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Stereochemistry of the Concerted Enolization Catalyzed by Δ^5-3-Ketosteroid Isomerase†

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ABSTRACT: The reaction catalyzed by Δ^5-3-ketosteroid isomerase has been shown to occur via the concerted enolization of the Δ^5-3-ketosteroid substrate to form a dienolic intermediate, brought about by Tyr-14, which hydrogen bonds to and protonates the 3-keto group, and Asp-38, which removes and axial (β) proton from C-4 of the substrate, in the same rate-limiting step [Xue, L., Talalay, P., & Mildvan, A. S. (1990) *Biochemistry 29*, 7491-7500; Kuliopulos, A., Mildvan, A. S., Shortle, D., & Talalay, P. (1989) *Biochemistry 26*, 3927-3937]. Since the axial C-4 proton is removed by Asp-38 from above the substrate, a determination of the complete stereochemistry of this rapid, concerted enolization requires information on the direction of approach of Tyr-14 to the enzyme-bound steroid. The double mutant enzyme, Y55F + Y88F, which retains Tyr-14 as the sole Tyr residue, was prepared and showed only a 4.5-fold decrease in k_{cat} (12 000 s^{-1}) and a 3.6-fold decrease in K_M (94 μM) for Δ^5-androstene-3,17-dione, in comparison with the wild-type enzyme. Deuteration of the aromatic rings of the 10 Phe residues further facilitated the assignment of the aromatic proton resonances of Tyr-14 in the 600-MHz TOCSY spectrum at 6.66 ± 0.01 ppm (3,5H) and at 6.82 ± 0.01 ppm (2,6H). Variation of the pH from 4.9 to 10.9 did not alter these shifts, indicating that the pK_a of Tyr-14 exceeds 10.9. Resonances assigned to the three His residues titrated with pK_a values very similar to those found with the wild-type enzyme. The binding of 19-nortestosterone, a product analogue and substrate of the reverse isomerase reaction, induced downfield shifts of -0.12 and -0.06 ppm of the 3,5- and 2,6-proton resonances of Tyr-14, respectively, possibly due to deshielding by the 3-keto group of the steroid, but also induced +0.29 to -0.41 ppm changes in the chemical shifts of 8 of the 10 Phe residues and smaller changes in 10 of the 12 ring-shifted methyl resonances, indicating a steroid-induced conformation change in the enzyme. NOESY spectra in H_2O revealed strong negative Overhauser effects from the 3,5-proton resonance of Tyr-14 to the overlapping 2α-, 2β-, or 6β-proton resonances of the bound steroid but no NOE's to the 4- or 6α-protons of the steroid. These observations indicate the NOE's to be from Tyr-14 to the 2α- and 2β-protons rather than to the 6β-proton of the steroid. Prolonged incubation of the enzyme–steroid complex in ^2H_2O resulted in deuteration of the 6β-, 4-, and 2α-positions of the steroid. The remaining 2β-proton retained 13% of the original NOE from Tyr-14, indicating that 87% of the NOE was to the 2α-proton. These observations are consistent with a specific and asymmetric orientation of Tyr-14 with respect to the bound steroid that places to phenolic ring of Tyr-14 beside C-2 and far from C-4 and C-6 of the steroid. This orientation permits Tyr-14 to form a hydrogen bond to the lone electron pair of the carbonyl oxygen of the steroid product that is trans to the C-4–C-5 double bond. On the basis of the geometry of this product complex and the fact that the axial C-4 β-proton of the substrate is removed by Asp-38 from above the steroid, we conclude that an orthogonal (rather than suprafacial or antarafacial) arrangement of the proton donor and proton acceptor, with respect to the bound substrate, is stereoelectronically appropriate for a rapid, concerted enolization.

The enzyme Δ^5-3-ketosteroid isomerase of *Pseudomonas testosteroni* catalyzes the isomerization of Δ^5-3-ketosteroids to Δ^4-3-ketosteroids by a stereospecific and conservative transfer of the 4β-proton to the 6β-position by way of an enolic intermediate (Batzold et al., 1976; Bantia & Pollack, 1986; Eames et al., 1990) (Figure 1). Primary, secondary, and combined deuterium kinetic isotope effects have established the enzyme-catalyzed enolization of the bound substrate to be concerted and rate limiting (Xue et al., 1990). The high velocity of this concerted enolization of 54 000 s^{-1} is brought about by the combined actions of Tyr-14, which protonates the 3-keto group, and Asp-38, which removes the axial (β) proton from C-4 of the substrate. Indeed, as revealed by the additive effects on catalysis of mutating Tyr-14 and Asp-38, the total catalytic power of the enzyme can be accounted for by general acid–base catalysis by these two residues in the concerted and rate-limiting enolization step of the isomerase reaction (Kuliopulos et al., 1989, 1990a).

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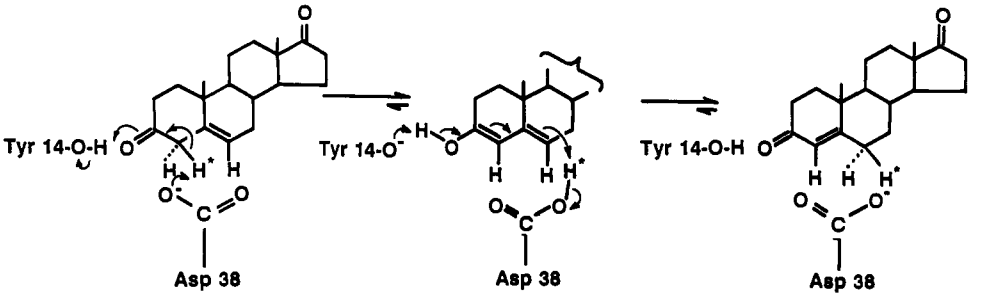

FIGURE 1: Mechanism of ketosteroid isomerase of *Ps. testosteroni* catalyzed conversion of Δ^5-3-ketosteroids to Δ^4-3-ketosteroids. In the forward reaction, Asp-38 deprotonates the steroid at the 4β-position and Tyr-14 protonates the 3-keto oxygen to produce the $\Delta^{3,5}$-dienol intermediate. Reketonization occurs by protonation at the 6β-position by Asp-38 and deprotonation of the oxygen at C3 by Tyr-14 to form the conjugated Δ^4-3-ketosteroid.

The rate equations of simple acid- and base-catalyzed enolization reactions contain a third-order term, indicative of a concerted process (Hand & Jencks, 1975). While in sterically constrained ketones the removal of the axial proton is strongly favored stereoelectronically (Corey & Sneen, 1956; Deslongchamps, 1983), the complete stereochemistry of such simple enolization reactions cannot be determined because of the lability of the enolic proton, although attempts are being made to construct geometrically constrained catalysts of this process (Rebek, 1988). The enzyme ketosteroid isomerase thus offers a unique opportunity to investigate the complete stereochemistry of a concerted and highly efficient enolization reaction. The detection of a tunneling contribution in the transfer of the axial 4β-proton from the substrate to Asp-38 during the enzyme-catalyzed enolization reaction requires a narrow barrier width, i.e., a close proximity of Asp-38 to the 4β-proton (Xue et al., 1990). Also detected was hydrogen bonding of Tyr-14 to the 3-keto group of 19-nortestosterone, on the basis of a red shift in the UV spectrum of the steroid bound to the wild-type enzyme and the absence of this red shift with the Y14F mutant (Kuliopulos et al., 1989). Model-building studies based on NMR docking of a spin-labeled steroid into the 2.5-Å X-ray structure of the enzyme indicate that, with a carboxylate oxygen of Asp-38 in direct contact with the 4β-proton of the bound substrate, Tyr-14 must move in order to donate a hydrogen bond to the 3-keto oxygen; i.e., a substrate-induced conformation change is required that alters the position of Tyr-14. This conformation change would permit Tyr-14 to approach the substrate most favorably, with minimal distortion of the enzyme, orthogonally with respect to Asp-38, somewhat less favorably in an antarafacial orientation, and least favorably in a suprafacial orientation due to steric interactions with neighboring residues (Figure 2; Kuliopulos et al., 1989; Xue et al., 1990). Because of the requirement for a substrate-induced conformation change, none of the limiting geometries in Figure 2 can be totally excluded by model building. The orthogonal arrangement of the catalytic residues with respect to the substrate would permit Tyr-14 to protonate a lone electron pair of the carbonyl oxygen, while the antarafacial and suprafacial orientations would allow protonation of the π electron pair. A distinction among the alternative geometries of Figure 2 is therefore of considerable theoretical interest.

