at the active site, possibly a lysine or tyrosine residue. Mutations of each of the four lysine residues K119L, K108Q, K92Q, and K60Q lowered specific activities only slightly to between 43% and 98% of the wild-type enzyme. Similarly, mutations of Tyr-55, Tyr-88, or both to phenylalanine led to only 2–4-fold reductions in catalytic activity. These findings suggest that despite the enormous difference between the pK_a value of Tyr-14 (11.6) and that of the 3-carbonyl group of the steroid (ca. pK_a −7), the reaction may rely on the concerted participation of Tyr-14 and Asp-38 only. The apparent pK_a value of 9.5 in the fluorescence titration of Tyr-14 and in kinetic measurements probably results from conformational changes of the enzyme. The unusually high pK_a value of Tyr-14 of 11.6 ± 0.2 was used to estimate a local dielectric constant of 18 ± 2 near this residue. UV absorption spectra of each of the three tyrosine double and single mutants reveal that the molar absorbances of Tyr-14 and Tyr-55 are 25–30% greater than those of Tyr-88 or of *N*-acetyltyrosine amide in solution and are red-shifted by 4–5 nm. Fluorescence studies show that the relative molar fluorescence intensity of Tyr-14 is unusually high (3.9) and that of Tyr-88 is much lower (0.42) in comparison to that of free *N*-acetyltyrosine amide. Consistent with the estimated dielectric constant of 18 ± 2 near Tyr-14, both the UV absorption spectrum and the fluorescence enhancement of Tyr-14 are mimicked by *N*-acetyltyrosine amide in 2-propanol, a solvent with a dielectric constant of 18.6. The fluorescences of Tyr-14 and Tyr-55 are strongly mutually quenched, whereas Tyr-88 has no effect on the fluorescence of these residues, and vice versa. The binding of 19-nortestosterone to the enzyme quenches nearly all of the fluorescence of Tyr-14 and Tyr-55, but only partially quenches the fluorescence of Tyr-88. These findings are consistent with the partial X-ray structure of isomerase which reveals that Tyr-14 and Tyr-55 are located in the hydrophobic active site while Tyr-88 is more distant and at least partially exposed to the medium.

The Δ^5-3-ketosteroid isomerase (EC 5.3.3.1) of *Pseudomonas testosteroni* promotes the highly efficient isomerization of a variety of Δ^5-3-ketosteroids to Δ^4-3-ketosteroids by an intramolecular, stereoselective, and conservative transfer of the 4β-proton to the 6β-position at a rate approaching the diffusion limit. The detailed mechanism of this reaction has been intensively studied in several laboratories [see reviews by Pollack et al. (1989) and Schwab and Henderson (1990)]. The gene coding for isomerase has been cloned and overexpressed in *Escherichia coli* (Kuliopulos et al., 1987a; Choi & Benisek, 1987). The enzyme is a tightly associated homodimer containing 125 amino acids per subunit. By means of X-ray diffraction, NMR, and other spectroscopic techniques, as well as site-directed mutagenesis, it has been established that two amino acid residues, Tyr-14 and Asp-38, are critically involved in the catalytic mechanism (Kuliopulos et al., 1989). Furthermore, substrate (primary and secondary), solvent, and combined substrate and solvent kinetic deuterium isotope effects (Xue et al., 1990) have demonstrated that at least two sequential steps (Figure 1) are involved in the reaction mechanism: (a) a rate-limiting, concerted enolization in which Asp-38 acts as a general base that removes the 4β-proton of the steroid, and Tyr-14 which approaches the substrate orthogonally with respect to Asp-38 (Kuliopulos et al., 1991), serves as the general acid that protonates or forms a low-barrier hydrogen bond (Cleland, 1992) with the 3-carbonyl oxygen of the steroid to form the enolic or enolate intermediate (Zeng et al., 1992); and (b) the rapid reketonization of this intermediate in which the functional roles of these two essential residues are reversed.

While Tyr-14 has been shown by NMR studies to approach closely to the bound substrate, the capability of Tyr-14 with a pK_a > 10.9 in the absence of substrate (Kuliopulos et al., 1991) to protonate the carbonyl group of the steroid (estimated pK_a −7) remains a vexing issue. Moreover, studies of both V_{max} and V_{max}/K_m as a function of pH showed that two apparent pK_a values of 4.2–5.6 and 9.4–9.7 are characteristic of the catalytic function of isomerase (Weintraub et al., 1970;

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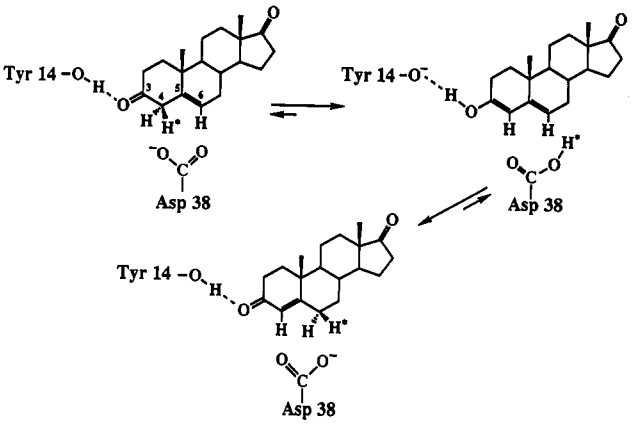

FIGURE 1: Two sequential steps involved in the isomerase reaction mechanism: (a) a rate-limiting, concerted enolization in which Asp-38 acts as a general base that removes the 4β-proton of the steroid, and Tyr-14, which approaches the substrate orthogonally with respect to Asp-38, serves as the general acid that protonates the 3-carbonyl oxygen of the steroid to form the enolic intermediate; and (b) the rapid reketonization of this intermediate in which the functional roles of these two essential residues are reversed (Kuliopulos et al., 1989, 1991; Xue et al., 1990).

Pollack et al., 1986).[1] The lower pK_a value can be reasonably attributed to the ionization of Asp-38. However, the pK_a of 9.6 ± 0.1 is inconsistent with our recent observations on Tyr-14 which has a pK_a value > 10.9 (Kuliopulos et al., 1991). This inconsistency suggests the possibility that another group(s) in the active site of the enzyme with a pK_a value near 9.5 may assist in the catalytic process. Other explanations such as a change in the rate-limiting step are possible but unlikely because the same anomalous apparent pK_a value is observed in fluorescence titrations in the absence of substrate (see below). Such cooperativity among catalytic residues is well-known in the serine proteases (Blow et al., 1969; Carter & Wells, 1988). Histidine is not a candidate since the three histidines of wild-type isomerase and of the Y55F/Y88F double mutant have pK_a values of 4.3, 5.8, and 7.8 and these residues are distant from the active site (Benisek & Ogez, 1982; Kuliopulos et al., 1987b, 1991). The N-terminal amino group is also not a candidate since its pK_a is 7.45 (Benisek & Ogez, 1982). Of the four lysine residues in isomerase, namely, Lys-60, Lys-92, Lys-108, and Lys-119, the last has not been located in the X-ray structure. The ϵ-NH_2 group of Lys-92 is 15.8 Å from C-4 of Tyr-14, and Lys-60 and Lys-108 are even more distant. However, a substrate-induced conformational change in the position of Tyr-14 is believed to occur (Kuliopulos et al., 1989, 1991) and could result in a tyrosine–lysine interaction. A recent survey of protein X-ray structures reveals a moderately high frequency of tyrosine–lysine in comparison to phenylalanine–lysine interactions.[2] Accordingly, we have investigated the possible roles of the four lysine residues in binding and catalysis by mutating these amino acids.

