Kinetic and Ultraviolet Spectroscopic Studies of Active-Site Mutants of Δ^5-3-Ketosteroid Isomerase†

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ABSTRACT: Δ^5-3-Ketosteroid isomerase (EC 5.3.3.1) of *Pseudomonas testosteroni* promotes the highly efficient isomerization of Δ^5-3-ketosteroids to Δ^4-3-ketosteroids by means of a direct and stereospecific transfer of the 4β-proton to the 6β-position, via an enolic intermediate. An acidic residue responsible for the protonation of the 3-carbonyl function of the steroid and a basic group concerned with the proton transfer have been implicated in the catalytic mechanism. Recent NMR studies with a nitroxide spin-labeled substrate analogue have allowed positioning of the steroid into the 2.5-Å X-ray crystal structure of the enzyme [Kuliopulos, A., Westbrook, E. M., Talalay, P., & Mildvan, A. S. (1987) *Biochemistry 26*, 3927–3937], thereby corroborating the approximate location of the steroid binding site deduced from a difference Fourier X-ray diffraction map of the 4-(acetoxymercuri)estradiol–isomerase complex [Westbrook, E. M., Piro, O. E., & Sigler, P. B. (1984) *J. Biol. Chem. 259*, 9096–9103]. The steroid lies in a hydrophobic cavity near Asp-38, Tyr-14, and Tyr-55. In order to assess the role of these amino acid residues in catalysis, the gene for isomerase was cloned, sequenced, and overexpressed in *Escherichia coli* [Kuliopulos, A., Shortle, D., & Talalay, P. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8893–8897], and the following mutants were prepared: Asp-38 to asparagine (D38N) and Tyr-14 and Tyr-55 to phenylalanine (Y14F and Y55F, respectively). The k_{cat} value of the D38N mutant enzyme is $10^{5.6}$-fold lower than that of the wild-type enzyme, suggesting that Asp-38 functions as the base which abstracts the 4β-proton of the steroid in the rate-limiting step. Threefold lower K_m values in all mutants indicate tighter binding of the substrate to the more hydrophobic sites. In comparison with the wild-type enzyme, the Y55F mutant shows only a 4-fold decrease in k_{cat} while the Y14F mutant shows a $10^{4.7}$-fold decrease in k_{cat}, suggesting that Tyr-14 is the general acid. The red shift of the ultraviolet absorption maximum of the competitive inhibitor 19-nortestosterone from 248 to 258–260 nm, which occurs upon binding to the wild-type enzyme [Wang, S. F., Kawahara, F. S., & Talalay, P. (1963) *J. Biol. Chem. 238*, 576–585], is mimicked in strong acid. This spectral shift was also observed with the D38N and Y55F mutants, but not on binding of the steroid to the Y14F mutant. These findings provide further evidence that the phenolic hydroxyl group of Tyr-14 is essential for protonation of the 3-carbonyl group of the steroid. Upon binding of 17β-estradiol to the wild-type enzyme and the D38N and Y55F mutants, the ultraviolet absorption spectrum of the steroid undergoes profound changes that resemble those observed upon ionization of the phenolic hydroxyl group in base. In contrast, binding of 17β-estradiol to the Y14F mutant does not affect the spectrum of the steroid, indicating that Tyr-14 is probably also required for deprotonation of the 3-hydroxyl group of the enolic steroid. From computer-modeling studies of substrate docked into the 2.5-Å X-ray crystal structure of the enzyme, we conclude that Asp-38 and Tyr-14, after a small change in orientation of the latter, are optimally positioned for a stereoelectronically favorable, antarafacial, enolization of the ketosteroid substrate in the rate-limiting first half of the isomerization reaction.

Δ^5-3-Ketosteroid isomerase (EC 5.3.3.1) of *Pseudomonas testosteroni* catalyzes the isomerization of a variety of Δ^5-3-ketosteroids to the conjugated Δ^4-3-ketosteroids by a stereospecific, intramolecular, cis, diaxial conservative transfer of the 4β-proton to the 6β-position at rates that approach the diffusion limit [see review by Batzold et al. (1976)]. The structure and catalytic mechanism of this enzyme have been under intensive study by physical and protein chemical methods in a number of laboratories [e.g., Benisek and Ogez (1982), Westbrook and Sigler (1984), Hearne and Benisek (1985), Bevins et al. (1986), Bounds and Pollack (1987), and Kuliopulos et al. (1987a,b)]. Furthermore, an approximate position for the steroid binding site has been deduced from a difference Fourier X-ray diffraction map of a complex between 4-(acetoxymercuri)estradiol and isomerase[1] (Westbrook et al., 1984). Spectroscopic, isotope-exchange, and kinetic evidence support the participation of an enolic intermediate in the reaction (Wang et al., 1963; Malhotra & Ringold, 1965). This enolization could reasonably be catalyzed by a proton donor group AH that protonates the carbonyl group of steroid and a proton acceptor group B that removes the 4β-proton and transfers it to the 6β-position (Figure 1). The involvement of an enolic intermediate rather than a concerted mechanism is also consistent with the fact that concerted, suprafacial, 1,3 proton transfers are symmetry forbidden (Woodward & Hoffmann, 1970; Alder et al., 1971).

We have recently shown, by NMR docking of the spin-labeled substrate analogue spiro[doxyl-2,3′-5′α-androstan]-17′β-ol into the 2.5-Å X-ray structure of isomerase[2] and by

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[1] Abbreviation: isomerase, Δ^5-3-ketosteroid isomerase.

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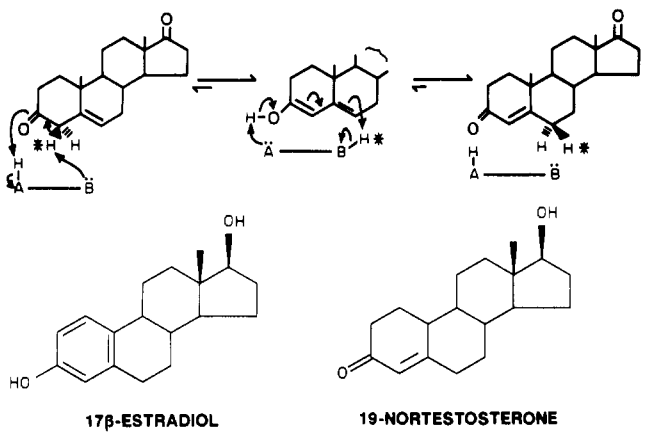


FIGURE 1: (Above) Mechanistic pathway for conversion of Δ^5-3-ketosteroids to Δ^4-3-ketosteroids by Δ^5-3-ketosteroid isomerase of *Ps. testosteroni*. The reaction is shown proceeding by way of a $\Delta^{3,5}$-dienol intermediate. The proton donor (HA) protonates the 3-carbonyl group, and the base (B) abstracts the 4β-proton (H*) which is transferred intramolecularly to the 6β-position. (Below) Structures of two linear competitive inhibitors of the enzyme: 19-nortestosterone and 17β-estradiol.

assuming that the substrate bound with its A and D rings reversed from those of the substrate analogue, that Asp-38 was appropriately positioned to function as the proton acceptor B and that either Tyr-55 or Tyr-14 could function as the proton donor (Kuliopulos et al., 1987b). The negative end of the dipole of the single 10-residue α-helix in the molecule (residues 8–17) was also invoked to facilitate the protonation of the steroid 3-carbonyl group. Tyr-14 and Tyr-55 were found to lie closer than Tyr-88 to the region of the steroid involved in catalysis. On the basis of computer modeling we favored Tyr-55 as the most likely candidate for protonation of the steroid 3-carbonyl group because it appeared to have more favorable orientation for hydrogen bonding (Kuliopulos et al., 1987b). However, it is possible that such favorable orientation of Tyr-14 could also be achieved by minor conformational changes in the enzyme such as might occur during substrate binding.

Although physical methods such as X-ray diffraction and NMR spectroscopy can contribute to our understanding of the topography of the catalytic and binding sites, only kinetic measurements can establish unequivocally the amino acid residues involved in the catalytic process. To further this goal, we have cloned and sequenced the gene for isomerase and overexpressed it in *Escherichia coli* (Kuliopulos et al., 1987a). Similar cloning efforts have also been reported by Choi and Benisek (1987). The present paper describes the effects of site-directed mutagenesis of Asp-38 to asparagine (D38N), Tyr-14 to phenylalanine (Y14F), and Tyr-55 to phenylalanine (Y55F) on the kinetic and spectroscopic properties of isomerase. This enzyme is particularly amenable to kinetic and spectroscopic studies since (a) reaction rates over the entire $10^{9.5}$-fold range between the uncatalyzed and enzyme-catalyzed reactions are easily measured spectroscopically and (b) binding of the linear competitive inhibitors 19-nortestosterone and 17β-estradiol to the enzyme results in characteristic spectral changes of these steroids that can be used to measure binding constants (Wang et al., 1963) and to detect the presence of proton donor/acceptor groups on the enzyme. A preliminary account of this work has been published (Kuliopulos et al., 1988).