The present ^{1}H NMR studies make use of the Y55F + Y88F double mutant of the isomerase in which the only remaining tyrosine residue is Tyr-14. The aromatic region of the NMR spectrum is further simplified by deuteration of the aromatic hydrogens of the ten phenylalanine residues, facilitating the assignment of the proton resonances of Tyr-14. Proximities between these protons and those of 19-nortestosterone, a product of the isomerase reaction, were obtained by

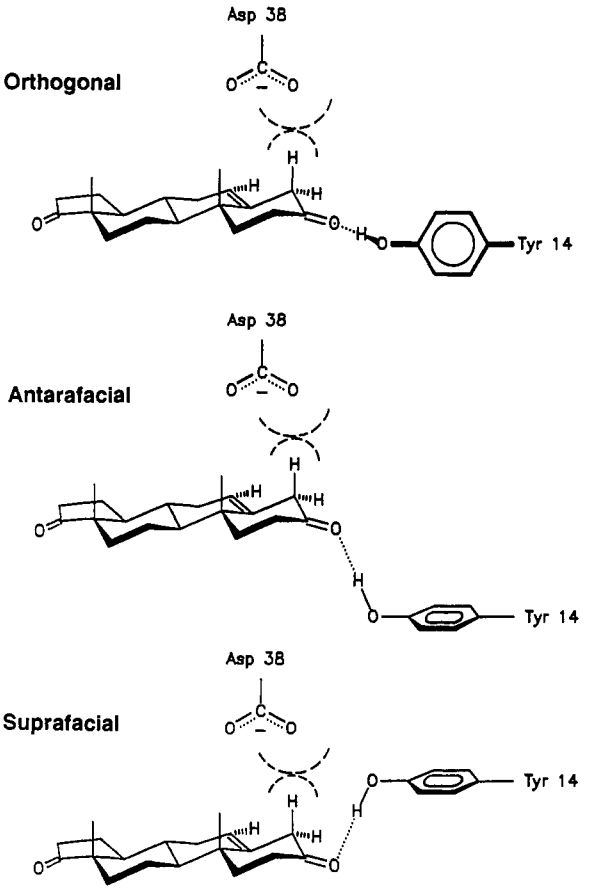

FIGURE 2: Three limiting orientations of Tyr-14 with respect to the bound substrate, based on NMR docking of the steroid into the 2.5-Å X-ray structure of isomerase (Kuliopulos et al., 1987b) and model building (Kuliopulos et al., 1989; Xue et al., 1990).

nuclear Overhauser effect spectroscopy. In assigning the resonances of the protons of 19-nortestosterone that were spatially near those of Tyr-14, advantage was taken of the fact that the isomerase promotes hydrogen exchange between ^2H_2O and protons of Δ^4-3-ketosteroid reaction products, including 19-nortestosterone, reflecting reversibility of the isomerase reaction (Wang et al., 1963). The proximities detected between protons of Tyr-14 and those of 19-nortestosterone are consistent with the orthogonal arrangement of the catalytic residues and argue against the alternative orientations (Figure 2).

Experimental Procedures

Materials

Phenylalanine, 98% enriched with ^{2}H in the ring (ring-D_5), was purchased from Cambridge Isotope Laboratories (Wo-

burn, MA). 19-Nortestosterone was purchased from Stearaloids, Inc., Wilton, NH, and purified by vacuum sublimation. Amino acids, niacin, and thiamin were obtained from Sigma.

Methods

Construction and Purification of the Y55F + Y88F Double Mutant Isomerases. The methods used to construct the Y55F + Y88F double mutant are described elsewhere (Kuliopulos et al., 1987a, 1989, 1990a). The nondeuterated Y55F + Y88F isomerase was expressed in the *Escherichia coli* JM101 strain as previously described (Kuliopulos et al., 1989). The double mutant isomerase, with deuterated aromatic protons of phenylalanine (D_5F-Y55F + Y88F),[1] was produced from *E. coli* grown in media consisting of minimal media salts as described by Maniatis et al. (1982) supplemented with 100 μM $CaCl_2$, 1 mM $MgSO_4$, and 0.2% glucose (w/v) with the further addition of 1 mg of biotin, 1 mg of thiamin, 200 mg of each amino acid (except Phe), 100 mg of ^{2}H-enriched phenylalanine, 50 mg of ampicillin, and 240 mg of IPTG per liter of cell culture. Cells were harvested when an A_{600} of 1.5 was reached. The unenriched and the D_5F-Y55F + Y88F isomerases were purified as previously described (Kuliopulos et al., 1989, 1990a) and stored as crystalline suspensions in 27% saturated ammonium sulfate at 4 °C.

Synthesis of 19-Nortestosterone 17β-Hemisuccinate. To increase the solubility of 19-nortestosterone, without significantly altering its interaction with the isomerase, the 17β-hemisuccinate ester was prepared as described by Benson et al. (1974). Initially, 2 g of 19-nortestosterone and 6 g of succinic anhydride (recrystallized from acidic anhydride) were dissolved in 60 mL of pyridine that had been dried over NaOH and heated to 100 °C with stirring under argon. After 6 h, when the reaction was complete, the mixture was cooled and slowly poured into 150 mL of cold 6 M HCl with gentle stirring. The precipitate was allowed to stand for 30 min, then collected and washed with 300 mL of cold 0.1 M HCl, and dissolved in 50 mL of 0.2 M Na_3PO_4. The solution was decolorized with charcoal, filtered, and acidified slowly with 1 M HCl. The precipitate was collected and recrystallized twice from a mixture of 70% (v/v) ethanol and H_2O.