[1] When the data of Weintraub et al. (1970) relating V_{max}/K_m to pH were analyzed by nonlinear regression curve fitting, comparable pK_a values of 4.4 ± 0.1 and 9.6 ± 0.1 were obtained. Such analysis of the V_{max} data of Weintraub et al. (1970) gave pK_a values of 5.45 ± 0.05 and 9.50 ± 0.05. Similarly from earlier measurements of reaction velocities (v) at two substrate concentrations, Wang et al. (1963) obtained pK_a values of 4.2–4.5 and 9.4–9.7 for the ascending and descending limbs, respectively, of plots of the velocity (v) with respect to pH. Pollack et al. (1986), in experiments in the low pH region, obtained a range of pK_a values from 4.57 to 5.5 depending on substrates and on whether the free enzyme (V_{max}/K_m) or enzyme–substrate complex (V_{max}) was examined. However, these authors did not make measurements above pH 9.

[2] S. A. Bryant, personal communication, 1992.

We have also studied the three individual tyrosine residues of the enzyme, Tyr-14, Tyr-55, and Tyr-88, by kinetic measurements and by UV absorption and by fluorescence spectroscopy of appropriate single and double mutants, enlarging upon previous studies of the wild-type enzyme and of some single tyrosine mutants (Wang et al., 1963; Kuliopulos et al., 1989). Since each subunit of the wild-type isomerase contains three tyrosine and eight phenylalanine residues but lacks tryptophan, the UV spectrum is dominated by tyrosine and phenylalanine absorptions and the fluorescence arises almost entirely from tyrosine. We report here on the spectroscopic properties of the three individual tyrosine residues of the enzyme and make deductions concerning their environments and interactions.

EXPERIMENTAL PROCEDURES

Materials. Δ^5-Androstene-3,17-dione was synthesized from dehydroepiandrosterone (Kawahara et al., 1962), and 19-nortestosterone was purified by crystallization and high vacuum sublimation. Phenylalanine, tyrosine, and tyrosine derivatives were obtained from Sigma and used without further purification. *E. coli* competent cells were obtained from Gibco-BRL (DH5αF′) and Stratagene (JM101). pUC19 was purified by using QIAGEN procedures and its Maxi column (Qiagen, Chatsworth, CA). Polynucleotide kinase, T4 DNA polymerase, T4 ligase, Sequenase, and all endonucleases were either from New England Biolabs or Gibco-BRL.

Site-Directed Mutagenesis. A 1.4-kb fragment of the plasmid pAK1370 that contained the isomerase gene (Kuliopulos et al., 1987a) was inserted into the *Hin*dIII and *Bam*HI sites of the phage M13mp18 (Norrander et al., 1983). Single-stranded uracil-containing template DNA complementary to the coding strand of isomerase was prepared according to the method of Kunkel (1985) by use of *E. coli* CJ236 (*dut*$^-$/*ung*$^-$) competent cells. The Y14F/Y88F double mutant was constructed by the use of Y88F uracil-containing DNA as template with the oligonucleotide containing the Y14F mutation. The mutations converting Lys-119 to leucine (K119L) were prepared with the degenerate 23-mer (corresponding to bases 345–367): 5′-GTTTGGCGAGC(TGA)GAATATTCACG-3′ (the codon that was changed is indicated by the underlined letters and the authenticity of the oligonucleotide was confirmed by DNA sequencing). This procedure also provided K119R and K119Q. Similarly, the preparations of the K108Q, K92Q, and K60Q mutants were performed by using the following oligonucleotides: 5′-TGGCGCCGGC-C(TGA)GGTGGTGAGCA-3′ (corresponding to bases 312–334), 5′-TCAGGGCCGCC(TGA)GACCGTGGTTG-3′ (corresponding to bases 264–286), and 5′-CAACTCGCTC-C(TGA)ACTGCCTTTGG-3′ (corresponding to bases 168–190), respectively. The desired clones were identified by sequencing the entire genes (Sanger et al., 1977). The mutated genes were then introduced into the *Eco*RI and *Hin*dIII sites of pUC19 for expression of the mutant enzymes (Kuliopulos et al., 1987a).

Protein Purification. The methods for overexpression and purification of the enzymes have been described (Kuliopulos et al., 1987a, 1989). All mutants were first purified on a Fast Flow Q-Sepharose (Pharmacia) column (20 cm × 2.8 cm) with a linear gradient from 1 mM to 100 mM Bis-Tris-propane–HCl, pH 7.0. Fractions containing isomerase were concentrated with Centriprep microconcentrators (Amicon) to at least 5 mg/mL with >90% purity. Crystallization and

recrystallization of isomerase was performed to obtain the pure enzymes. All lysine mutants (K119L, K108Q, K92Q, K60Q) and the Y14F/Y88F double mutant were crystallized at 22–27% saturation of ammonium sulfate at pH 7.0. The purity of the enzymes was assessed by SDS–polyacrylamide gel electrophoresis and staining with Coomassie Blue R 250.

Protein Concentrations. These were determined by the method of Goodwin and Morton (1946), assuming a_m for tyrosinate at 293 nm of 2390 M^{-1} cm^{-1} (Kuliopulos et al., 1989).

Ultraviolet Spectroscopy. All UV absorbance measurements were made at 25 °C with a Beckman DU-7 spectrophotometer in 1.0-cm quartz cuvettes in a total volume of 0.5–1.0 mL. Spectra of isomerase were measured in 50 mM Tris-HCl, pH 7.5, against a blank containing the same buffer. Spectra of the individual tyrosines (Tyr-14, Tyr-55, Tyr-88) were obtained from double mutants containing only a single tyrosine residue and were compared with appropriate subtractions of spectra of wild-type and single and double mutant enzymes. The UV spectra of 19-nortestosterone on binding to isomerase mutants were obtained as described (Kuliopulos et al., 1989). One binding site for 19-nortestosterone was obtained per subunit of the mutants, in agreement with the stoichiometry of binding to the wild-type enzyme (Penning et al., 1980). The protein concentrations determined from the stoichiometry of 19-nortestosterone binding were in good agreement with those derived from the UV absorption measurements (Goodwin & Morton, 1946). The dissociation constants, K_D, were determined by computer fitting of the titration curves as described (Kuliopulos et al., 1989).