[2] E. M. Westbrook, R. L. Stanfield, and R. Kahn, The 2.5-Å Crystal Structure of Δ^5-3-Ketosteroid Isomerase, in preparation.

Experimental Procedures

Site-Directed Mutagenesis. The KSI gene coding for isomerase has been cloned, overexpressed, and sequenced as described (Kuliopulos et al., 1987a). A 1.4-kb fragment from the plasmid pAK1370 that contained the isomerase gene 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* CJ 236 (dut^-/ung^-) competent cells. Deoxyoligonucleotides containing the desired base changes were synthesized and used for site-directed mutagenesis according to the method of Zoller and Smith (1983). The mutation converting Tyr-14 to phenylalanine (Y14F) was obtained with the 17-mer deoxyoligonucleotide (corresponding to bases 31–47): 5′-GTACAGCGCTTTGTGGC-3′ (the base change is indicated by the underlined letter). The Asp-38 to asparagine mutant (D38N) was made by using the 17-mer (corresponding to bases 104–120): 5′-CGGTGGAAAACCCGTG-3′. Similarly, the Tyr-55 to phenylalanine mutant (Y55F) was obtained with the 21-mer (corresponding to bases 154–174): 5′-CGTGAGTTTTTCGCGAACTCG-3′. The 21-mer contained an additional silent mutation G at position 168 which created a *Nru*I site (TCGCGA) within the coding sequence of the gene. Clones of M13mp18 that contained the desired mutations were identified by screening with ^{32}P-labeled mutant oligonucleotides or, in the case of the Y55F mutation, by the presence of a new *Nru*I site. The presence of the desired mutations and the absence of adventitious base changes were then verified by sequencing the entire gene by the dideoxynucleotide method (Sanger et al., 1977) with use of a primer 14 bp upstream of the ATG start site, [^{35}S]dATP, and Sequenase (United States Biochemical Corp.). Mutated DNA was isolated as the double-stranded replicative form from M13mp18 and reintroduced into the *Eco*RI, *Hin*dIII sites of the plasmid pUC 19 for expression of the mutant enzymes (Kuliopulos et al., 1987a).

Purification of Protein. The procedure for overexpression and purification to homogeneity (5–10-fold) of the isomerase enzymes was carried out as described (Kuliopulos et al., 1987a) with the following modifications. After sonic disruption of the cell pellet, poly(ethylenimine) (Polymin P, BRL, Bethesda, MD) was added to the supernatant fraction, and the mixture was centrifuged. The isomerase activity in the supernatant fluid was then precipitated between 35% and 65% saturation with ammonium sulfate. The precipitate was dialyzed against three changes of 2 L of 1 mM Tris-HCl, pH 7.0, overnight or, alternatively, desalted in the same buffer by passage through a Sephadex G-25M column (Pharmacia, PD-10). Isomerase was then purified by chromatography on a Pharmacia Mono Q anion-exchange column with the use of a linear gradient from 1 to 150 mM Tris-HCl at pH 7.0. The fractions containing the isomerase activity were then further purified on a gel filtration column (Pharmacia, Superose 12) with 50 mM Tris-HCl and 100 mM NaCl at pH 7.5 as the elution buffer. The purity of isomerase was assessed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and staining with Coomassie Blue R 250. For the wild-type enzyme, the specific activity also served as an index of purity.

Protein Concentrations. The protein concentrations of solutions of wild-type and mutant enzymes were derived from absorbance measurements at 280 nm. It has been established by independent means that a solution of the pure wild-type enzyme isolated from *Ps. testosteroni* containing 1.00 mg of

protein/mL has an absorbance of 0.336 ± 0.004 at 280 nm (Benson et al., 1975). The corresponding values for the cloned wild-type and the three mutant enzymes were obtained by measuring the tyrosine content of enzyme solutions by the method of Goodwin and Morton (1946). Measurement of the absorbances of these solutions at 293 nm at neutral pH and again after addition of NaOH to a concentration of 0.1 N (pH 13), at which the protein is denatured and the phenolic groups of tyrosine residues become fully ionized, provides an accurate measure of the tyrosine concentrations, since the wild-type and mutant enzymes contain no tryptophan. We used the extinction coefficient of 2390 M^{-1} cm^{-1} at 293 nm for the difference spectrum of tyrosine at neutrality and pH 13 (Goodwin & Morton, 1946; Kawahara et al., 1962). The absorbances of the neutral enzyme solutions at 280 nm were also determined. Assuming a subunit weight of 13 399 for the wild-type enzyme and that the number of moles of tyrosine per enzyme subunit is 3 in both wild-type and D38N and 2 in both Y14F and Y55F, the following absorbances at 280 nm were determined for solutions containing 1.00 mg of enzyme/mL: 0.327 for cloned wild-type enzyme (in good agreement with the value of 0.336 determined for the isolated wild-type enzyme); 0.343 for D38N; 0.226 for Y14F; and 0.245 for Y55F.

Small-Zone Gel Chromatography. A Sephacryl 200 high-resolution (Pharmacia, S200 HR) column (0.9 × 30 cm) was used. Elution buffers of varied ionic strengths ranging for 5 to 50 mM Tris-HCl, pH 7.5, also containing 25–250 mM NaCl, were used.

Kinetic Studies. Specific activity measurements were made by monitoring the change in absorbance at 248 nm and at 25 °C in 3.0-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 amounts used in the assays were in the range of 0.2 ng for wild type, 0.4 ng for Y55F, 2.0 μg for Y14F, and 30.0 μg for D38N. Enzymes were diluted appropriately in 1% bovine serum albumin, pH 7.0, just prior to use. The k_{cat} and K_m values of each enzyme were determined at three different pH values. Buffers used were 33 mM potassium phosphate at pH 6.5 and 50 mM Tris-HCl at pH 7.5 and 8.5. Substrate was added at concentrations of 11.6, 23.1, 34.9, 58.2, 81.5, and 116.4 μM. The final concentration of methanol was 3.3% by volume. The inhibitions of Y14F by either 17β-estradiol or 19-nortestosterone were measured in 50 mM Tris-HCl, pH 7.5, and substrate concentrations of 29.1, 58.2, and 116.4 μM. The concentrations of 17β-estradiol were 10.0, 23.3, and 33.3 μM, and those of 19-nortestosterone were 16.7, 33.3, and 66.7 μM. For the inhibition experiments, both inhibitors and substrates were added in methanol (final concentration 4% by volume). The kinetic data were analyzed by double reciprocal plots, and the K_m and V_{max} values were obtained by use of a Wilkinson hyperbolic weighted least-squares program (Wilkinson, 1961). The differing volumes of methanol used in the above experiments were dictated by a compromise between the inhibition of the enzyme by this solvent (Weintraub et al., 1980; Falcoz-Kelly et al., 1968) and the need to maintain steroid solubility.

Ultraviolet Spectroscopy. Ultraviolet absorption measurements were made at 25 °C with a Beckman DU-7 spectrophotometer in matched 1.0-cm quartz cuvettes in total volumes of 500–510 μL. The initial spectrum of the isomerase solution in 50 mM Tris-HCl and 100 mM NaCl, pH 7.5, was measured against a blank containing the same buffer. Wild-type and mutant enzymes were titrated with steroid by making successive additions of steroid solutions in methanol (2 or 10 mM), and the absorbances were corrected for the small volume changes that took place. The spectrum of the bound steroid was derived from these spectra by subtracting the absorption of the total enzyme, and of the residual free steroid, which was calculated from the dissociation constant. These spectra were obtained at 16 μM total steroid and 31–43 μM enzyme under conditions in which more than 40% of the steroid was bound.

To determine K_D values, changes in absorption (ΔA) at the respective absorption maxima in the difference spectra were measured as a function of steroid concentration. The absorption maxima in such difference spectra were determined by subtracting the spectra of both total steroid and total enzyme from the spectrum of a mixture containing the same amounts of both steroid and enzyme (Wang et al., 1963). Plots of ΔA with respect to total steroid concentration gave typical hyperbolic saturation curves that were fit by the function

$$\frac{\Delta A}{\Delta A_{max}} = \frac{K_D + E_t + S_t - [(K_D + E_t + S_t)^2 - 4E_tS_t]^{1/2}}{2E_t} \quad (1)$$

In eq 1, K_D is the dissociation constant, E_t is the concentration of total enzyme expressed in terms of subunits of mol wt 13 399 and is equal to $E_b + E_f$, where subscripts b and f refer to the bound and free species, respectively. S_t is the concentration of the total steroid and is equal to $S_b + S_f$. ΔA_{max} is the maximal change in absorption observed as S_t approaches infinity. Alternatively, the data could be fit by a Scatchard plot of (S_b/E_tS_f) against S_b/E_t. S_b and S_f were obtained from the values of ΔA and ΔA_{max}. Because of the limited solubility of 17β-estradiol in aqueous media, ΔA_{max} for this steroid was initially estimated from the limiting value of the slope of ΔA as a function of S_t, as S_t approached zero and visually iterated to optimize the fit. In those cases in which the affinity of steroid for enzyme was high, the reported values for K_D should be regarded as upper limit estimates.