NMR Sample Preparation. The isomerase was prepared for the NMR experiments by passage through a PD-10 desalting column (Pharmacia) to remove the ammonium sulfate and exchanged into 5 mM Tris-d_{11}-HCl, pH* 7.5, in 99.95% ^2H_2O with the use of Centriprep-10 filter units (Amicon, Waltham, MA). Slowly exchanging amide NH protons were exchanged for deuterium as previously described (Benisek & Ogez, 1982; Kuliopulos et al., 1987b). The isomerase was stored as a lyophilized powder at -80 °C and dissolved prior to use in either 99.95% ^2H_2O or in distilled and deionized water that had been passed through Chelex 100. The final protein concentration was determined by the method of Goodwin and Morton (1946), which measured tyrosine concentration, assuming an absorption of 0.157 at 280 nm for a solution containing 1 mg/mL isomerase in a 1-cm path-length cuvette. Volumes were 400–600 μL, and enzyme samples were found to retain at least 85% of their activity after 7 days in the NMR spectrometer at 300 K. 19-Nortestosterone 17β-hemisuccinate was neutralized to pH 7.0 with a slight excess of NaOH, dried in Eppendorf tubes on a Speed Vac concentrator (Savant), and dissolved directly into the enzyme solution. In titration experiments, 6.00 mM 19-nortestosterone in 99.96% methanol-d_4 was added in 1–10-μL aliquots to the enzyme solution, while the 1D and 2D NMR spectra of the enzyme were monitored. After prolonged enzyme-catalyzed deuteration of 19-nortestosterone 17β-hemisuccinate to improve resolution, the steroid was separated from the isomerase by centrifugation through a Centricon C10 ultrafilter (Amicon Corp., Lexington, MA) with a porosity of 10000 Da, at 3000 rpm for 15 min in a Beckman GPR refrigerated centrifuge.

NMR Spectroscopy. All ^{1}H NMR experiments were performed at 600.13 MHz at 300 K on a Bruker AM-600 NMR spectrometer. The 1D NMR spectra of the enzyme were recorded by using a 90° pulse of 10-μs duration with quadrature detection, a sweep width of 8 kHz, 64K data points, 64–256 transients, and low-power presaturation of the H_2O signal (16 mW) or of the residual solvent resonance in ^2H_2O (0.63 mW). All 1D and 2D NMR studies of isomerase were carried out in 5 mM Tris-d_{11}-HCl at pH 7.66–7.75. For consistency, the pH titration of isomerase was performed in 5 mM Tris-d_{11}-HCl in the pH range 4.94–10.89 at 27 °C. The pH of the sample was measured directly in 5-mm NMR tubes with an Orion 3-mm pH probe and a Radiometer Model M84 pH meter. The pH of the sample was measured before and after each spectrum was acquired and found to vary by less than 0.02 pH unit. The observed chemical shifts of histidine resonances were fit to the Hill equation allowing the pK_a, the limiting chemical shifts of the protonated and neutral species, and the number of protons n to vary by utilizing a nonlinear least-squares program (Markley, 1975).

All 2D NMR spectra were acquired in the phase-sensitive mode by using time-proportional phase incrementation (TPPI) (Marion & Wüthrich, 1983) and processed on an Aspect 3000 computer. TOCSY experiments (Braunschweiler & Ernst, 1983) were performed by using the MLEV17 pulse sequence (Bax & Davis, 1985), and the solvent resonance was suppressed by presaturation at 5 mW. In the TOCSY experiments all pulses were generated with the decoupler. Fast switching was employed between the power levels used for saturating the water resonance and for subsequent pulses. The parameters for acquisition of TOCSY spectra included a 0.7-s relaxation delay, a 0.5-s acquisition time, a 26.1-ms mixing time, an 8064-Hz sweep width, 4096 time-domain data points in F_2, and 300–512 time domain data points in F_1. NOESY spectra (Jeener et al., 1979; Anil Kumar et al., 1980) were acquired at mixing times of 100 and 300 ms with presaturation of the water resonance by using pulses of 6.3 mW in H_2O and 0.63 mW in ^2H_2O during the relaxation delay and mixing time. Optimization of the receiver phase was performed to reduce base-line roll and to minimize phase corrections in F_2. The parameters for acquisition of NOESY spectra included a 1.0-s relaxation delay, a 0.4-s acquisition time, a 10000-Hz sweep width, 4096 time-domain data points in F_2, and 300–1024 time-domain data points in F_1. The 2D spectra were processed by Fourier transformation in F_2, and polynomial base-line correction in F_2, followed by Fourier transformation in F_1. All proton chemical shifts are reported with respect to external DSS.

Results and Discussion

Preparation and Properties of the D_5F-Y55F + Y88F and Y55F + Y88F Isomerases. To facilitate the assignment of the proton resonances of Tyr-14, the two other tyrosines of isomerase, Tyr-55 and Tyr-88, were mutated to phenylalanine. The DNA sequence of the Y55F + Y88F construct was verified and was found to contain no adventitious mutations. To further simplify the assignment of the ring protons of Tyr-14, the enzyme was prepared from *E. coli* JM101 that

[1] Abbreviations: D_5F-Y55F + Y88F, the isomerase double mutant Y55F + Y88F with deuterated aromatic hydrogens of all ten Phe residues; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; 19NTHS, 19-nortestosterone 17β-hemisuccinate; pH*, pH measured in ^2H_2O.