Fluorescence Spectroscopy. Fluorescence was measured on a Perkin Elmer luminescence spectrometer Model LS 50 in 2-mL systems containing 50 mM Tris-HCl, pH 7.5, at 25 °C. Samples were excited at 278 nm, and the emission intensities were acquired at 307 nm. The small Raman scatter arising from the buffer solution was subtracted. Measurements of the fluorescence quenching of the wild-type and mutant enzymes by 19-nortestosterone were done by adding the enzymes to a buffer solution containing various amounts of 19-nortestosterone in a final concentration of 1.5% methanol (by volume). In agreement with the UV titrations, plots of fluorescence intensity with respect to 19-nortestosterone concentration revealed one steroid binding site per enzyme subunit. Hence, the fluorescence titrations were fit by the hyperbolic function

$$F = (F_f/2E_t)\{E_t - K_D - NT_t + [(E_t + K_D + NT_t)^2 - (4NT_tE_t)]^{1/2}\} + (F_b/2E_t)\{E_t + K_D + NT_t - [(E_t + K_D + NT_t)^2 - (4NT_tE_t)]^{1/2}\}$$

where F is the fluorescence intensity, K_D is the dissociation constant, E is enzyme concentration, NT is 19-nortestosterone concentration, and the subscripts f, b, and t refer to the free, bound, and total species, respectively. The fit of the titration curve to the data was optimized to yield K_D, F_f, and F_b.

pK_a Titrations. The pK_a value of Tyr-14 was determined by absorbance or fluorescence measurements of the Y55F/Y88F double mutant (containing Tyr-14 only). The changes in absorbance at 295 nm of the enzyme (37.9 μM) resulting from tyrosine ionization were measured in a series of 50 mM buffer solutions: Tris-HCl, pH 7.5–9.0; CHES, pH 8.7–9.8; CAPS, pH 9.5–10.8; potassium phosphate, pH 11–12.8. The pH values were measured after the addition of the enzyme to each buffer solution. In the pH range of 11.0–12.8, the absorbances increased with time in a first-order manner. Data obtained in this range were therefore extrapolated to both zero time and infinite time by a first-order function. Fluorescence titrations of the Y55F/Y88F double mutant as a function of pH were also carried out. Time-dependent changes in fluorescence were observed in the pH ranges 9–10 and 11–12. Zero time and infinite time fluorescence intensities were obtained by fitting the measurements to first-order decay curves.

Kinetic Studies. Specific activity measurements were made by monitoring the change in the absorbance at 248 nm in 0.6-mL systems containing 50 mM Tris-HCl, pH 7.5, and 58.2 μM 5-androstene-3,17-dione in methanol (1.7% final concentration). Enzyme concentrations in the assay solutions were always 8.33 pM for the lysine mutants. Stock solutions of these enzymes were diluted to the appropriate concentration in 1% bovine serum albumin, pH 7.0. Michaelis constants were determined at pH 7.5 in 50 mM Tris-HCl, in the presence of 3.3% methanol and substrate concentrations of 14.55, 29.1, 58.2, 87.3, and 116.4 μM. The kinetic data were analyzed by a nonlinear regression analysis of the double-reciprocal plots using the program ENZFITTER written by R. J. Leatherbarrow (Elsevier-BIOSOFT, Cambridge, U.K., 1987).

RESULTS AND DISCUSSION

Kinetic Properties of Tyrosine and Lysine Mutants of Isomerase. Previous studies (Kuliopulos et al., 1989) demonstrated that among the single mutations of the three tyrosine residues (Y14F, Y55F, and Y88F) the catalytic activity of only the Y14F enzyme was severely damaged (Table I). For the present experiments, all combinations of tyrosine double mutants (Y14F/Y88F, Y14F/Y55F, and Y55F/Y88F) were prepared and purified by repeated crystallization from neutral ammonium sulfate solutions. The crystal forms of all of the mutants were indistinguishable from that of wild-type isomerase, indicating no major differences in conformation or molecular packing. The kinetic properties of these mutants (Table I) confirm the earlier findings (Kuliopulos et al., 1989) that mutations of Tyr-55 or Tyr-88, alone or in combination, have only small effects on catalytic activity. Thus, the k_{cat}/K_m values of Y88F, Y55F, and Y55F/Y88F are 92, 62, and 82%, respectively, of that of the wild-type isomerase. These results strengthen the earlier conclusions (Kuliopulos et al., 1989) that Tyr-55 and Tyr-88 play no proton-donating or -accepting role in the catalytic process and show that their modification also has little effect on substrate binding (K_m values are similar). We also infer that mutation of either or both catalytically unimportant tyrosine residues probably does not significantly perturb the conformation of the active site of the enzyme.

Interestingly, the Y14F/Y55F double mutant (containing Tyr-88 only) has about 30 times higher catalytic activity than does the Y14F/Y88F double mutant enzyme (containing Tyr-55 only) or the Y14F single mutant (containing Tyr-55 and Tyr-88). The higher activity of the Y14F/Y55F mutant enzyme may be ascribed to a mechanism similar to that deduced for the Y14F single mutant, in which the dissociation of the carbanion intermediate from the enzyme is partially rate-limiting (Xue et al., 1991). This dissociation may be accelerated in the presence of Y55F as a second mutation.

The crystal forms of all four lysine mutants (K60Q, K92Q, K108Q, and K119L) were also indistinguishable from that of wild-type isomerase. Mutations of the four lysine residues had very little effect on the catalytic efficiency of the isomerase; the k_{cat}/K_m values were 74–97% of that of the wild-type enzyme

Table I: Kinetic Parameters of Isomerase Mutants[a]

enzyme	tyrosine residues present	sp act. (μmol/(mg·min))	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	relative k_{cat}/K_m
wild type[b]	14 + 55 + 88	45 300	53 600	340 ± 20	1.58×10^8	1.00
		Tyrosine Mutants				
Y55F[b]	14 + 88	22 800	12 700	130 ± 15	9.77×10^7	0.62
Y14F[b]	55 + 88	2	1	123 ± 13	9.35×10^3	$10^{-4.2}$
Y88F	14 + 55	36 300	24 350	168 ± 20	1.45×10^8	0.92
Y14F/Y88F	55	2	1	140 ± 15	8.75×10^3	$10^{-4.2}$
Y14F/Y55F	88	63	32	132 ± 12	2.43×10^5	$10^{-2.8}$
Y55F/Y88F	14	22 600	12 200	94 ± 7	1.30×10^8	0.82
		Lysine Mutants				
K60Q	14 + 55 + 88	30 220	25 810	220 ± 12	1.17×10^8	0.74
K92Q	14 + 55 + 88	35 710	28 900	188 ± 9	1.54×10^8	0.97
K108Q	14 + 55 + 88	35 160	28 920	189 ± 25	1.53×10^8	0.97
K119L	14 + 55 + 88	28 600	23 230	190 ± 12	1.23×10^8	0.78

[a] All kinetic measurements were made at 25 °C in 0.6-mL systems containing 50 mM Tris-HCl and 3.3% methanol by volume. The specific activities were obtained under standard assay conditions with 58.2 μM 5-androstene-3,17-dione. The k_{cat} values were derived from V_{max} intercepts of double-reciprocal plots of velocity with respect to substrate concentration. [b] Values determined by Kuliopulos et al. (1989).