Spectra of 19-nortestosterone in HCl ranging from 1 to 10 M or in H_2SO_4 ranging from 0.1 to 10 M were measured against equivalent concentrations of the acids alone. Similarly, spectra of 17β-estradiol in 0.3 M NaOH were measured against equivalent concentrations of the base.

Results and Discussion

Gene Sequence, Homogeneity, and Molecular Properties of Mutant Enzymes. The three newly constructed mutant genes of isomerase that code for Y14F, D38N, and Y55F were identified by hybridization to their respective oligonucleotides, and the entire genes were sequenced. Only the desired changes were found in the coding sequence, and the recently reported nucleotide sequence of the isomerase structural gene was completely confirmed (Kuliopulos et al., 1987a). The mutant enzymes were then overexpressed in *E. coli* JM 101 and purified to near homogeneity. Even when overloaded, the purified Y14F and D38N enzymes showed only a single band on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The final Y55F protein preparation, however, had a contaminating high molecular weight component consisting of ≤2% of the total protein (Figure 2). All three mutant enzymes had the same mobility as wild type on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The abnormally rapid mobility of the wild-type isomerase subunit (revised calculated mol wt = 13 399), which migrates at an apparent mol wt of 10 300, has been discussed (Benson et al., 1975).

When the wild-type and three mutant enzymes were subjected to electrophoresis on native gels at pH 8.1, Y14F and Y55F migrated at rates identical with wild type, whereas

Table I: Kinetic Constants of Ketosteroid Isomerase and Its Mutants[a]

enzyme	sp act.[b] (μmol min^{-1} mg^{-1})	k_{cat}[c] (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	rel k_{cat}	rel K_m
WT	45300	53600	340 ± 20	1.58×10^8	1.00	1.00
Y55F	22800	12700	130 ± 15	9.77×10^7	0.24	0.38
Y14F	2.28	1.18	123 ± 13	9.59×10^3	$10^{-4.7}$	0.36
D38N	0.255	0.130	102 ± 22	1.27×10^3	$10^{-5.6}$	0.30

[a] All assays were done at 25.0 °C. [b] The specific acivities were determined under standard assay conditions in 3.0-mL systems containing 50 mM Tris-HCl, pH 7.5, and 58.2 μM 5-androstene-3,17-dione in methanol (1.7% final volume). [c] The k_{cat} values were derived from V_{max} intersects of double reciprocal plots of velocity with respect to substrate concentration, under conditions of the standard assay, except that the methanol concentration was raised to 3.3% by volume. Since 1.7% methanol inhibits the enzyme by 9% while 3.3% methanol inhibits by 23%, the k_{cat} values here recorded are somewhat lower than those calculated from the specific activities, which were determined in 1.7% methanol.

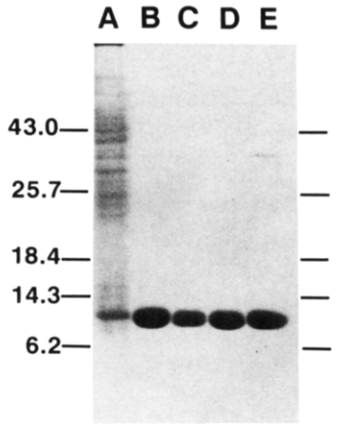


FIGURE 2: Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate of recombinant wild-type and mutant isomerases and of an extract of *E. coli* containing the plasmid that encodes the isomerase gene (pAK1370). The 17% polyacrylamide gel contained 0.1% sodium dodecyl sulfate and was stained with Coomassie Blue R 250. All preparations were boiled in 0.1% sodium dodecyl sulfate prior to electrophoresis. Molecular weights ($\times 10^{-3}$) are shown on the left. Lanes: (A) centrifuged cell pellet of *E. coli* JM 101 grown in the presence of 0.67 mM thiogalactoside and containing pAK1370; (B) 38.4 μg of wild-type isomerase purified by ammonium sulfate precipitation, anion-exchange chromatography, and gel filtration chromatography; (C) 22.0 μg of Y14F mutant isomerase prepared as for lane B; (D) 27.9 μg of D38N mutant isomerase prepared as for lane B; (E) 36.6 μg of Y55F mutant isomerase prepared as for lane B.

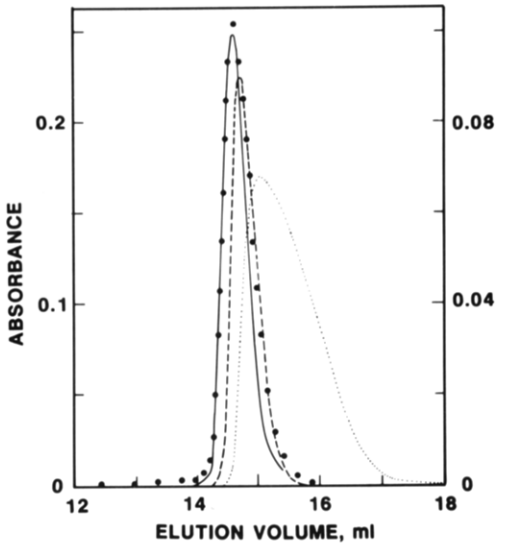


FIGURE 3: Elution profiles of small zone chromatographies of wild-type and mutant isomerases on a gel filtration column (Superose 12, Pharmacia): (—) 192 μg of wild-type isomerase; (- - -) 440 μg of Y14F isomerase; (···) 490 μg of D38N isomerase; (●●●) 732 μg of Y55F isomerase. The absorbance at 280 nm is plotted against elution volume for each sample: (right ordinate) for wild-type enzyme; (left ordinate) for mutant enzymes. The elution buffer was 100 mM NaCl and 50 mM Tris-HCl, pH 7.5, and the flow rate was 0.4 mL/min. The retarded elution and spreading of the trailing boundary of the D38N peak could be nearly eliminated by lowering the ionic strength of the elution buffer.

D38N migrated slightly more slowly. The slower migration of D38N toward the cathode was expected since it had one fewer negatively charged amino acid as compared to the wild type. Similarly, D38N eluted from the anion-exchange column (Mono Q, Pharmacia) at lower ionic strength (70 mM Tris-HCl, pH 7.0), compared to the wild-type, the Y14F, and the Y55F enzymes, which required 90 mM Tris-HCl, pH 7.0, for elution.

The molecular weights of the mutant enzymes under native conditions were determined by small-zone gel filtration on Sephacryl 200 HR. As shown in Figure 3, Y14F and Y55F had essentially the same apparent molecular weight as the wild-type enzyme, which exists as a homodimer at subunit concentrations ranging from $\leq 4.5 \times 10^{-11}$ to 1.5×10^{-4} M (Benson et al., 1975). Under these conditions (50 mM Tris-HCl and 100 mM NaCl, pH 7.5) D38N eluted consistently later than the other three proteins and exhibited significant spreading of the trailing edge of the solute zone. Although this abnormally slow mobility may be interpreted as resulting from dissociation of the D38N dimer (Ackers, 1970), the retardation of D38N was moderately affected by ionic strength. Thus, the D38N mutant migrated more slowly than wild type in 250 mM NaCl, with an elution volume 26% greater than that of wild type, while in 5 mM Tris-HCl and 25 mM NaCl, pH 7.5, the elution volume of D38N was only 18% greater than that of wild type. Furthermore, the abnormal migration of D38N was abolished in the presence of steroids that bind to the active site of the enzyme, such as 12.5 μM 17β-estradiol or 40 μM 19-nortestosterone. In the presence of these steroids, the elution volume of D38N differed by less than 2% from that of wild type. Thus, the retarded elution of D38N from Sephacryl 200 HR, in comparison to the wild-type enzyme and the other mutants, probably resulted from hydrophobic interaction of the D38N mutant with the gel matrix at the more hydrophobic steroid binding site, although dissociation to monomers cannot be excluded.

Kinetic Properties. The specific activities, k_{cat}, and K_m values of each of the purified mutants were compared to those of the recombinant wild-type enzyme purified by the same method (Table I). At pH 7.5 the Y14F and D38N mutations resulted in profound decreases in k_{cat} to values $10^{4.7}$- and $10^{5.6}$-fold lower, respectively, than that of the wild-type enzyme, while the Y55F mutation caused only a 4-fold decrease in k_{cat}. In all three cases, however, the substrate bound more tightly to the mutant enzymes as reflected in an approximately 3-fold lowering of K_m relative to that of wild type at pH 7.5. The lower K_m values of the mutants make it unlikely that the residual enzyme activity originates from contaminating wild-type enzyme arising from errors in translation. These results are consistent with the conclusion that Tyr-14 acts as the acid catalyst or proton donor (HA in Figure 1) and that Asp-38

Table II: Effect of pH on Kinetic Constants of Ketosteroid Isomerase Mutants

enzyme	pH[a]	k_{cat}[b] (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	rel k_{cat}	rel K_m
Y55F	6.5	11800 ± 700	117 ■ 11	10.1×10^7	1.00	1.00
	7.5	12700 ± 900	130 ± 15	9.77×10^7	1.08 ± 0.07	1.11 ± 0.13
	8.5	13600 ± 1300	137 ± 21	9.93×10^7	1.15 ± 0.11	1.17 ± 0.13
Y14F	6.5	1.42 ± 0.11	168 ± 19	8.45×10^3	1.00	1.00
	7.5	1.18 ± 0.07	123 ± 13	9.59×10^3	0.83 ± 0.05	0.73 ± 0.08
	8.5	1.12 ± 0.13	100 ± 19	11.2×10^3	0.79 ± 0.09	0.60 ± 0.11
D38N	6.5	0.124 ± 0.009	61.1 ± 9.8	2.03×10^3	1.00	1.00
	7.5	0.130 ± 0.016	102 ± 22	1.27×10^3	1.05 ± 0.13	1.67 ± 0.36
	8.5	0.312 ± 0.046	250 ± 48	1.25×10^3	2.52 ± 0.37	4.09 ± 0.79

[a] Buffers used were 33 mM potassium phosphate, pH 6.5, and 50 mM Tris-HCl, pH 7.5 and pH 8.5. [b] The conditions under which the k_{cat} values were determined are stated in the legend of Table I.