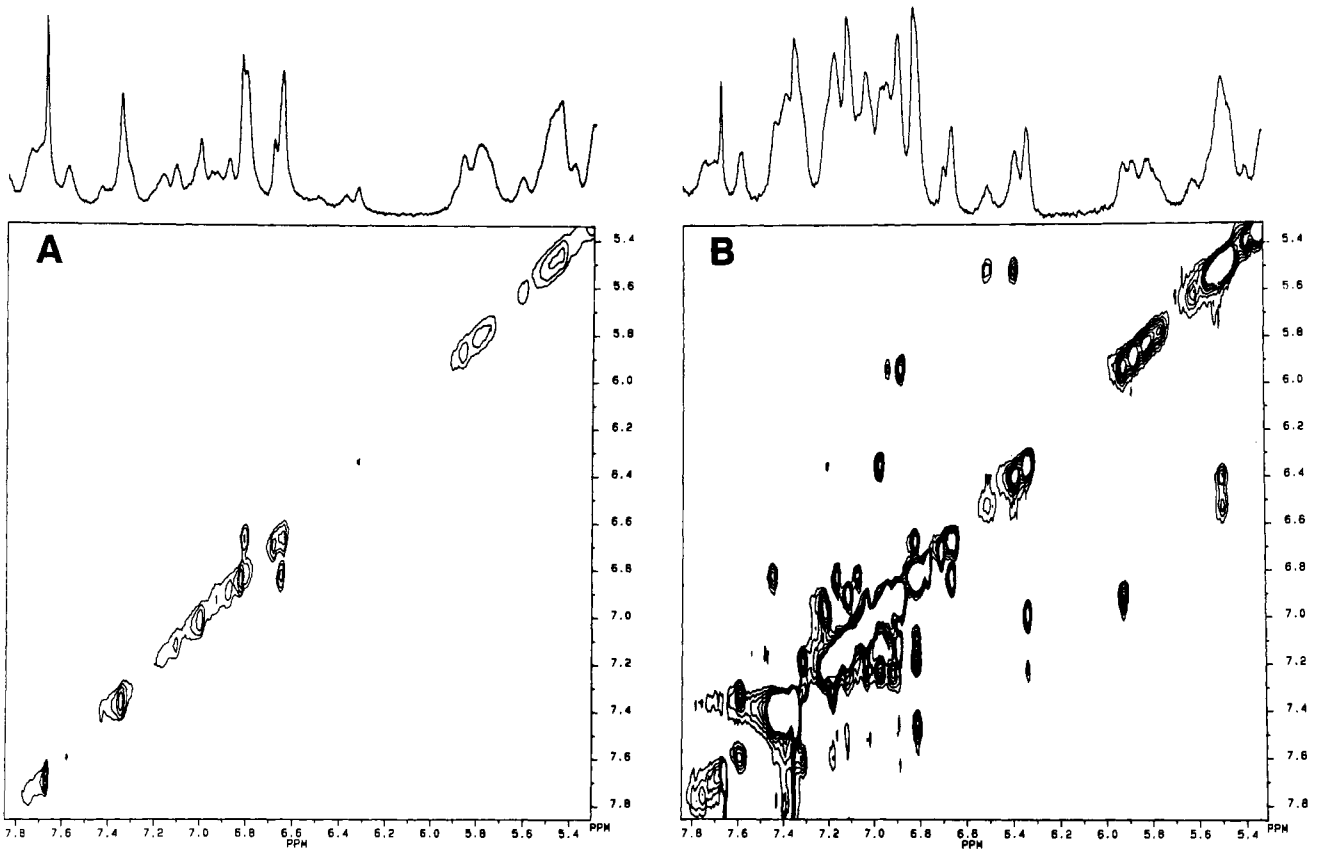

FIGURE 3: High-resolution ^1H NMR spectra of partially deuterated and fully protonated forms of the Y55F + Y88F double mutant of ketosteroid isomerase in ^2H_2O. (A) TOCSY and 1D spectra of D_5F-Y55F + Y88F isomerase (152 μM sites) in 5 mM Tris-d_{11}-HCl, pH* 7.66 and 99.95% ^2H_2O, 27 °C. The 1D spectrum was acquired at 600.1 MHz, with a pulse width of 10 μs, a sweep width of 8064.5 Hz, an acquisition time of 4.063 s, 320 transients, and presaturation of the residual HDO resonance by using a decoupler power of 5 mW. A line broadening of 1 Hz was applied to the FID in order to improve the signal-to-noise ratio. The TOCSY spectrum was recorded with 128 transients of 4K data points per t_1. (B) TOCSY and 1D spectra of nondeuterated Y55F + Y88F isomerase (184 μM sites). The conditions were identical with those in (A), except that the pH* was 7.77, the number of transients was 168, and a decoupler power of 1.3 mW was used for the 1D spectrum.

had been grown in media in which the aromatic ring protons of phenylalanine were deuterated. As had been previously shown (Torchia et al., 1990), it is not necessary to use strains of *E. coli* that are obligate auxotrophs for selected amino acids in order to incorporate isotopically labeled amino acids into the protein. When supplied with an abundance of a particular amino acid, strains of *E. coli* such as JM101 will preferentially use the exogenous amino acid rather than synthesize that amino acid de novo, especially in the case of the more complex amino acids such as phenylalanine. Thus, we were able to obtain 20 mg of pure isomerase per liter of media, 86% enriched in deuteriophenylalanine on the basis of the relative intensities of resolved proton resonances of Phe in 1D NMR spectra (vide infra). Both the deuterated (D_5F-Y55F + Y88F) and protonated (Y55F + Y88F) isomerases were recrystallized three times in order to ensure homogeneity. The recrystallized enzymes migrated as single bands in SDS–polyacrylamide gel electrophoresis stained with Coomassie Blue. The purified enzymes had identical specific activities of 22 600 μmol min^{-1} mg^{-1}, a k_{cat} of 12 000 s^{-1}, and a K_M of 94 ± 7 μM for the substrate Δ^5-androstene-3,17-dione. The value of k_{cat} is only 4.5-fold lower and that of K_M is 3.6-fold lower than the corresponding kinetic parameters of the wild-type enzyme (Kuliopulos et al., 1989), indicating that little damage has been done. The ultraviolet absorption spectrum of the Y55F + Y88F double mutant confirmed the absence of two of the three tyrosines normally found in wild-type enzyme, as indicated by the loss of 60% of the molar extinction of tyrosine absorbance at 279 nm relative to that of wild-type. Independent measurements of the absolute concentrations of the double mutant determined by the dry weight of the lyophilized enzyme were found to be within 16% of the values determined by tyrosine concentration.

^1H NMR Spectrum of the Double Mutant in ^2H_2O. 1D and 2D TOCSY spectra of the enzyme with the Phe rings deuterated (Figure 3A) are compared with those of the totally protonated enzyme (Figure 3B). In Figure 3A, the only resolvable connectivity in the aromatic region is due to coupling of the 3,5- with the 2,6-proton resonances of Tyr-14, since no Trp is present, the Phe's have been deuterated, and the aromatic protons of the three His residues have only very small couplings. From the positions of the cross peaks, the chemical shifts of the 3- and 5-proton resonances of Tyr-14 are at 6.66 ppm and those of the 2- and 6-proton resonances are at 6.82 ppm. The overlap of these pairs of resonances indicates that the ring of Tyr-14 is mobile or, less likely, that the magnetic environments of the symmetric pairs of protons are coincidentally identical. Similarly, in Figure 3B, the 10 Phe residues that are fully protonated each have only three resonances, indicating local mobility. Table I summarizes the chemical shifts of the Phe residues. Integration of the 1D spectra of D_5F-Y55F + Y88F isomerase and protonated Y55F + Y88F isomerase (Figure 3) indicates the Phe rings to be 86% deuterated in the former.

Increasing the pH in a titration from 4.9 to 10.9 had no effect on the chemical shifts of the ring resonances of Tyr-14, indicating that its pK_a value was greater than 10.9. This high pK_a value may well result from the hydrophobic environment of Tyr-14 (Kuliopulos et al., 1987b, 1990). The resonances previously assigned to the three histidine residues were titrated

Table I: Effect of 19-Nortestosterone on the Aromatic and Ring-Shifted Methyl Resonances of Y55F + Y88F Isomerase[a]

	enzyme alone[b]			+19-nortestosterone[c]					
resonance[d]	δ_a (ppm)	δ_b (ppm)	δ_c (ppm)	δ_a (ppm)	δ_b (ppm)	δ_c (ppm)	$\Delta\delta_a$ (ppm)	$\Delta\delta_b$ (ppm)	$\Delta\delta_c$ (ppm)
F1	7.582	7.314[e]	7.180	7.660	7.207[e]	7.148	−0.078	0.107	0.032
F2	7.206	6.972[e]	6.335	7.613	6.832[e]	6.435	−0.407	0.140	−0.100
F3	7.438	6.802		7.494	6.773		−0.056	0.029	
F4	7.432[e]	7.388	7.336	7.450[e]	7.390	7.337	−0.018	−0.002	−0.001
F5	7.218	7.059	6.920[e]	7.210	7.056	6.700[e]	0.008	0.003	0.220
F6	7.167	7.073	6.813[e]	7.163	7.000	6.794[e]	0.004	0.073	0.019
F7	7.133	7.117	6.893[e]	7.135	7.053	6.848[e]	−0.002	0.064	0.045
F8	6.946	6.875[e]	5.920	6.947	6.828[e]	5.635	−0.001	0.047	0.285
F9	6.514	6.398	5.491[e]	6.573	6.385	5.517[e]	−0.059	0.013	−0.026
Tyr-14	6.661[f]	6.821[g]		6.780[f]	6.882[g]		−0.119	−0.061	

ring-shifted methyl groups	δ_a (ppm) (enzyme alone)[b]	δ_a (ppm) (+19-nortestosterone)[c]	$\Delta\delta_a$ (ppm)	ring-shifted methyl groups	δ_a (ppm) (enzyme alone)[b]	δ_a (ppm) (+19-nortestosterone)[c]	$\Delta\delta_a$ (ppm)
M1	0.583	0.616	−0.033	M7	0.326	0.224	0.102
M2	0.568	0.439	0.129	M8	0.198	0.339	−0.141
M3	0.551	0.555	−0.004	M9	0.041	−0.043	0.084
M4	0.495	0.522	−0.027	M10	−0.156	−0.294	0.138
M5	0.454	0.410	0.044	M11	−0.387	−0.373	−0.014
M6	0.358	0.393	−0.035	M12	−0.502	−0.522	0.020