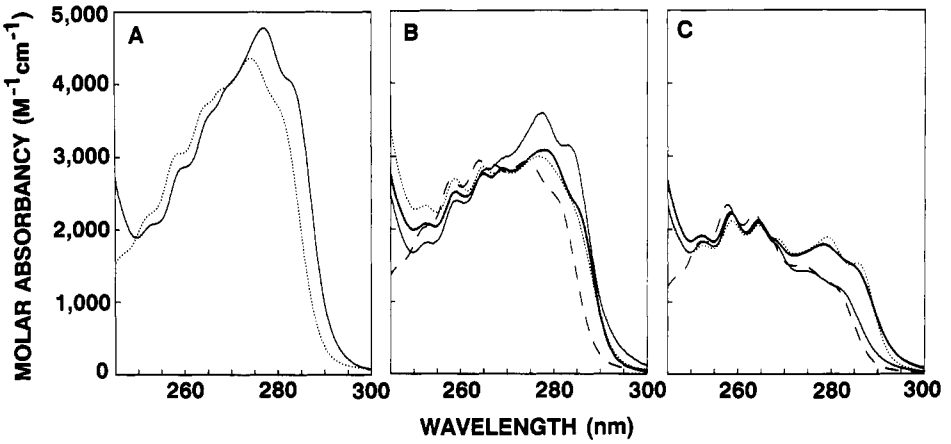

FIGURE 2: Comparison of the UV spectra of isomerase and its mutants with those of corresponding amino acid mixtures. (Panel A) Wild-type enzyme (—), and a mixture of phenylalanine and tyrosine in a molar ratio of 8:3 (···); (panel B) single tyrosine mutants Y14F (···), Y55F (—), and Y88F (—) and mixture of phenylalanine and tyrosine in a molar ratio of 9:2 (– –); (panel C) double tyrosine mutants Y55F/Y88F (···), Y14F/Y88F (—), and Y14F/Y55F (—) and a mixture of phenylalanine and tyrosine in a molar ratio of 10:1 (– –). All spectra were measured in 50 mM Tris-HCl, pH 7.5, against blanks containing the same buffer.

(Table I). The possibility that any of the lysine residues is involved in proton donation to the substrate, directly or via Tyr-14, is therefore remote. Since few, if any, other charged residues are present in the active site of isomerase, according to the partial X-ray structure, we infer that the highly efficient catalysis promoted by isomerase may rely only on the concerted participation of Tyr-14 and Asp-38.

Ultraviolet Absorption Spectra of Isomerase Mutants. Since isomerase lacks tryptophan, the UV absorption spectra in the 250–300-nm range are similar to those of the corresponding molar mixture of three tyrosines and eight phenylalanines, with the expected slight red shift of the absorbance peaks in the protein, as shown in Figure 2 (Kawahara et al., 1962). Thus, the principal absorption peak of free tyrosine at 274 nm is shifted to 277.4 nm in the wild-type isomerase and also intensified by about 11%. Similarly, the UV spectra of the three single tyrosine mutant enzymes (Y14F, Y55F, and Y88F) and the three double tyrosine mutant enzymes (Y14F/Y55F, Y14F/Y88F, and Y55F/Y88F) were compared with solutions containing the corresponding combinations of free *N*-acetyltyrosine amide and *N*-acetylphenylalanine ethyl ester in concentration ratios of 2:9 and 1:10 for the single and double mutants, respectively (Figure 2). The principal absorbance of tyrosine in Y88F is 25% higher, whereas those of Y14F and Y55F are only 5% higher than the corresponding absorbances of the free amino acid mixtures (Table II). Interestingly, the absorption characteristics of the tyrosine residues in the double mutants were different. For instance, molar absorbances of the tyrosine residues of Y55F/Y88F (containing Tyr-14 only) and of Y14F/Y88F (containing Tyr-55 only) are 25–31% more intense than that of Y14F/Y55F (containing Tyr-88 only), which is comparable

Table II: Molar Absorbance Coefficients (a_m) and Absorption Maxima (λ_{max}) of Isomerase Mutants and of Solutions Containing Various Ratios of Tyrosine to Phenylalanine[a]

tyrosine residues in enzyme	Tyr:Phe ratio	λ_{max} (nm)	a_m (M^{-1} cm^{-1})	a_m/mol of Tyr (M^{-1} cm^{-1})	relative a_m values[b]
14 + 55 + 88 (WT)	3:8	277.4	4778	1593	1.11
55 + 88 (Y14F)	2:9	277.4	2997	1499	1.04
14 + 88 (Y55F)	2:9	277.8	3041	1521	1.05
14 + 55 (Y88F)	2:9	277.6	3598	1799	1.25
14 (Y55F/Y88F)	1:10	279.3	1889	1889	1.31
55 (Y14F/Y88F)	1:10	278.0	1789	1789	1.24
88 (Y14F/Y55F)	1:10	274.0	1426	1426	0.99
model system[c]					
3 Tyr:8 Phe	3:8	274.0	4363	1454	1.01
2 Tyr:9 Phe	2:9	274.0	2927	1463	1.01
1 Tyr:10 Phe	1:10	274.0	1471	1471	1.02

[a] Ultraviolet absorption measurements were made in 50 mM Tris-HCl, 100 mM NaCl, pH 7.5. The contribution of phenylalanine to the peptide linkage is negligible at 277.4 nm. [b] Calculated at the absorption maximum of each protein using the value of a_m = 1440 M^{-1} cm^{-1} for tyrosine at 274 nm. [c] Mixtures of *N*-acetyltyrosine amide and *N*-acetylphenylalanine ethyl ester.

to that of the corresponding amino acid mixture. Furthermore, the absorption maximum of Tyr-88 in Y14F/Y55F is at 274.5 nm, near that of the free amino acid, while that of Tyr-55 in Y14F/Y88F and that of Tyr-14 in Y55F/Y88F are red-shifted to 278 and 279.3 nm, respectively. These red shifts and intensity enhancements of Tyr-14 and Tyr-55, and their absence in Tyr-88, are entirely consistent with the highly hydrophobic environment of the former residues which are located in the active site, and with at least partial exposure of Tyr-88 to the aqueous environment.[3] In order to simulate the hydrophobic environment of the active site, the spectra of *N*-acetyltyrosine amide (λ_{max} 274.4 nm) were examined in various mixtures of water and methanol (dielectric constant 30) and 2-propanol (dielectric constant 18.6). Larger red shifts and higher absorptions were observed when the proportions of the alcohols were increased. In 95% 2-propanol the absorption was enhanced by 23% and shifted to 277.7 nm, while in 95% methanol it was enhanced by 15% and also shifted to 277.7 nm. Clearly, not only Tyr-14 but also Tyr-55 is located in a very hydrophobic region, which is consistent with the partial X-ray structure (Figure 3A).

Measurement of the pK_a Value of Tyr-14 in the Y55F/Y88F Double Mutant Enzyme. The Y55F/Y88F double mutant enzyme, which has almost full catalytic activity (k_{cat}/K_m is 82% of that of wild type), provides an ideal system for measuring the pK_a value of Tyr-14 without interference from other tyrosine residues. The pK_a of the phenolic hydroxyl group of Tyr-14 was determined by two methods: ultraviolet absorption and fluorescence quenching. Titration of *N*-acetyltyrosine ethyl ester and *N*-acetyltyrosine amide with base gave identical pK_a values of 10.20 ± 0.03 by both methods. In fluorescence titrations, a two-state excited-state proton-transfer process may be observed for aromatic alcohols, especially in solutions containing high concentrations of proton acceptors such as 100 mM acetate (Hercules & Rogers 1959; Davenport et al., 1986). However, this phenomenon was not observed with these model compounds under our experimental conditions.