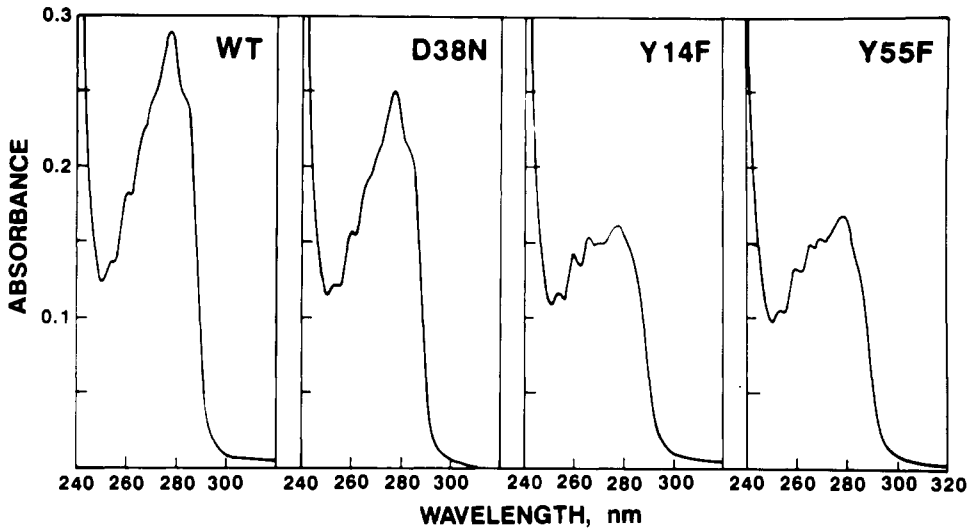


FIGURE 4: Ultraviolet absorption spectra of wild-type and mutant isomerases. Concentrations for each enzyme were as follows: wild-type (WT), 0.80 mg/mL; D38N, 0.67 mg/mL; Y14F, 0.69 mg/mL; Y55F, 0.74 mg/mL. Wild-type and mutant isomerases were purified as described in the legend of Figure 2. All measurements were taken in 100 mM NaCl and 50 mM Tris-HCl, pH 7.5, at 25.0 °C in 1.0-cm quartz cuvettes against solvent blanks.

acts as the base catalyst or proton acceptor (B in Figure 1). Tyr-55 does not participate significantly in the rate-limiting step of the isomerase forward reaction.

With trypsin (Sprang et al., 1987; Craik et al., 1987) and with staphylococcal nuclease (Mildvan et al., 1988), the 10^4-fold decreases in k_{cat} resulting from mutations of residues that function as general bases are partially restored by raising the pH, and these rate enhancements are proportional to hydroxyl ion concentration. Thus with these enzymes, hydroxyl ion can partially replace the proton-accepting residue. It has been demonstrated in several laboratories that k_{cat} of isomerase is largely independent of pH over the range of 6–9 (Wang et al., 1963; Weintraub et al., 1970; Pollack et al., 1986). Hence, the effects of pH on the kinetic parameters of the three mutant enzymes were also examined over the limited pH range from 6.5 to 8.5 (Table II). Outside this pH range, the measurements are complicated by significant contributions from nonenzymatic reaction rates. While k_{cat} of the D38N mutant increased slightly over this range, the change was much smaller than the 100-fold increase in hydroxyl ion concentration, and the k_{cat}/K_m value actually decreased because of a 4-fold increase in K_m (Table II).

An opposite effect of pH on k_{cat} might be expected for a mutant enzyme, such as Y14F, which lacked the proton donor, if H_3O^+ could replace the catalytic residue. Little or no decrease in k_{cat} of Y14F occurred on raising the pH from 6.5 to 8.5, while the k_{cat}/K_m value increased slightly over this pH range. Similarly, little effect of pH on either k_{cat} or K_m of the highly active Y55F mutant was detected (Table II). Hence the active site of isomerase appears to be inaccessible to both H_3O^+ or OH^-, a conclusion consistent with the conservative transfer of the 4β-proton to the 6β-position of the steroid in the isomerase reaction (Talalay & Wang, 1955; Malhotra & Ringold, 1965).

Ultraviolet Absorption Spectra of Isomerase Mutants. The ultraviolet absorption spectra of the three mutant proteins were also examined in order to characterize their properties, to confirm the aromatic amino acid composition, to assess their purity, and to provide a method for determining protein concentrations. Each subunit of wild-type isomerase contains three tyrosine, eight phenylalanine, and no tryptophan residues. Consequently, the near-ultraviolet absorption spectrum is dominated by tyrosine and phenylalanine absorptions, and indeed this spectrum is almost identical with the additive spectral contributions of these aromatic amino acids (Kawahara et al., 1962).

As expected, the spectrum of the D38N mutant was nearly identical with that of the wild-type enzyme (Figure 4), with the characteristic absorption maximum of tyrosine in peptide linkage at 277 nm and a shoulder at 283 nm. The four smaller absorption peaks of the phenylalanine residues observed in the wild-type enzyme at 252.5, 258.0, 265.0, and 270.0 nm were very similar in D38N, which had corresponding absorption maxima at 252.5, 259.0, 265.5, and 269.0 nm. Like the wild-type enzyme, the D38N mutant had an absorption minimum at 250 nm. The other two mutants, Y14F and Y55F, had strikingly different spectra that were consistent with simple additivity of the contributions of their two tyrosine and nine phenylalanine residues to the spectrum. The fine structural details arising from the phenylalanine absorption peaks at 253,

Table III: Ultraviolet Absorption Characteristics of 19-Nortestosterone and 17β-Estradiol on Binding to Ketosteroid Isomerase and Its Mutants and in the Presence of Acid or Base

steroid	enzyme or reagent	λ_{max} (nm)	$\Delta\lambda_{max}$ (nm)	ϵ_{max} (M^{-1} cm^{-1})	$\Delta\epsilon_{max}$[a] (M^{-1} cm^{-1})
19-nortestos-	none	248		16 100	
terone	wild-type	260	12	17 800	1 700
	Y55F	257	9	15 100	−1 000
	Y14F	248	0	15 800	−300
	D38N	260	12	17 400	1 300
	HCl	258[b]	10		
	(10 M)	285[c]	37		
	H_2SO_4	258[c]	10		
	(10 M)	285[b]	37		
17β-estradiol	none	220		7 200	
		278		2 000	
	wild-type	238.5	18.5	18 300	11 100
		290	12	5 500	3 500
	Y55F	235.5	15.5	12 400	5 200
		284	6	3 800	1 800
		289	11	3 700	1 700
	Y14F	220	0	7 200	
		282	4	1 300	−700
	D38N	238	18	21 800	14 600
		290	12	5 600	3 600
	NaOH	238	18	9 600	2 400
	(0.3 M)	295	17	2 900	900
	HCl	216	−4	10 000	2 800
	(10 M)	277	−1	2 300	300

[a] $\Delta\epsilon_{max}$ are the differences in molar absorption coefficients between bound (or acid/base-treated) steroid and free steroid at their respective maxima. [b] Peak in spectrum. [c] Shoulder in spectrum.