[a] Obtained at 27 °C in 5 mM tris-d_{11}-HCl and 99.95% ^2H_2O, pH* 7.77. [b] The Y55F + Y88F subunit concentration was 184 μM. [c] The Y55F + Y88F subunit concentration was 176 μM and the 19-nortestosterone concentration was 270 μM, conditions under which 98% of the enzyme was bound with steroid. This represents the end point of a stepwise titration with 19-nortestosterone, monitoring all of the chemical shift changes. [d] The phenylalanine ring protons were assigned according to a shared spin system as described in the text. The tenth phenylalanine (F10) could not be assigned. [e] Resonance arising from the ring 3,5-protons of phenylalanine, assigned on the basis of the shared cross peaks of the ring 3,5-protons with both the ring 2,6- and 4-protons in the TOCSY spectra. [f] Ortho 3,5-protons of tyrosine. [g] Meta 2,6-protons of tyrosine.

Table II: pH Parameters of the Histidine Resonances of the D_5F-Y55F + Y88F Isomerase[a]

	2-proton				4-proton	
His[b]	δ_{H^+}[c] (ppm)	δ_{OH^-}[c] (ppm)	pK_a	n_{H^+}[c]	δ_{H^+}[c] (ppm)	δ_{OH^-}[c] (ppm)
H1		7.35 ± 0.01	4.3 ± 0.2[d]	1.0 ± 0.2		7.01 ± 0.01
H2	8.75 ± 0.01	7.65 ± 0.01	5.83 ± 0.01	0.9 ± 0.1	6.82 ± 0.02	6.69 ± 0.01
H3	8.58 ± 0.01	7.70 ± 0.01	7.80 ± 0.01	1.0 ± 0.2	6.90 ± 0.02	6.70 ± 0.01

[a] Obtained at 27 °C, in 5 mM Tris-d_{11}-HCl and 99.95% ^2H_2O, in the pH* range of 4.94–10.89. The enzyme concentration was 200 μM in subunits. [b] Histidines are not unequivocally assigned. However, on the basis of docking a spin-labeled steroid into the active site of wild-type isomerase, H3, H2, and H1 [by the nomenclature of Benisek and Ogez (1982)] have been tentatively assigned to His-6, His-122, and His-100, respectively (Kuliopulos et al., 1987b). [c] δ_{H^+} and δ_{OH^-} are the limiting chemical shifts of the protonated and neutral species, respectively, and n_{H^+} is the Hill coefficient. The pH parameters of each resonance were fit to the Hill equation (Markley, 1975). [d] At pH 4.94 the resonances of H1 had only been partially titrated. Hence, the pK_a obtained for H1 can only be considered an estimate. At pH values below 4.94 the enzyme is rapidly denatured.

over this range of pH values with titration parameters (Table II) very similar to those previously described for the wild-type enzyme (Benisek & Ogez, 1982; Kuliopulos et al., 1987b), providing further evidence against significant structural changes in the double mutant enzyme.

Effect of 19-Nortestosterone on ^1H NMR Spectra of the Y55F + Y88F Double Mutant. Titration of the partially deuterated enzyme with 19-nortestosterone, an analogue of the product and a substrate of the reverse isomerase reaction, caused downfield shifts of -0.12 and -0.06 ppm of the 3,5- and the 2,6-proton resonances of Tyr-14, respectively (Figure 4A, Table I), possibly due to the direct deshielding effect of the 3-keto group that occurs in a conical region surrounding the carbonyl axis (Apsimon et al., 1970). If so, then the 3-keto group of the steroid is closer to the 3,5-protons and to the phenolic −OH of Tyr-14 than to the 2,6-protons. Figure 4B shows the end points in a titration of the fully protonated enzyme with 19-nortestosterone 17β-hemisuccinate, obtained by monitoring the TOCSY spectra throughout the titration. The binding of the steroid produced +0.29 to −0.41 ppm changes in the chemical shifts of 8 of the 10 Phe residues (Figure 4B, Table I) and smaller changes in the chemical shifts of 10 of the 12 ring-shifted methyl groups (Table I), indicating a steroid-induced conformation change in the enzyme. Such a conformation change, altering the environment of Tyr-14, would provide an alternative and indirect explanation for the small downfield shifts of the aromatic resonances of Tyr-14, induced by the binding of 19-nortestosterone. Evidence supporting the direct effect of the carbonyl group was provided by the nuclear Overhauser effect, which allowed the detection of proximities between protons of enzyme-bound 19-nortestosterone and those of Tyr-14 (vide infra).

The data of Figure 4A indicated the tight binding of 19-nortestosterone 17β-hemisuccinate to the enzyme, but the overall changes in chemical shift were too small for determination of the dissociation constant. Analysis of the effects of the steroid on the larger changes in chemical shifts of the Phe and ring-shifted methyl resonances (Figure 4B, Table I) yielded a stoichiometry of one binding site per enzyme monomer with a dissociation constant of 2.5 ± 1.5 μM for the enzyme–steroid complex. Because the enzyme concentration greatly exceeded the dissociation constant, the stoichiometry was confirmed by the observation of an end point in the NMR titration curve. These values are comparable to those found by spectroscopic titrations of the Y55F + Y88F double mutant enzyme and the Y55F single mutant enzyme with 19-nortestosterone (n = 1, K_d = 1.0 ± 0.2 μM; Kuliopulos et al., 1989, 1990b), which show slightly tighter binding of this steroid than found with the wild-type enzyme (n = 1, K_d = 5.5 ± 1.0 μM; Kuliopulos et al., 1989), presumably due to greater hydrophobicity at the active site of the mutant enzymes.