In the titration of Y55F/Y88F with base, the UV absorbance changes at 295 nm and the magnitude of fluorescence quenching were time-dependent at higher pH values. Consequently, single and precise measurements could not be obtained. The results shown in Figure 4 were obtained by extrapolation of the time-dependent measurements to either zero time or infinite time. The UV absorption titrations of the Y55F/Y88F enzyme were monitored and gave zero time and infinite time pK_a values for Tyr-14 of 11.82 ± 0.07 and 11.46 ± 0.05, respectively (Figure 4A). Fluorescence titrations under similar conditions showed biphasic sigmoidal behavior with two apparent pK_a values of 9.50 ± 0.08 and 11.50 ± 0.09 on the basis of the analysis of the infinite time measurements (Figure 4B). However, extrapolation of the same measurements to zero time (Figure 4C) yielded a nonsigmoidal and highly cooperative decrease in fluorescence between pH 9.5 and 10.0 to a plateau followed by a further decrease at higher pH values. In Figure 4B, the first pK_a value of 9.5 is consistent with the titration of a group involved in catalysis as shown by the pH–activity profile (Weintraub et al., 1970).[1] The second

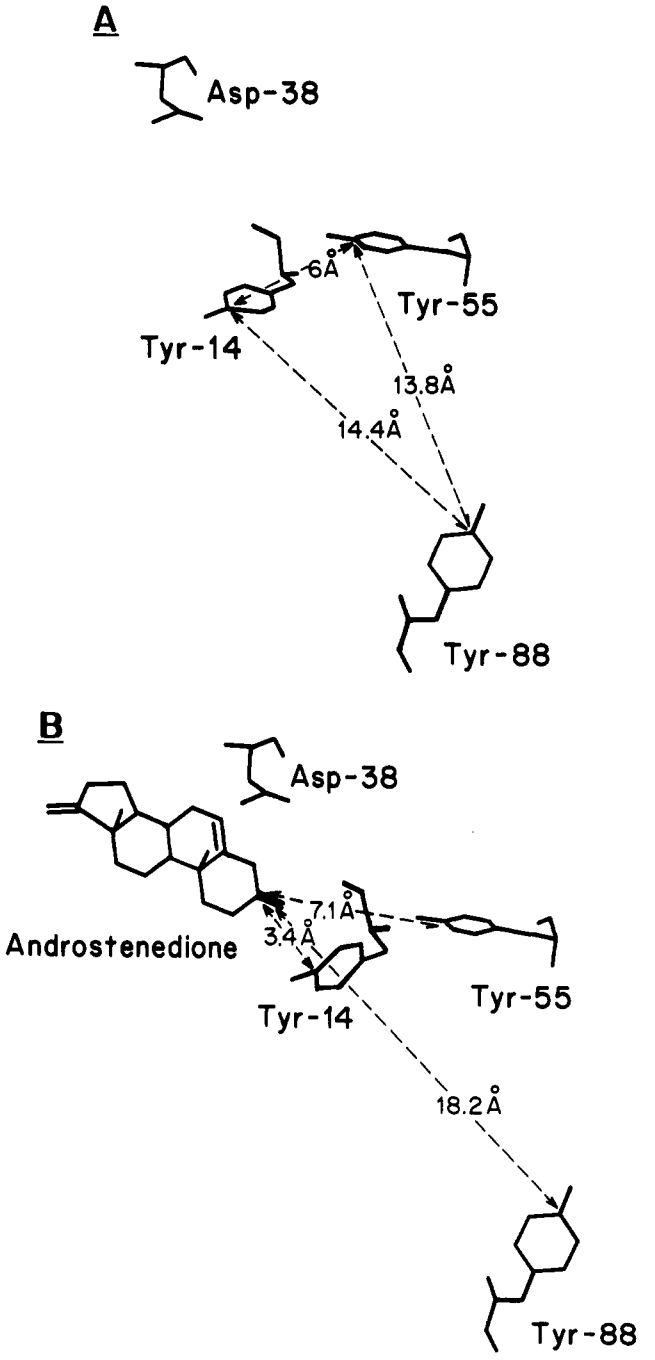

FIGURE 3: Residues at or near the active site of ketosteroid isomerase based on the 2.5-Å X-ray structure of the enzyme (Kuliopulos et al., 1987b). (A) Distances between C-4 of tyrosine residues in the free enzyme. (B) Distances from C-4 of tyrosine residues to C-3 of the substrate 5-androstene-3,17-dione based on NMR docking (Kuliopulos et al., 1987b) and nuclear Overhauser effect studies (Kuliopulos et al., 1991).

pK_a value of 11.5 is consistent with the results of the UV titration of Tyr-14 and previous NMR studies in which the pK_a value of this residue was shown to be > 10.9 (Kuliopulos et al., 1991). The issue is whether the apparent pK_a of 9.5 represents the titration of an amino acid residue or reflects a conformational change of the enzyme induced by the simultaneous deprotonation of several residues. These findings prompted a search for the presence of another amino acid near the active site, possibly a lysine or tyrosine residue, and led to the investigation of the lysine and tyrosine mutants described above. These and previous studies (Kuliopulos et al., 1989) gave no indication of the involvement of such amino

[3] The contribution of the individual tyrosine residues to the absorption spectrum of isomerase in the 270–300-nm region has also been analyzed by arithmetic subtractions of the spectra of wild-type enzyme and appropriate single (Y14F, Y55F, and Y88F) and double (Y14F/Y55F, Y14F/Y88F, and Y55F/Y88F) mutants. The results provide evidence for interactions between Tyr-14 and Tyr-55 as manifested by mutual blue shifts of 1.4–2.4 nm in their absorption maxima, and the lack of such interactions between Tyr-88 and Tyr-14 or Tyr-55.