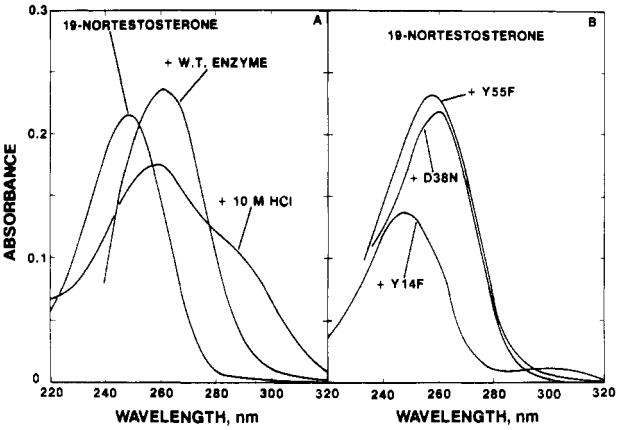
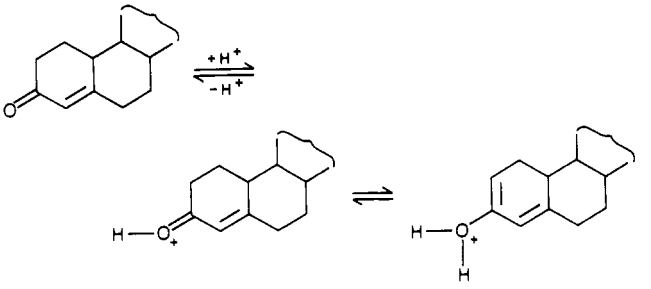


FIGURE 5: Effects of isomerase, its mutants, and strong acid on the ultraviolet absorption spectrum of 19-nortestosterone. The following spectra are shown: (A) 19-nortestosterone in buffer; 19-nortestosterone bound to 43.1 μM wild-type isomerase; 19-nortestosterone in 10 M HCl; (B) 19-nortestosterone bound to 39.4 μM Y14F isomerase; 19-nortestosterone bound to 31.2 μM D38N isomerase; 19-nortestosterone bound to 41.1 μM Y55F isomerase. When enzyme was present, the spectra of total isomerase and free steroid were subtracted from the spectrum of the steroid–enzyme mixture. The total concentrations of 19-nortestosterone were 13.4 μM in buffer and in acid and 15.9 μM in the presence of each enzyme. Under these conditions, on the basis of the measured K_D values (Table IV), the concentrations of steroid bound to wild type, Y14F, D38N, and Y55F were 13.4, 8.6, 12.2, and 15.3 μM, respectively. The buffer was 100 mM NaCl and 50 mM Tris-HCl, pH 7.5. The methanol concentration was 0.8% by volume.

259, 265, and 269 nm were more pronounced since the underlying broad tyrosine peak was decreased in intensity.

The spectra of the mutants at pH 7.0 were analyzed by measuring the tyrosine and phenylalanine absorption maxima at 277 and 259.5 nm, respectively. These absorption peaks correspond to those of the free amino acids at 275 nm (ϵ = 1230 M^{-1} cm^{-1}) and 257.5 nm (ϵ = 177 M^{-1} cm^{-1}), respectively. The absorption of free tyrosine at 257.5 nm is 0.333 of its maximum at 275 nm. We calculated that the phenylalanine to tyrosine ratios of the four proteins were as follows: wild type, 2.3; D38N, 2.1; Y14F, 4.0; and Y55F, 3.4. These results are in reasonable agreement with expected ratios of 2.67 (8 Phe/3 Tyr) for wild type and D38N and 4.50 (9 Phe/2 Tyr) for Y14F and Y55F and are inconsistent with other integral ratios for 11 aromatic residues. Both Y14F and Y55F also had absorption minima at 250 nm.

Effects of Isomerase Mutants and Strong Acid on the Ultraviolet Absorption Spectrum of 19-Nortestosterone. It has been shown that the ultraviolet absorption peak of the linear competitive inhibitor 19-nortestosterone (248 nm in aqueous solution) undergoes a red shift in complex with wild-type isomerase (Wang et al., 1963). A 12-nm red shift to 260 nm was also observed when 19-nortestosterone was bound to the recombinant wild-type enzyme (Table III). We were able to mimic the enzyme-induced spectral shift of 19-nortestosterone by placing this steroid in 10 M HCl (Figure 5A). Thus, the absorption maximum of the steroid in 10 M HCl was shifted to 258 nm, and a shoulder at 285 nm was also observed. When the steroid was added to a stronger acid, namely, 10 M H_2SO_4, the peak at 258 nm decreased, becoming a shoulder on a larger peak which absorbed maximally at 285 nm (Table III). The spectral changes in acid were stable for at least 24 h at all concentrations of acid and could be completely abolished by neutralization with equivalent amounts of NaOH. The absorption spectrum of 19-nortestosterone in acid suggests the sequential formation of two species, one of which is a precursor of the other. The first species (λ_{max} = 258 nm) is likely to be the protonated carbonyl form of the steroid, whereas the second is a more highly conjugated species, such as a dienol. Since the latter species absorbs at 285 nm and has almost the same extinction coefficient as the parent compound, it is likely to be a homoannular dienol with double bonds at 2(3) and 4(5) rather than a heteroannular structure with double bonds at 3(4) and 5(6), since the latter would be expected to absorb at lower wavelengths and have a higher extinction coefficient than the parent compound (Fieser & Fieser, 1959; Dorfman, 1953).



The alternative homoannular Δ3(4)-5(10) dienol is less likely since testosterone, with a 19-methyl group at position 10, shows a spectrum identical with that of 19-nortestosterone in strong acid.

By monitoring the change in λ_{max}, we found that spectral titrations of 19-nortestosterone with HCl or H_2SO_4 gave a pK_a value of −2.3 ± 0.2 for the protonation of the 3-carbonyl oxygen of the steroid on the basis of standard acidity functions (Gordon & Ford, 1972; Lowry & Richardson, 1981).

Upon complexation of 19-nortestosterone to the Y55F and D38N mutant enzymes, red shifts of the absorption maximum from 248 to 257 and 260 nm, respectively, were observed similar to those obtained with the wild-type enzyme (Figure 5, parts A and B; Table III). These spectral changes presumably also resulted from the protonation of the 3-carbonyl oxygen of the steroid. In contrast, the Y14F mutant had a

Table IV: Characteristics of Ultraviolet Difference Spectra of 19-Nortestosterone and 17β-Estradiol on Binding to Ketosteroid Isomerase and Its Mutants. Dissociation (K_D) and Inhibitor (K_i) Constants

steroid	enzyme	λ_{max}[a] (nm)	$\Delta\epsilon_b$ (M^{-1} cm^{-1})	K_D (μM)	K_i (μM)
19-nortes-	wild-type	270	11300	5.5 ± 1.0	5.2,[b] 13.0[c]
tosterone	Y55F	270	6960	1.0 ± 0.2	
	Y14F	290	800	26.5 ± 13.0	30 ± 13
	D38N	265	8970	5.5 ± 2.0	
17β-estra-	wild-type	238	16900	0.8 ± 0.3	4.5,[d] 10[b]
diol		295	4030	1.7 ± 0.6	
	Y55F	232	12300	4.0 ± 3.0	
		288	2000	6.7 ± 3.5	
	Y14F				43 ± 5
	D38N	238	17500	0.10 ± 0.05	
		292	4800	0.10 ± 0.05	

[a] These wavelengths are the maxima of the difference spectra (enzyme–steroid complex minus total enzyme minus total steroid). [b] In 33 mM potassium phosphate, pH 7.0, and 1.7% methanol at 25 °C (Wang et al., 1963). [c] In 20 mM Tris-HCl, pH 7.5, and 3.3% methanol at 21 °C (Falcoz-Kelly et al., 1968). [d] In 50 mM Tris-HCl, pH 7.8, and 5% methanol at 37 °C (Goldman, 1968).

negligible effect on the spectrum even though it is known to bind to this steroid (Table IV). We conclude that the phenolic hydroxyl group of Tyr-14 is responsible for the protonation of the 3-carbonyl oxygen of the steroid. Furthermore, the very low catalytic activity of the Y14F mutant enzyme provides strong evidence for the critical participation of Tyr-14 as the proton donor in the catalytic process.

The ability of Tyr-14 ($pK_a \sim 9$) to protonate the carbonyl group of a ketosteroid ($pK_a \sim -2.3$ in H_2O) may result from several properties of the active site of isomerase. First, the proximity of the bound substrate to the negative end of the helix dipole arising from residues 8–17 in an otherwise hydrophobic cavity could raise the pK_a value of the steroid. Second, the hydrogen bond from Tyr-14 to the carbonyl group of the substrate may be sufficiently strong to effect partial protonation in the ground state.

From the spectral titrations, we were able also to calculate the dissociation constants for the 19-nortestosterone–isomerase complexes for each enzyme (Wang et al., 1963). Such spectral titrations, for the wild-type and the three mutant enzymes, are shown in Figure 6 and could be fit only with $n = 1$. The K_D and $\Delta\epsilon_b$ values are listed in Table IV. Wild type and D38N had K_D values of 5.5 μM, whereas Y55F bound 19-nortestosterone 5-fold more tightly with a K_D of 1.0 μM. Y14F, on the other hand, bound steroid 5-fold more weakly than did the wild type and had a K_D of 26.5 μM. The large differences in the extinction coefficients of the steroid–enzyme complexes were readily apparent as indicated by the asymptotic ΔA values of the titration curves (Figure 6). The complex between 19-nortestosterone and the wild-type enzyme had the largest $\Delta\epsilon_b$ of 11300 M^{-1} cm^{-1}, and the corresponding values for D38N and Y55F were 80% and 62% of the wild-type absorption coefficient. The $\Delta\epsilon_b$ value of the complex between Y14F and 19-nortestosterone was only 7% of that of the wild-type complex. The latter was measured at a λ_{max} of 290 nm where only protein tyrosine residues but not the inhibitor contributed to the absorption. Although the Y14F titration data could be fit with a K_D of 26.5 μM, the observed absorption differences were small and gave rise to much scatter. A kinetic determination of the K_i value (30 μM) for 19-nortestosterone with Y14F agreed with the K_D value (see Table IV).