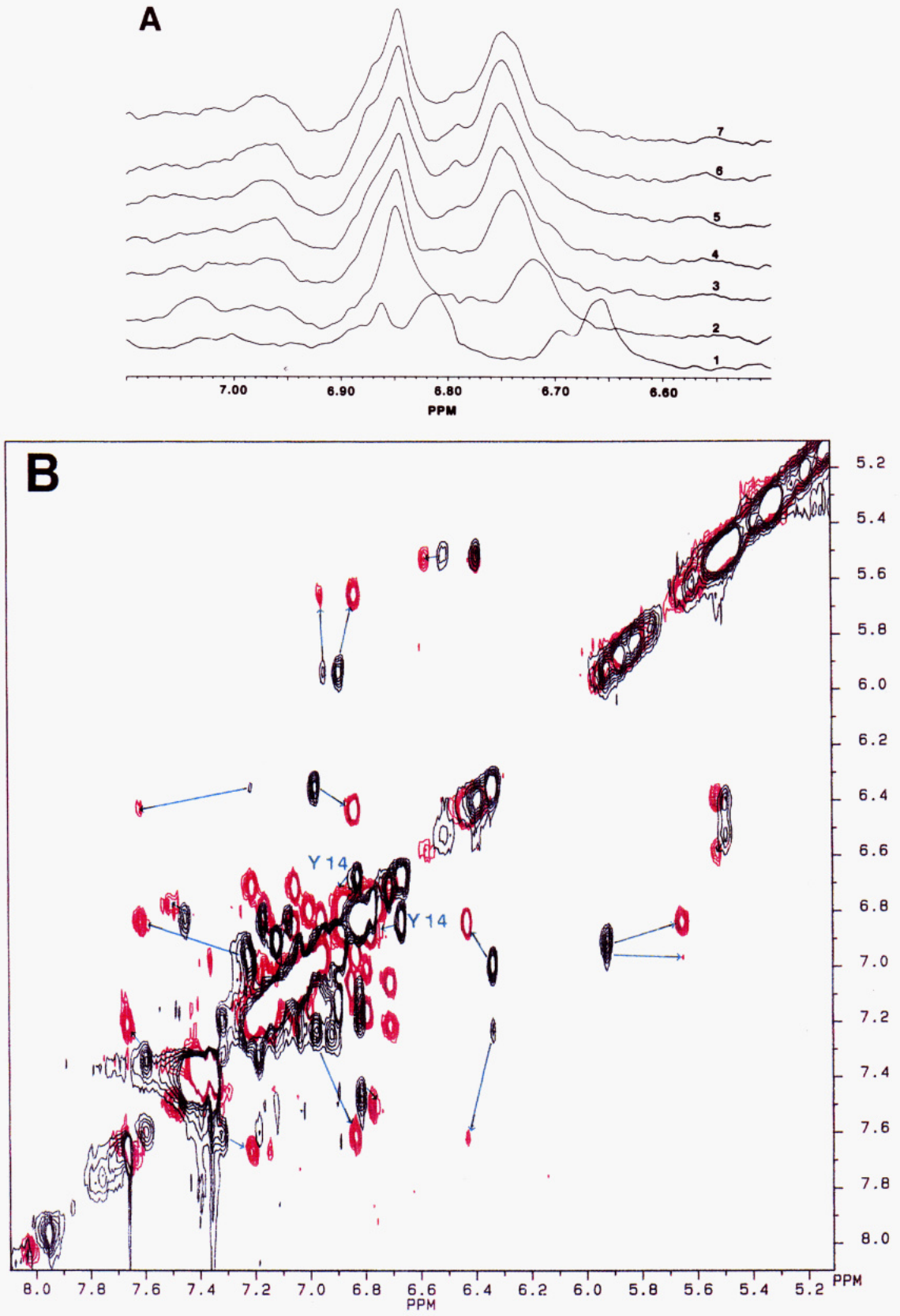

FIGURE 4: Effect of 19-nortestosterone 17β-hemisuccinate on the ^{1}H NMR spectrum of the Y55F + Y88F mutant of ketosteroid isomerase. (A) 19-Nortestosterone 17β-hemisuccinate titration of the D_5F-Y55F + Y88F mutant of isomerase showing effects on the chemical shifts of the aromatic proton resonances of Tyr-14. The conditions were identical with those listed in the legend to Figure 3A except that the pH* was 7.15, a line broadening of 5 Hz was applied, and 256 transients were used for each trace. Spectrum 1: 152 μM isomerase (KSI) sites alone. Spectrum 2: 152 μM (KSI) + 31 μM 19-nortestosterone 17β-hemisuccinate (19NTHS). Spectrum 3: 152 μM KSI + 63 μM 19NTHS. Spectrum 4: 151 μM KSI + 94 μM 19NTHS. Spectrum 5: 151 μM KSI + 125 μM 19NTHS. Spectrum 6: 150 μM KSI + 156 μM 19NTHS. Spectrum 7: 149 μM KSI + 309 μM 19NTHS. (B) Effect of 19-nortestosterone 17β-hemisuccinate on the aromatic resonances of Y55F + Y88F isomerase in the TOCSY spectrum. Black: enzyme alone under conditions described in the legend to Figure 3B. Red: enzyme (176 μM sites) together with 19-nortestosterone 17β-hemisuccinate (270 μM). Conditions were otherwise as described in the legend to Figure 3B. The green arrows connecting the resonances of unbound enzyme with those of the enzyme–steroid complex are based on a stepwise titration.

Table III: Chemical Shifts of Proton Resonances of 19-Nortestosterone 17β-Hemisuccinate in the Absence and Presence of Enzyme[a]

steroid proton	MeOH/H_2O[b]	solvent H_2O[c]	H_2O + enzyme[d]
1α	1.51	1.53	1.52
1β	2.29	2.24	2.25
2α	2.32	2.35	2.35
2β	2.32	2.35	2.35
4	5.85	5.87	5.88
6α	2.52	2.49	2.48
6β	2.36	2.33	2.35
7α	1.05	1.05	1.02
7β	1.87	1.85	1.83
8β	1.46	1.47	1.48
9α	0.86	0.90	0.88
10β	2.21	2.24	2.24
11α	1.83	1.80	1.79
11β	1.30	1.28	1.26
12α	1.17	1.16	1.14
12β	1.77	1.74	1.72
14α	1.10	1.11	1.09
15α	1.66	1.64	1.63
15β	1.38	1.37	1.36
16α	2.12	2.09	2.08
16β	1.55	1.55	1.54
17α	4.58	4.59	4.56
18-CH_3	0.87	0.85	0.83
21-CH_2	2.43	2.44	2.43
22-CH_2	2.56	2.56	2.54

[a] From DQF COSY and NOESY experiments. Shifts ± 0.01 ppm are from external DSS. [b] 15 mM 19-nortestosterone 17β-hemisuccinate in 50% methanol-d_4/50% ^2H_2O, pH* 7.09. [c] 2 mM 19-nortestosterone 17β-hemisuccinate in 90% H_2O/10% ^2H_2O and 5 mM Tris-d_{11}-HCl, pH 7.50. [d] 6.24 mM 19-nortestosterone 17β-hemisuccinate in the presence of 200 μM D_5F-Y55F + Y88F double mutant of isomerase. Other components as in footnote *c*.

Assignment of Proton Resonances of 19-Nortestosterone 17β-Hemisuccinate. Table III summarizes the assignments of the proton resonances of 19-nortestosterone 17β-hemisuccinate determined by 1D decoupling experiments as well as by 2D COSY, *J*-resolved, and NOESY spectroscopy. The NOESY spectrum (not shown) revealed weak, positive cross peaks indicating NOE's between protons of the steroid ≤3 Å apart.

2D NOESY Studies of the D_5F-Y55F + Y88F Double Mutant of Isomerase Complexed with 19-Nortestosterone in H_2O. The 2D NOESY spectrum of the partially deuterated enzyme (200 μM) complexed with 19-nortestosterone 17β-hemisuccinate (6.0 mM) shows strong negative cross peaks corresponding to the chemical shifts of the protons of 19-nortestosterone alone. These reflect intramolecular transferred NOE's of the enzyme-bound steroid, and the locations of these cross peaks confirm the chemical shifts of the resonances of 19-nortestosterone and indicate the absence of significant changes in chemical shifts due to the presence of the enzyme (Table III).