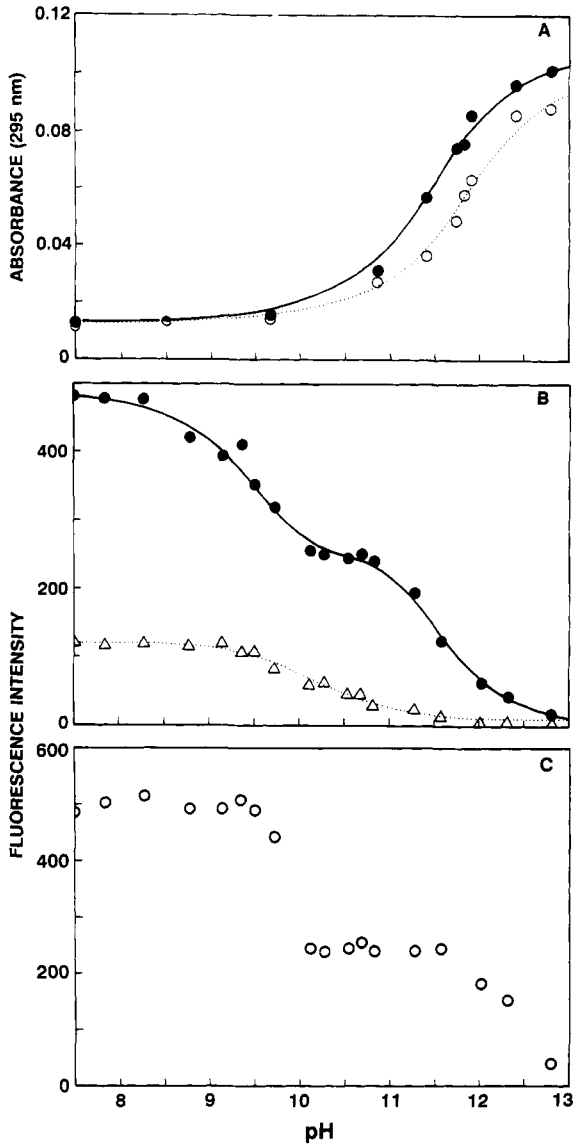

FIGURE 4: UV and fluorescence titrations with respect to pH of Tyr-14 in the Y55F/Y88F double mutant enzyme. (A) UV absorbances of enzyme (37.85 μM) at 295 nm as a function of pH. The pH values were measured after the addition of the enzyme. Between pH 11.2 and 11.8, the UV absorbances increased with time. Data obtained in this range were extrapolated to zero time (○) and infinite time (●) by a first-order function. The pK_a values used to fit the data were 11.82 ± 0.07 (zero time) and 11.46 ± 0.05 (infinite time). (B) Fluorescence titration of the enzyme as a function of pH (●). Time-dependent decreases in fluorescence were observed in the pH ranges 9–10 and 11–12. Fluorescence intensities (in arbitrary units) extrapolated to infinite time were obtained by fitting the measurements to first-order curves. The infinite time points were fit by two pK_a values of 9.50 ± 0.08 and 11.50 ± 0.09. The lower titration curve (△) is that of *N*-acetyltyrosine amide at the same concentration which was fit with a pK_a of 10.20 ± 0.03. (C) When the measurements described in (B) were extrapolated to zero time, the fluorescence values could not be fit to a sigmoidal function. They show a steep decline between pH 9.5 and 10.0 as well as a second decline at higher pH values.

acid residues (Table I), suggesting a conformation change of the enzyme with an apparent pK_a value of 9.5. During the pH titration, after incubation of the enzyme at pH values over the range 8.2–10.7 for short periods, subsequent assay of the enzyme at pH 7.5 showed full restoration of catalytic activity, indicating that the conformation change at pH 9.5 is reversible. Prior incubation of the enzyme at pH values above 10.7 resulted in the irreversible loss of activity.

The 1.4 ± 0.2 unit higher pK_a value of Tyr-14 (11.6 ± 0.2) compared with tyrosine in water (10.2) probably results from the hydrophobic environment of this residue in the enzyme, i.e., a lower local dielectric constant near Tyr-14. Assuming this to be the case, an estimate of the local dielectric constant near Tyr-14 (ϵ_{prot}) can be made by using the Born approximation (Rashin & Honig, 1985; Friedman & Krishan, 1973):

$$\Delta\Delta G^\circ = \frac{163.9(1/\epsilon_{prot} - 1/\epsilon_{water})}{r}$$

where ϵ_{water}, the dielectric constant of water, is 77.9 at 25 °C, and r, the cavity radius of the delocalized tyrosinate anion, is 3.79 Å. This value is based on the X-ray structure of *N*-acetyltyrosine (Koszelak & van der Helm, 1981) and the cavity radii of oxygen and of carbon (Rashin & Namboodiri, 1987). The 1.4 ± 0.2 unit increase in pK_a value of Tyr-14 in the enzyme corresponds to a $\Delta\Delta G^\circ$ of 1.9 ± 0.2 kcal/mol. This yields an ϵ_{prot} value of 18 ± 2, which is similar to the dielectric constant of 2-propanol (18.6) in which the red shift and intensification of the ultraviolet absorption maximum of *N*-acetyltyrosine amide approximate those of Tyr-14.

Fluorescence Studies of Tyrosine Residues of Isomerase. It is well-known that free tyrosine has high fluorescence quantum yields in solution but that when tyrosine is incorporated into the peptide linkage of proteins the fluorescence is often quenched, and in some proteins it is profoundly depressed. The reduction of tyrosine fluorescence in proteins has been attributed to quenching by proton transfer to a nearby proton acceptor in the excited state or the ground state or to efficient energy transfer from tyrosine to tryptophan (Cowgill, 1976). Since tryptophan is not present, and the fluorescence yield of phenylalanine is very low, the fluorescence of wild-type isomerase is almost entirely due to its three tyrosine residues (Tyr-14, Tyr-55, and Tyr-88). The availability of all combinations of single and double mutants of these tyrosine residues provided the opportunity to determine the fluorescence of each tyrosine residue alone and in the presence of the other tyrosine residues. The relative molar fluorescence intensities of the wild-type and each mutant enzyme were related to the fluorescence of *N*-acetyltyrosine amide to which the value of unity was assigned.

Measurement of the relative molar fluorescence intensities of the three double mutant enzymes (each containing only a single tyrosine residue) provided direct information on the fluorescence of each tyrosine residue independently. The intrinsic fluorescences of the three residues when present alone are markedly different (Table III and Figure 5). The relative molar fluorescences of Tyr-14, Tyr-55, and Tyr-88 are 3.90, 1.21, and 0.42, respectively, compared to that of *N*-acetyltyrosine amide in aqueous solution under identical conditions. The fluorescence of Tyr-14 is therefore extraordinarily high. The measured relative intensity of fluorescence of the wild-type enzyme is 4.45 (for three residues) which is somewhat lower than the calculated numerical sum (5.53) of the fluorescences of three independent tyrosine residues, suggesting that interactions occur between the fluorescence processes of at least some of these residues in the protein.

The unusually high fluorescence of Tyr-14 can be mimicked by placing *N*-acetyltyrosine amide in hydrophobic solvents. For example, the fluorescence of *N*-acetyltyrosine amide in methanol is 1.85 times and in 2-propanol is 3.85 times that in aqueous solution. Both the unusually high fluorescence and the red-shifted ultraviolet absorption spectrum of Tyr-14 are thus mimicked by *N*-acetyltyrosine amide in 2-propanol (ϵ = 18.6), consistent with the local dielectric constant near

Table III: Relative Molar Fluorescence Intensity of Tyrosine Residues in Wild-Type and Mutant Enzymes[a]

enzyme	tyrosine residues present		relative molar fluorescence	
	number	positions	obsd[b]	calcd[c]
N-acetyltyrosine amide (standard)	1		1.00	
wild-type isomerase	3	14 + 55 + 88	4.45	5.53
Y14F	2	55 + 88	1.63	1.63
Y55F	2	14 + 88	4.33	4.32
Y88F	2	14 + 55	4.05	5.11
Y55F/Y88F	1	14	3.90	
Y14F/Y88F	1	55	1.21	
Y14F/Y55F	1	88	0.42	

[a] All fluorescence experiments were done in 2-mL systems containing 50 mM Tris-HCl, pH 7.5, 25 °C. Samples were excited at 278 nm and fluorescence emission was recorded at 307 nm. [b] Expressed per mole of enzyme subunit relative to a value of 1.00 for *N*-acetyltyrosine amide of equivalent molarity. [c] Calculated relative molar fluorescence intensity based on the sum of the fluorescence intensities of the appropriate double mutants.