Effects of Isomerase Mutants on the Ultraviolet Absorption Spectrum of 17β-Estradiol. 17β-Estradiol is another linear competitive inhibitor for isomerase that may be considered an analogue of the dienol intermediate. The spectrum of 17β-estradiol at neutral pH has two absorption maxima at 220 and 278 nm, which are characteristic of phenols (Figure 7A).

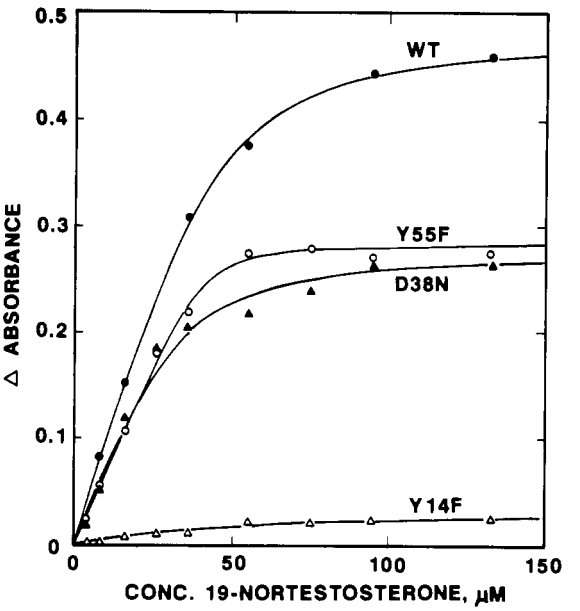


FIGURE 6: Ultraviolet spectral titrations of the direct binding of 19-nortestosterone to wild-type and mutant isomerases. A difference spectrum was obtained for each titration by subtracting the spectral contributions of both total steroid and total enzyme from that of a mixture of steroid and enzyme. This difference spectrum was then used to determine the absorption maximum of the difference spectrum from which the ΔA could be obtained as a function of total steroid concentration. These absorption maxima were at 270, 290, 265, and 270 nm for wild type, Y14F, D38N, and Y55F, respectively. The theoretical titration curves were calculated with $n = 1$ in all cases and the K_D values were 5.5, 26.5, 5.5, and 1.0 μM for wild type, Y14F, D38N, and Y55F, respectively. Solutions of 510-μL final volume contained isomerase ranging from 31.2 to 43.1 μM and were constant for the individual titrations. Buffer was 50 mM Tris-HCl and 100 mM NaCl, pH 7.5, and the final methanol concentration was 2.0% by volume. Aliquots of the steroid were added as 2.0 or 10.0 mM solutions in methanol, and ΔA was corrected for the slight volume changes.

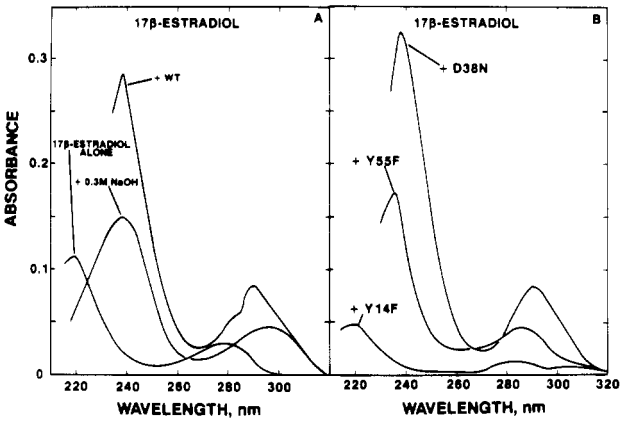


FIGURE 7: Effects of isomerase mutants and base on the ultraviolet absorption spectrum of 17β-estradiol. The following spectra are shown: (A) 17β-estradiol in buffer; 17β-estradiol bound to 43.1 μM wild-type (WT) isomerase; 17β-estradiol in 0.3 M NaOH; (B) 17β-estradiol bound to 39.4 μM Y14F; 17β-estradiol bound to 31.2 μM D38N; 17β-estradiol bound to 41.1 μM Y55F. When enzyme was present, the spectra of total isomerase and free steroid were subtracted from the spectrum of the steroid–enzyme mixture. The total concentrations of 17β-estradiol were 15.6 μM in buffer and in base and 15.9 μM in the presence of each enzyme. Under these conditions, on the basis of the measured K_D (K_i) values (Table IV), the concentrations of steroid bound to wild type, Y14F, D38N, and Y55F were 15.6, 6.8, 14.9, and 13.8 μM, respectively. The buffer and methanol concentrations were as stated in the legend to Figure 5.

Wang et al. (1963) demonstrated that the spectrum of 17β-estradiol was dramatically changed upon binding to the wild-type isomerase in neutral solution. The peak at 220 nm was intensified 2.5-fold and red-shifted by 18.5 nm to 238.5 nm, whereas the 278-nm peak was intensified 2.7-fold and red-shifted by 12 nm to 290 nm. That these spectral changes originated from effects of the protein on the steroid, rather than vice versa, was established by analogous observations with 17β-dihydroequilenin, a steroid differing in structure from 17β-estradiol by the presence of additional aromaticity in ring B. The ultraviolet absorption spectrum of this steroid has a series of absorption maxima, including those at 324 and 337 nm, which are widely separated from those of the protein. The enzyme-induced spectral shifts of 17β-estradiol and 17β-dihydroequilenin observed at neutral pH resembled those obtained when the phenolic hydroxyl groups of these steroids were ionized in 0.3 M NaOH (Wang et al., 1963). These observations on the spectral changes of 17β-estradiol that occur on binding to isomerase were confirmed with the cloned wild-type enzyme (Figure 7A). As shown in Figure 7A, in 0.3 M NaOH the two principal absorption maxima of 17β-estradiol were red-shifted by 18 nm for the 220-nm peak and by 17 nm for the 278-nm peak. The two peaks were also intensified but only by 1.3- and 1.4-fold, respectively (Table III).

The effects of the three mutant enzymes on the spectrum of 17β-estradiol were also examined. The results paralleled those in which the 19-nortestosterone–enzyme complexes were studied. Both the D38N and the Y55F mutants caused spectral changes in 17β-estradiol similar to those induced by the wild-type enzyme (Figure 7B). The magnitudes of the red shifts of the absorption peaks are listed in Table III. In contrast, the Y14F–17β-estradiol complex had a spectrum essentially identical with that of 17β-estradiol alone. Since Y14F binds 17β-estradiol, as shown by competitive inhibition (Table IV), the absence of spectral change indicates that Y14F was incapable of ionizing the bound 17β-estradiol; presumably the hydroxyl oxygen of Tyr-14 is essential for removal of a proton from the 3-hydroxyl group of 17β-estradiol. The remarkable ability of Tyr-14 both to deprotonate the hydroxyl group of 17β-estradiol and, as discussed above, to protonate the carbonyl group of 19-nortestosterone (which differs by 12 pK_a units) may result from the proximity and unusually strong hydrogen-bonding interactions of Tyr-14 with the substituents at the 3-positions of each steroid.

Direct binding studies were also carried out on the three mutant enzymes by spectral titrations with 17β-estradiol (Figure 8) in order to obtain the dissociation constants (K_D) and the changes in extinction due to steroid binding, i.e., the $\Delta\epsilon_b$ values for the steroid–enzyme complexes (Table IV). Since 17β-estradiol is much less soluble in water than is 19-nortestosterone, an estimate of the asymptotic ΔA_{max} was obtained by plotting ΔA values against inhibitor concentration and calculating the limiting slope as $S_t \rightarrow 0$, which is equal to $\Delta\epsilon_b$, when the enzyme concentration exceeds the dissociation constant. The K_D value obtained for the complex between 17β-estradiol and the wild-type enzyme (0.8 $\pm$ 0.3 μM) was 5-fold lower than the K_D for Y55F and 8-fold greater than the K_D for D38N. Since negligible changes occurred in the spectrum of 17β-estradiol on complexation to Y14F, a K_D value could not be obtained. However, the K_i value of 43 $\pm$ 5 μM indicated that the steroid binding was 50-fold weaker to the Y14F mutant than to the wild-type enzyme. As observed in the titration experiments with 19-nortestosterone, the $\Delta\epsilon_b$ of bound 17β-estradiol varied significantly for each mutant enzyme. The $\Delta\epsilon_b$ value of 17 500 M^{-1} cm^{-1} at 238 nm obtained for D38N