The NOESY spectrum also reveals a negative cross peak between the 3,5-proton resonance of Tyr-14 at 6.74 ppm and a resonance at 2.35 ppm assigned to the overlapping signals of the 2α-, 2β-, and 6β-protons of enzyme-bound 19-nortestosterone (Figure 5), reflecting proximity between Tyr-14 and one or more of these steroid protons. This cross peak is also detectable at a mixing time of 0.1 s and becomes ~3-fold stronger at a mixing time of 0.3 s, indicating approximate linearity, i.e., the absence of a lag in the development of the NOE, consistent with only a minimal contribution of spin diffusion. No cross peaks from the 2,6-proton resonances of Tyr-14 (6.89 ppm) to those of 19-nortestosterone are detected at 0.1 s, providing further evidence against spin diffusion. Moreover, no cross peaks are detected between the 3,5-protons

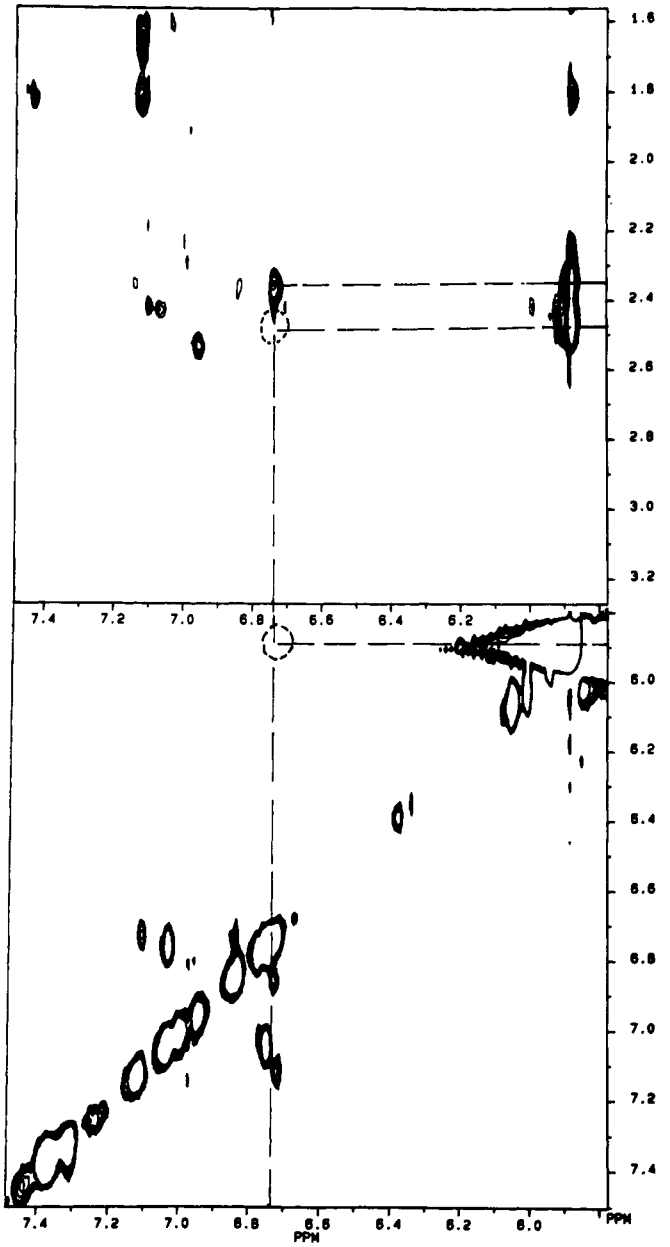

FIGURE 5: NOESY spectrum of D_5F-Y55F + Y88F isomerase complexed with 19-nortestosterone 17β-hemisuccinate. The isomerase concentration was 246 μM and 19NTHS was 7.39 mM (30-fold excess) in 5 mM Tris-d_{11}-HCl, pH 7.75, and 90% H_2O/10% ^2H_2O, at 27 °C. The mixing time was 300 ms and the acquisition time was 205 ms. A total of 256 transients of 4K data points per t_1 increment was used for a total of 416 t_1's. The water resonance was presaturated during the mixing time and relaxation delay (0.7 s) at 6.3 mW. Note the intermolecular NOE traced by the dashed lines from 6.74 ppm (3,5-protons of Tyr-14) to 2.35 ppm (2α-, 2β- and/or 6β-protons of 19NTHS) but none to 2.48 ppm (6α-proton of 19NTHS, dashed circle) or to 5.88 ppm (4-proton of 19NTHS, dashed circle).

of Tyr-14 and the 6α-proton (at 2.48 ppm) or the 4-proton (at 5.88 ppm) of bound 19-nortestosterone at a mixing time of 0.3 s (Figure 5). These observations argue against an NOE from Tyr-14 to the 6β-proton resonance, which underlies those of the 2α- and 2β-protons, and are consistent with a specific and asymmetric orientation of Tyr-14 with respect to the bound substrate analogue. This orientation places the phenolic ring of Tyr-14 *beside* the 2-position of the steroid and far from the 4- and 6-positions, permitting hydrogen bonding of the phenolic OH to the lone electron pair of the carbonyl oxygen, which is trans to the C-4–C-5 double bond, and also explains the steroid-induced chemical shift changes of the resonances

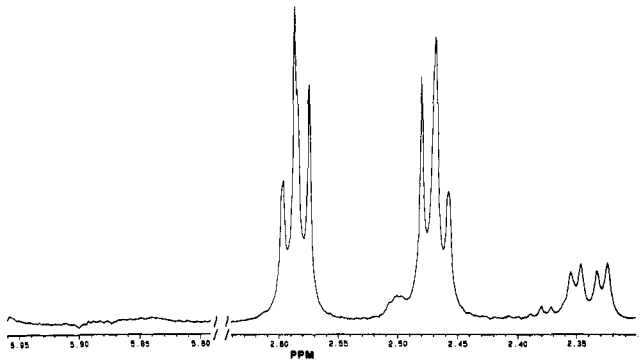

FIGURE 6: Portion of the 1D ^1H NMR spectrum of 19NTHS after 200 h in ^2H_2O in the presence of D_5F-Y55F + Y88F. The enzyme was removed by ultrafiltration to improve resolution. Conditions are otherwise as given the legend to Figure 5. The residual signal at 2.34 ppm is assigned to the 2β-proton (see text), and the succinate methylene protons at 2.47 and 2.58 ppm were used as intensity standards. Note the absence of the resonance at 5.87 ± 0.02 ppm indicating deuteration at the 4-position of the steroid.

of Tyr-14 (Table I). The absence of NOE's from the 3,5-proton resonances of Tyr-14 to the 4-proton and the 6α-proton of the steroid rules out a position for the phenolic ring symmetrically placed *below* or *above* the A-ring of the steroid where it might hydrogen bond to the π electrons. More detailed information on this enzyme–substrate interaction is provided in a subsequent section.

1D and 2D NMR Studies of the Y55F + Y88F Double Mutant of Isomerase Complexed with 19-Nortestosterone in ^2H_2O. On dissolving a sample of the protonated Y55F + Y88F double mutant enzyme (200 μM) and 19-nortestosterone 17β-hemisuccinate (6.0 mM) in ^2H_2O, the 4-proton resonance (at 5.88 ppm) and the 6β-proton resonance (at 2.35 ppm) became largely deuterated within an hour. Deuteration of the 6β-proton resonance was confirmed by the loss of a cross peak in the NOESY spectrum between the 6β-resonance and the adjacent 8β-proton resonance at 1.48 ppm (not shown). No deuteration of the 6α-proton resonance at 2.48 ppm occurred after 45 and 90 h, with two samples, a finding confirmed by the persistence in the NOESY spectrum of the cross peak with the 7α-proton resonance at 1.02 ppm and the 7β-proton resonance at 1.83 ppm. Similarly, no deuteration of the 10β-proton resonance at 2.24 ppm was noted, on the basis of the persistence in the NOESY spectrum of its cross peak with the 11α-proton resonance at 1.79 ppm, and no other protons of 19-nortestosterone were deuterated. After 200 h in ^2H_2O, the entire 2α- and 6α-proton resonances and ~30% of the 2β-proton resonance at 2.35 ppm became deuterated.[2] The residual resonance at 2.35 ± 0.01 ppm, constituting 0.7 proton, was assigned to the 2β-proton on the basis of its large coupling to the axial proton resonance at position 1 (J = 13.0 ± 0.5 Hz) and its smaller coupling to the equatorial proton at position 1 (J = 5.2 ± 0.5 Hz) (Figure 6).