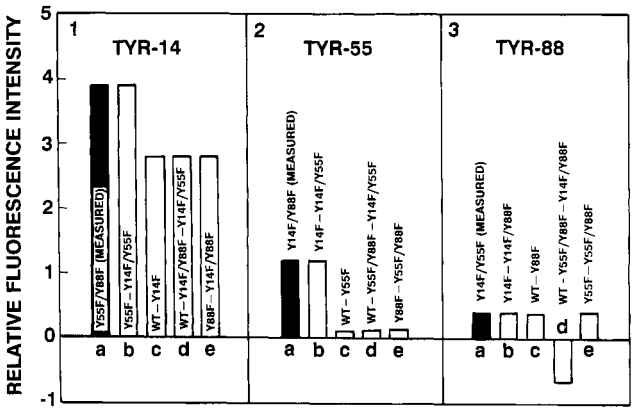

FIGURE 5: Molar fluorescence intensities of Tyr-14 (panel 1, bar a), Tyr-55 (panel 2, bar a), and Tyr-88 (panel 3, bar a) were measured in the corresponding double mutant isomerases, i.e., Y55F/Y88F, Y14F/Y88F, and F14F/Y55F, respectively (Table III). The values are expressed relative to the fluorescence of equivalent concentrations of free *N*-acetyltyrosine amide. Note that Tyr-14 has nearly four times the fluorescence of free *N*-acetyltyrosine amide. These measured values were compared with numerical subtractions of the fluorescence measurements of the wild-type enzyme (WT) and appropriate single and double mutants, as follows. (Panel 1) Tyr-14: (a) Y55F/Y88F (measured); (b) Y55F minus Y14F/Y55F; (c) WT minus Y14F; (d) WT minus Y14F/Y88F minus Y14F/Y55F; (e) Y88F minus Y14F/Y88F. (Panel 2) Tyr-55: (a) Y14F/Y88F (measured); (b) Y14F minus Y14F/Y55F; (c) WT minus Y55F; (d) WT minus Y55F/Y88F minus Y14F/Y55F; (e) Y88F minus Y55F/Y88F. (Panel 3) Tyr-88: (a) Y14F/Y55F (measured); (b) Y14F minus Y14F/Y88F; (c) WT minus Y88F; (d) WT minus Y55F/Y88F minus Y14F/Y88F; (e) Y55F minus Y55F/Y88F. Note that the mutual quenching effect between Tyr-14 and Tyr-55 has the magnitude of 1.1 when used to calculate the fluorescence intensities of both Tyr-14 (cf. 1a or 1b with 1c, 1d, and 1e) and Tyr-55 (cf. 2a or 2b with 2c, 2d, and 2e). The "negative" fluorescence value (3d) for Tyr-88 calculated by subtracting the fluorescence intensity of the two double mutants (Y55F/Y88F and Y14F/Y88F) from that of the wild type is therefore to be expected since the mutual quenching effect between Tyr-14 and Tyr-55 is subtracted twice in this calculation. The magnitude of this calculated "negative" fluorescence (–0.66) corresponds to the expected value (0.42 – 1.1 = –0.68).

Tyr-14 of 18 ± 2 estimated above from the pK_a of Tyr-14.

The fluorescence intensities of the individual tyrosine residues in isomerase were also obtained by numerical subtractions of the fluorescences of wild-type and various mutant enzymes. Thus, the fluorescence of Tyr-14 was calculated by subtracting the fluorescence of Y14F/Y55F (containing Tyr-88 only) from that of Y55F (containing Tyr-14 and Tyr-88). The results of such measurements and calculations (Figure 5) provide further insight into the interactions among the three tyrosine residues. When both Tyr-14 and Tyr-55 are simultaneously present, the observed fluorescences of these residues are not simply additive. For example, the relative molar fluorescence intensity of the Y88F mutant (which contains both Tyr-14 and Tyr-55) is 4.05, which differs from the value of 5.11 obtained for the sum of the fluorescences of Y55F/Y88F (Tyr-14 only) and Y14F/Y88F (Tyr-55 only). The molar fluorescence intensities of Tyr-14 in the presence and absence of Tyr-55 are 2.8 and 3.9, respectively (see Figure 5, 1b–1e). Similarly, the fluorescence intensities of Tyr-55 in the presence and absence of Tyr-14 are 0.13 and 1.21, respectively (Figure 5, 2b–2e). Thus, there is mutual quenching of fluorescence between Tyr-14 and Tyr-55. In contrast, the relatively low fluorescence of Tyr-88 (0.42) is not affected by Tyr-14 or Tyr-55 (Figure 5, 3b, 3c, 3e). Presumably, Tyr-14 and Tyr-55, both located in the hydrophobic active site, strongly quench each other, whereas Tyr-88, which is distant from the active site, has no effect on the fluorescence of the other tyrosine residues, and vice versa. The low fluorescence of Tyr-88 in isomerase might stem from its proximity to Glu-87, which could serve as an efficient proton acceptor from Tyr-88 in the excited state. As indicated by the X-ray structure of isomerase (Figure 3A), the independence of the fluorescence of Tyr-88 may be explained by its long distance from Tyr-14 and Tyr-55, and the mutual quenching effects of Tyr-14 and Tyr-55 could be attributable to their proximity. This interpretation is also supported by the ultraviolet spectroscopy.[3] The internal consistency of the directly measured tyrosine fluorescence values with those obtained by calculation argues strongly for the structural and conformational similarities of the wild-type and mutant enzymes.

UV Spectral Studies of the Interactions of 19-Nortestosterone with Mutant Isomerases. The competitive inhibitor 19-nortestosterone (K_i = 5.2 μM), a product analogue, has served as a useful probe for the active site of isomerase and for measuring the enzyme–steroid binding affinity. When 19-nortestosterone binds to isomerase, its 248-nm absorption peak is red-shifted by 10–12 nm (Wang et al., 1963; Kuliopulos et al., 1989). This red shift can be mimicked by exposing the steroid to strong acids (10 M HCl or 10 M H_2SO_4) which protonate the 3-carbonyl group (Kuliopulos et al., 1989). Similar red shifts were observed with both the D38N and Y55F mutant enzymes, but not with the Y14F, Y14F/Y88F, and Y14F/Y55F mutants. These findings have led us to conclude that the Tyr-14 hydroxyl group partially donates its proton or participates in strong hydrogen bonding to the 3-carbonyl oxygen of bound 19-nortestosterone in the ground state of the enzyme–steroid complex. NMR experiments with the complex between 19-nortestosterone and the Y55F/Y88F mutant enzyme disclosed strong negative nuclear Overhauser effects from the ortho protons of Tyr-14 to the 2α- and 2β-protons, but not the 4- or 6α-protons of the steroid, indicating a close approach of Tyr-14 to the edge of the bound steroid (Kuliopulos et al., 1991). UV resonance Raman spectroscopic studies of the 19-nortestosterone complex with the Y55F/Y88F double mutant enzyme also indicated that Tyr-14 interacts with the 3-carbonyl group of 19-nortestosterone, polarizing the ene–one system (Austin et al., 1992). Although the 230-nm excitation Raman spectra do not show changes characteristic of strong hydrogen bonding of the hydrogen of the tyrosine hydroxyl group, it was speculated that this hydrogen bond may have been compensated by a second