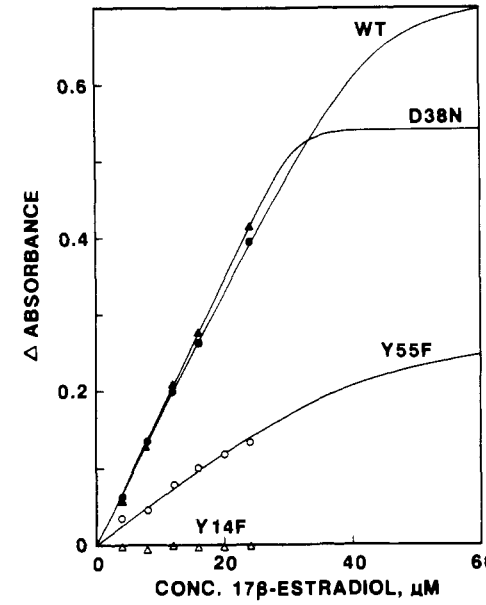


FIGURE 8: Ultraviolet spectral titrations measuring the direct binding of 17β-estradiol to wild-type and mutant isomerases. The absorption maxima for each titration were determined as described in the legend to Figure 6. The value used here was 238 nm in all cases. The theoretical titration curves were calculated with $n = 1$ and $K_D = 0.8$, 0.10, and 4.0 μM for wild-type, D38N, and Y55F, respectively (Table IV). An estimate of the asymptotic ΔA_{max} was obtained by plotting ΔA against 17β-estradiol concentration and calculating the limiting slope as the steroid concentration approaches zero, which is equivalent to $\Delta\epsilon_b$ when the enzyme concentration exceeds the K_D.

was nearly identical with that observed with the wild-type enzyme. The $\Delta\epsilon_b$ of 17β-estradiol bound to the Y55F mutant was 70% of that obtained with the wild-type enzyme. The $\Delta\epsilon_b$ values obtained for the 288–295-nm peak showed similar trends, with the $\Delta\epsilon_b$ value for D38N being 20% greater than the value for the wild-type enzyme and the $\Delta\epsilon_b$ for Y55F being only 50% of the wild-type value (Table IV).

Modeling of Substrate-Induced Conformation Changes at the Active Site of Isomerase. Our previous suggestion that Tyr-55 might be the proton donor was based on the fact that its phenolic hydroxyl group was oriented toward the bound steroid, as determined by docking of the spin-labeled substrate analogue spiro[doxyl-2,3′-5′α-androstan]-17′β-ol into the 2.5-Å X-ray structure of isomerase. Tyr-14, which is closer to the bound steroid, is not properly oriented (Figures 9 and 10) (Kuliopulos et al., 1987b). Further docking studies using the known X-ray structure of the substrate 5-androstene-3,17-dione (Carrell et al., 1978) indicate that, at the deepest possible penetration of the substrate into the active site, the distance from its 3-carbonyl oxygen to the hydroxyl oxygen of Tyr-55 (3.7 Å) precludes hydrogen bonding. This distance could not be decreased to the required 3 Å without major distortion of the protein backbone.

The present kinetic and ultraviolet spectral data strongly implicate Tyr-14 as the proton donor AH and Asp-38 as the proton acceptor B. The binding of the substrate must therefore induce a conformational change in the enzyme, reorienting Tyr-14 to permit its hydroxyl group to donate a hydrogen bond to the 3-carbonyl oxygen of the bound substrate. The minimal changes in enzyme conformation necessary to achieve such an interaction were investigated by molecular modeling using the MOGLI program (A. Dearing) on an Evans and Sutherland PS 330 computer graphics system. The substrate was positioned into the steroid binding site so as to maximize its overlap with the previously determined position of the spin-labeled steroid (Kuliopulos et al., 1987b) and rotated about its long axis to

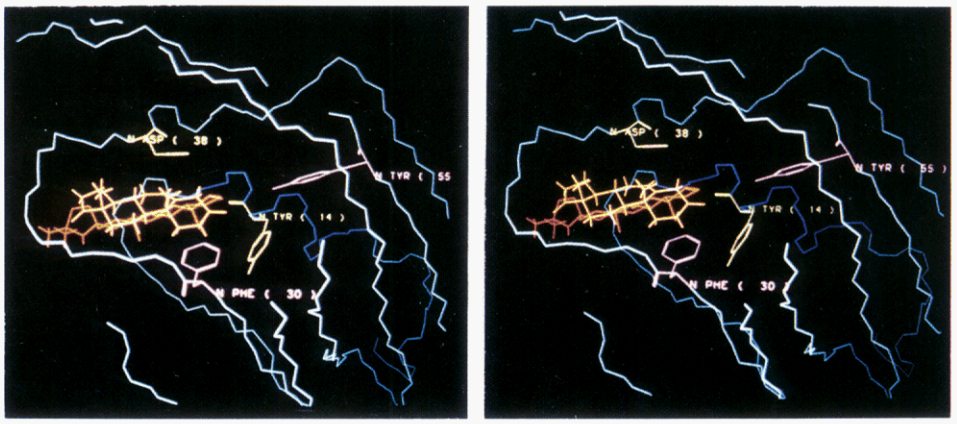


FIGURE 9: Steroid binding site of isomerase determined by NMR docking of the spin-labeled steroid spiro[doxyl-2,3′-5′α-androstan]-17′β-ol (orange) into the 2.5-Å X-ray crystal structure of the enzyme (Kuliopulos et al., 1987b). Superimposed onto the spin-labeled steroid is the substrate 5-androstene-3,17-dione (yellow) oriented with its A and D rings reversed.

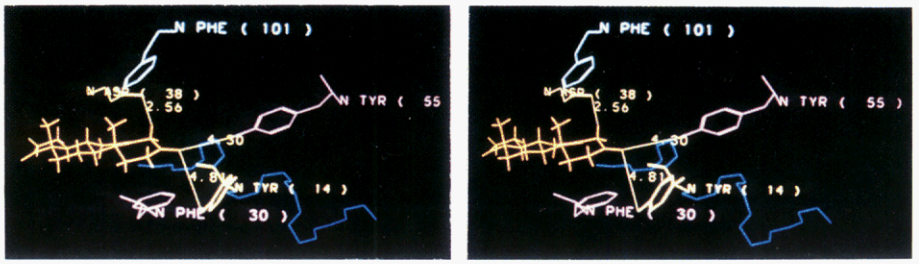


FIGURE 10: Details of the steroid binding site of isomerase showing initial distances between critical active-site residues and the optimally positioned substrate 5-androstene-3,17-dione. A carboxylate oxygen of Asp-38 (green) is in molecular contact (2.6 Å) with the 4β-proton of the substrate.

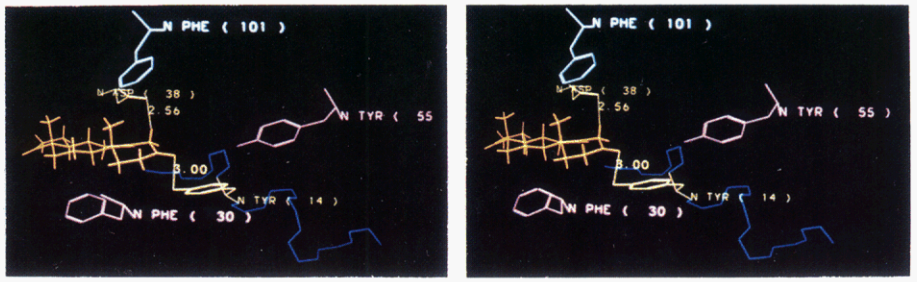


FIGURE 11: Active site of isomerase showing the minimal conformational change in the enzyme needed to explain the kinetic and ultraviolet spectroscopic results. Tyr-14 (green) has been rotated 21° about its Cα–Cβ bond to bring its hydroxyl oxygen into H-bonding distance (3.0 Å) from the 3-carbonyl oxygen of the substrate. In order to accommodate this movement of Tyr-14, Phe-30 (pink) has been rotated 92° about its Cα–Cβ bond and 36° about its Cβ–Cγ bond.