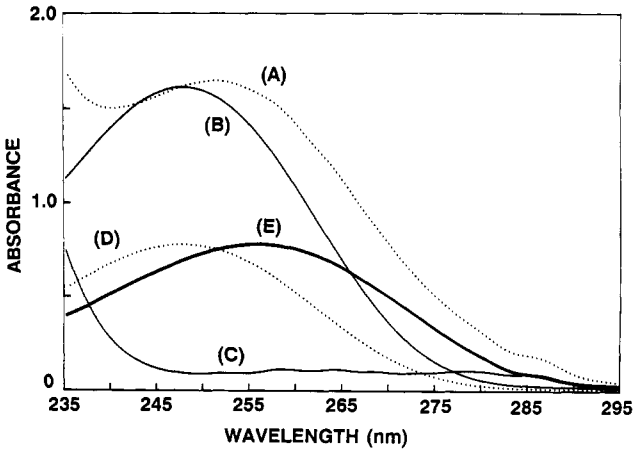

FIGURE 6: Ultraviolet absorption spectrum of 19-nortestosterone bound to the Y55F/Y88F mutant of isomerase. (A) Absorption spectrum of a mixture of enzyme (50 μM) and 19-nortestosterone (99 μM) against a buffer blank (50 mM Tris-HCl, pH 7.5). (B) Spectrum of 99 μM 19-nortestosterone. (C) Spectrum of 50 μM enzyme. (D) Calculated spectrum of free (47.8 μM) 19-nortestosterone. This is the concentration bound in the enzyme-steroid mixture (A), on the basis of a K_D = 2.3 μM (Table IV). (E) Calculated spectrum of enzyme-bound steroid (47.8 μM) in mixture A; i.e., 96% of the enzyme and 48.8% of the steroid are liganded.

Table IV: Fluorescence Quenching of Wild-Type and Mutant Isomerases by 19-Nortestosterone[a]

enzyme	tyrosine residues present	quenching of fluorescence (%)	K_D[b] (μM)
wild type	14 + 55 + 88	94 ± 2	4.4 ± 0.4
Y88F	14 + 55	94 ± 2	3.3 ± 0.6
Y55F	14 + 88	94 ± 3	3.5 ± 0.5
Y14F	55 + 88	91 ± 1	35 ± 5
Y55F/Y88F	14	97 ± 2	2.3 ± 0.3
Y14F/Y55F	88	74 ± 4	71 ± 14
Y14F/Y88F	55	91 ± 1	17 ± 0.5

[a] All experiments were carried out in 2-mL systems containing 50 mM Tris-HCl, pH 7.5, and 1.4% methanol at 25 °C. Enzymes were added to cuvettes containing buffer solutions and various concentrations of 19-nortestosterone. Samples were excited at 278 nm, and fluorescence emission was recorded at 307 nm. [b] The K_D values were calculated by curve fitting with the equation presented in Experimental Procedures.

hydrogen bond to Tyr-14 from another protein residue (Austin et al., 1992).

The UV difference spectrum of the 19-nortestosterone–Y55F/Y88F complex revealed an 8-nm red shift of the steroid (from 248 nm to 256 nm), but the extinction coefficient of 16 300 M^{-1} cm^{-1} was unchanged (Figure 6). Thus, the Y55F/Y88F mutant, which contains Tyr-14 as the sole tyrosine residue, retains the capacity to red-shift the UV spectrum of the steroid. These findings indicate that neither Tyr-55 nor Tyr-88 interacts with 19-nortestosterone, in accord with the results of NMR docking studies of the substrate into the X-ray structure of the enzyme (Figure 3B). As a further test of whether Tyr-55 might serve as an alternate proton donor to the steroid carbonyl group when Tyr-14 is absent, a red shift of 19-nortestosterone was sought in the presence of the Y14F/Y88F mutant. No significant red-shifted spectrum of 19-nortestosterone was observed in a solution containing 100 μM Y14F/Y88F enzyme and 20 μM 19-nortestosterone. Under these conditions, 83% of the steroid is bound to the enzyme, on the basis of a K_D value of 17 μM (Table IV). Therefore, participation of Tyr-55 as proton donor to the steroid 3-carbonyl group can be ruled out.

Fluorescence Studies of the Interactions of 19-Nortestosterone with Mutant Isomerases. Wang and co-workers (1963) have shown that addition of 19-nortestosterone to wild-type isomerase quenches the tyrosine fluorescence in a concentration-dependent manner.[4] The fluorescence of Tyr-14 in the Y55F/Y88F double mutant was quenched almost completely by 19-nortestosterone, and a K_D value of 2.3 ± 0.3 μM was obtained. The mechanism of this quenching process can be readily understood if a hydrogen bond existed between the phenolic hydroxyl of Tyr-14 and the 3-carbonyl oxygen of the steroid. These conclusions are also consistent with NMR studies on the 19-nortestosterone–Y55F/Y88F complex and the UV spectra of the enzyme-bound steroid, in which 8–10-nm red shifts of the peak were observed in enzymes containing Tyr-14. The fluorescences of Tyr-55 and Tyr-88, when isolated in appropriate double mutants, were quenched by 91% and 74%, respectively (Table IV), in accord with their greater distances from the enzyme-bound steroid (Figure 3B). Quenching by 19-nortestosterone was approximately additive when 2 or 3 tyrosines were present, since Tyr-14 contributes most of the fluorescence (Table III).

The 4–16-fold higher dissociation constants of 19-nortestosterone from mutants lacking Tyr-14 (Table IV) indicate that Tyr-14 is required for the tight binding of steroids by the enzyme. Especially noteworthy is the Y14F/Y55F double mutant which shows weaker steroid binding (Table IV) and greater catalytic activity (Table I) than the Y14F single mutant. Both of these properties are consistent with a more rapid rate-limiting dissociation of the carbanion intermediate from the Y14F/Y55F double mutant.

CONCLUSIONS

These studies establish that, despite its 18 unit difference in pK_a value from that of the carbonyl oxygen of the steroid substrate, Tyr-14 probably functions alone as a general acid catalyst and that the interaction of the phenolic proton of Tyr-14 with the substrate is facilitated only by concerted deprotonation of the axial 4β-proton by Asp-38. Such concerted general acid–base catalysis, invoked to explain large pK_a differences for many enzymes involved in enolization reactions (Gerlt & Kozarich, 1991), has been established for wild-type ketosteroid isomerase by combined kinetic isotope effect studies (Xue et al., 1990). The active site is hydrophobic with an estimated dielectric constant of 18 ± 2 on the basis of the elevated pK_a value of 11.6 ± 0.2 for Tyr-14. Consistent with this dielectric constant, the ultraviolet absorption and fluorescence properties of *N*-acetyltyrosine amide in 2-propanol (dielectric constant, 18.6) approximate those of Tyr-14. The pK_a of 9.5 found both in the kinetic data and in the first phase of the fluorescence titration of Tyr-14 probably reflects a partial and reversible conformational change of the enzyme.

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[4] The fluorescence quenching of wild-type isomerase by 19-nortestosterone was first observed by Wang et al. (1963), who reported a maximum fluorescence quenching of about 75%. In the present experiments, saturating concentrations of 19-nortestosterone quenched 94% of the fluorescence of this enzyme. The earlier results of Wang et al. (1963) can probably be attributed to impurities in the small amounts of enzyme available in 1963.

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