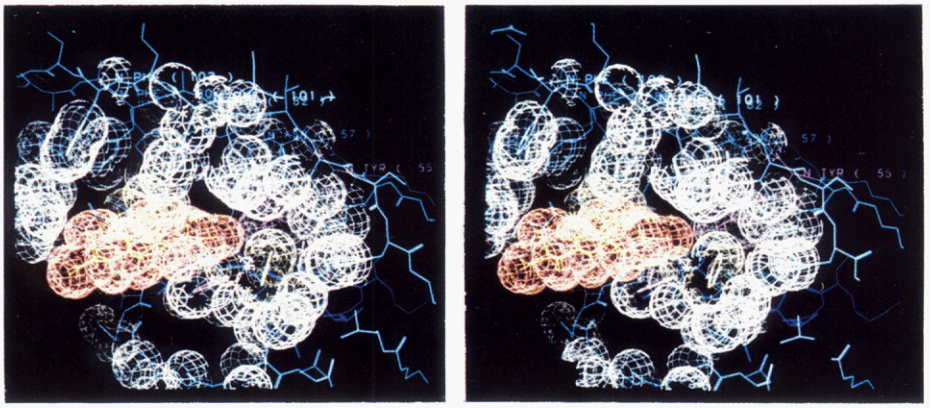


FIGURE 12: van der Waals surfaces of enzyme residues and substrate (yellow) in the conformationally altered form of isomerase (Figure 11) showing the accommodation of substrate by the enzyme.

permit molecular contact of the 4β-hydrogen with a carboxylate oxygen of Asp-38 (Figure 9). The side chain of Tyr-14 was then rotated by 21° about its Cα–Cβ bond, thereby decreasing the distance from the phenolic oxygen of Tyr-14 to the 3-carbonyl oxygen of the steroid from 4.8 to 3.0 Å, thus permitting hydrogen bonding (Figures 10 and 11). Such movement of Tyr-14 required a 92° rotation of Phe-30 about its Cα–Cβ bond and a 36° rotation about its Cβ–Cγ bond in order to avoid steric overlap with the newly positioned Tyr-14 ring. The altered position of Phe-30 was in a cavity below the

steroid substrate (Figure 11). This hydrogen-bonding distance could be further decreased without additional changes in the positions of other amino acid residues or the position of the substrate. This arrangement provides a binding site that accommodates the steroid without significant van der Waals overlap (Figure 12) and offers a possible explanation for the upfield shifts of five aromatic resonances of the enzyme that were observed on binding of 17β-estradiol (Benisek & Ogez, 1982) since these could arise from ring current effects of the aromatic A ring of 17β-estradiol on the resonances of Tyr-14, Phe-30, and Phe-101. Although these minimal spatial rearrangements seem plausible, they await conformation by X-ray diffraction and NOE studies of a binary complex of isomerase with a substrate analogue.

It is of mechanistic interest that the interaction of Tyr-14 with the carbonyl group of the substrate can occur only from the α-face, i.e., from below the steroid, while Asp-38 interacts with the 4β-hydrogen on the opposite β-face, i.e., above the steroid. Thus, in order to achieve hydrogen bonding to the substrate, Tyr-14 can be positioned only antarafacially with respect to Asp-38 in the enzyme–substrate complex.

Mechanism. Enolization of the substrate is a reasonable first step in the ketosteroid isomerase reaction since the overall reaction, a suprafacial 1,3 (actually 4β to 6β) proton transfer, cannot be concerted (Woodward & Hoffmann, 1970; Alder et al., 1971), and there is experimental evidence for such an intermediate [see review by Batzold et al. (1976)]. The present findings strongly support the roles of Tyr-14 as the proton donor AH and Asp-38 as the proton acceptor B in the isomerase reaction. Four crucial observations support the role of Tyr-14 as the general acid. First, mutation of this residue to phenylalanine decreased the k_{cat} of isomerase by a factor of $10^{4.7}$. Second, this mutation blocked the ability of isomerase to protonate the carbonyl group of 19-nortestosterone. Third, this mutation blocked the ability of isomerase to deprotonate the hydroxyl group of 17β-estradiol. Fourth, the antarafacial position of Tyr-14, with respect to Asp-38 in the enzyme–substrate complex, provides a stereoelectronically favorable arrangement for a concerted, trans enolization of the substrate (Figure 13). The favorable geometry for a concerted antarafacial enolization on isomerase does not, however, prove a concerted mechanism since the trans-elimination reactions catalyzed by enolase, aconitase, and fumarase proceed with carbanion intermediates (Hanson & Rose, 1975; Rose & Hanson, 1976; Dinovo & Boyer, 1971; Blanchard & Cleland, 1980; Porter & Bright, 1980; Schloss et al., 1980). Suffice it to say that, by arranging a trans enolization, nature has avoided steric or electronic barriers to the rate-limiting first step of the ketosteroid isomerase reaction. Such a geometry would also nicely explain the stereoselective alkylation of Asp-38 by 3β- but not by 3α-epoxysteroids (Bounds & Pollack, 1987). The failure of H_3O^+ to replace Tyr-14 as a proton donor is in accord with the low accessibility of the active site to solvent, as manifested by proton conservation in the overall reaction (Wang et al., 1963; Malhotra & Ringold, 1965).

The profound decrease of k_{cat} by a factor of $10^{5.6}$ upon mutation of Asp-38 to asparagine (Table I), together with the inhibitory effects of affinity labeling (Martyr & Benisek, 1973, 1975; Benisek et al., 1980, 1984; Hearne & Benisek, 1985; Pollack et al., 1979, 1986; Kayser et al., 1983; Bevins et al., 1980, 1984; Bounds & Pollack, 1987), and chemical modification of this residue (Benisek et al., 1980) indicate that Asp-38 is the proton acceptor in the isomerase reaction. The inability of OH^- to replace Asp-38 is also consistent with the low solvent accessibility of the active site. The ability of the

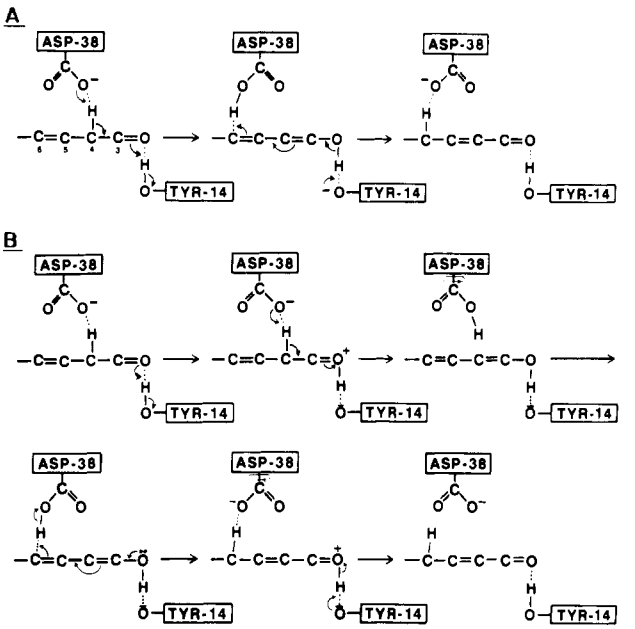


FIGURE 13: Alternative concerted (A) and stepwise (B) mechanisms of isomerase showing the roles of antarafacially located general acid Tyr-14 and general base Asp-38 in effecting enolization and proton transfer, respectively. Partial structures of 5- and 4-androstene-3,17-dione are shown, consisting of C-3, C-4, C-5, and C-6.

catalytically impaired D38N mutant to protonate 19-nortestosterone and to deprotonate 17β-estradiol suggests that the functioning of Tyr-14 does not require the prior deprotonation of the substrate by Asp-38. Hence, if the enolization of substrate is nonconcerted, protonation by Tyr-14 probably precedes deprotonation by Asp-38, which would yield an oxycarbonium ion rather than a carbanion intermediate. An oxycarbonium intermediate could be stabilized by both Asp-38 and the negative end of the dipole of the α-helix which extends from residues 8 through 17. A carbanion intermediate is highly unlikely, as indicated by the inhibitory properties of both the 2,4-dinitro and the 2,4-dibromo derivatives of 17β-estradiol. Thus the K_i of the dinitro derivative increased from 11 to 54 μM when the pH was increased from 5.4 to 6.0 (pK_a = 4.45), and the K_i of the dibromo derivative increased from 8 to 26 μM on increasing the pH from 6.0 to 8.1 (pK_a = 7.81). Analysis of these results suggests little or no inhibition by the anionic species.[3] Hence, the mechanism of the initial enolization is either concerted or stepwise, with carbonyl protonation preceding deprotonation at C-4.

The ketonization of this dienol intermediate to yield the product is likely to be stepwise rather than concerted. The reason is that the phenolate of Tyr-14 is the most likely proton acceptor, and its antarafacial location with respect to Asp-38 is stereoelectronically unfavorable for such a concerted 1,5 process. We considered the possibility that the phenolate of Tyr-55, which is suprafacially positioned with respect to Asp-38, could deprotonate the enol in concert with protonation of the steroid at C-6 and that this step becomes partially rate limiting in the Y55F mutant. However, Tyr-55 appears to be too far from the enol to interact, and the Y55F mutant retains the ability to deprotonate 17β-estradiol. Hence, a reasonable mechanism for the ketonization of the dienol intermediate and the formation of product involves prior protonation of C-6 of the steroid by Asp-38 to form a cationic enol, followed by deprotonation of this species by Tyr-14, thereby avoiding a carbanion intermediate.

[3] A. Kuliopulos and L. Xue, preliminary observations.

Finally, it should be noted that the product of the factors by which k_{cat} has been decreased in the Y14F and D38N mutants ($10^{10.3}$) is comparable to the overall rate acceleration produced by the parent enzyme ($10^{9.5}$). Hence, the mechanism involving general acid–base catalysis by Tyr-14 and Asp-38 in a hydrophobic environment may offer a quantitative as well as qualitative description of catalysis by this enzyme.

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Registry No. Asp, 56-84-8; Tyr, 60-18-4; Asn, 70-47-3; Phe, 63-91-2; isomerase, 9031-36-1; 19-nortestosterone, 434-22-0; 17β-estradiol, 50-28-2; 5-androstene-3,17-dione, 571-36-8.

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