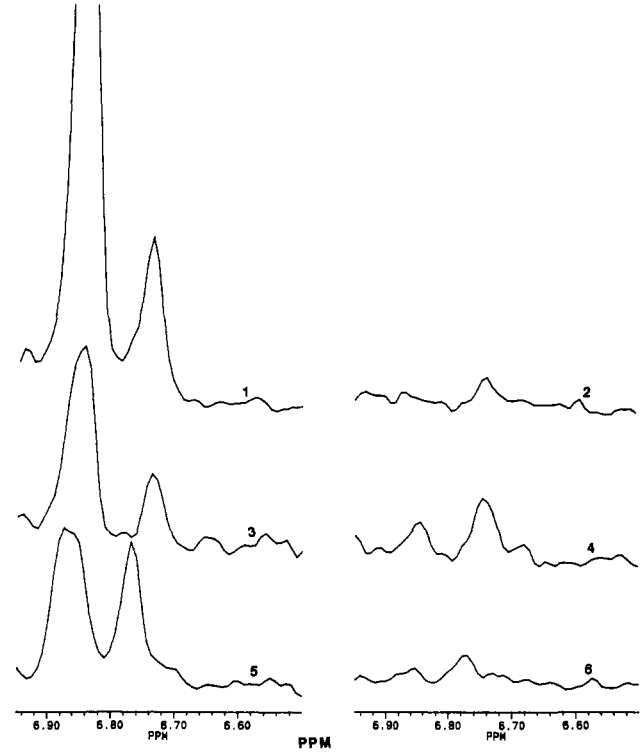

FIGURE 7: Quantitation of the nuclear Overhauser effects from the 3,5-protons of Tyr-14 to the 2α- and 2β-protons of 19-nortestosterone 17β-hemisuccinate. Spectrum 1: NOESY in H_2O at a mixing time of 100 ms. Slice from 2,6-protons of Tyr-14 at 6.86 ± 0.01 ppm showing an NOE to 3,5-protons of Tyr-14 at 6.75 ± 0.01 ppm, which was used as a distance standard. Spectrum 2: NOESY in H_2O at mixing time of 100 ms. Slice from 2α-, 2β- and 6β-resonances of 19NTHS at 2.35 ± 0.01 ppm showing NOE to 3,5-protons of Tyr-14 at 6.75 ± 0.01 ppm, which was used to measure distance. Spectrum 3: as in spectrum 1 but with a mixing time of 300 ms. Spectrum 4: as in spectrum 2 but with a mixing time of 300 ms. Spectrum 5: NOESY in ^2H_2O at a mixing time of 300 ms. Otherwise as in spectrum 1. Spectrum 6: NOESY in ^2H_2O at a mixing time of 300 ms. Slice from residual 2β-proton resonance of 19NTHS at 2.35 ± 0.01 ppm used to measure distance. Conditions were as described in the legends to Figures 5 and 6, except that spectral smoothing was used to facilitate integration by the cutting and weighing of peaks. The large range in relative magnitudes of the NOE's leads to an error of only ±0.3 Å from these data.

Interproton Distances between Tyr-14 and 19-Nortestosterone 17β-Hemisuccinate. Although 70% of the 2β-proton resonance remained at 2.35 ppm after 200 h in ^2H_2O, the intermolecular NOE between this resonance and the 3,5-proton resonance of Tyr-14 (at 6.74 ppm) was attenuated to 9.4% of its original intensity (Figure 7). Correcting for the partial deuteration, this result indicates that 13% of the original NOE from Tyr-14 was to the 2β-proton and 87% was to the 2α-proton of the bound steroid. Since NOE intensities are inversely related to the sixth power of the interproton distances, this result implies that a 3- (or 5-) proton of Tyr-14 is $(0.87/0.13)^{1/6}$ or 1.37 ± 0.07-fold farther from the 2β-proton than from the 2α-proton.

This ratio of distances, which is more accurately determined than absolute interproton distances, was used, together with three internal standards, to estimate the absolute distances from the 3- (or 5-) proton of Tyr-14 to the 2α- and 2β-protons of enzyme-bound 19-nortestosterone, on the basis of the intensities of the cross peaks in the NOESY spectra obtained at mixing times of 0.1 and 0.3 s (Figure 7). The internal standards were the intensities of the NOE's between the vicinal 3,5- and 2,6-protons of Tyr-14 (Figure 7) at a distance of 2.35

[2] The slow deuteration of the 6β-position of 19-nortestosterone 17β-hemisuccinate probably occurs by multiple reversals of the isomerase reaction, permitting slow exchange with solvent (Wang et al., 1963). The slow deuteration at the 4-position requires multiple reversals and incomplete stereospecificity in the abstraction of the 4β-proton in the forward isomerase reaction, as previously reported (Viger et al., 1981). The very slow deuteration at the 6α-position requires multiple reversals and incomplete stereospecificity at the 6-position as previously described (Viger et al., 1981). The very slow deuteration at the equatorial 2α-position may result from the fact that the A-ring of 19-nortestosterone can exist in two conformations that interconvert the equatorial and axial positions at C-2 (Precigoux et al., 1975), together with the possibility of occasional inverted binding of the steroid to isomerase (Bevins et al., 1984, 1986). The lack of detectable exchange of the 10β-proton argues against the formation of the Δ^{5-10}-3-ketosteroid.

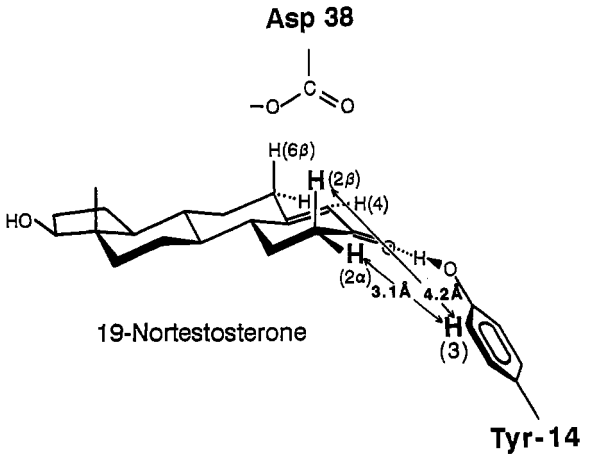

FIGURE 8: Position of Tyr-14 with respect to enzyme-bound 19-nortestosterone consistent with interproton distances calculated from NOE's and with hydrogen bonding to the 3-keto group detected by UV spectroscopy (Kuliopulos et al., 1989).

± 0.04 Å as found in the X-ray structure of *N*-acetyltyrosine (Koszelak & van der Helm, 1981) and between the geminal 7α- and 7β-, and 16α- and 16β-, protons of 19-nortestosterone 17β-hemisuccinate at distances of 1.75 ± 0.03 Å based on neutron diffraction studies of geminal protons (Fung et al., 1976). The distances from the 3- (or 5-) proton of Tyr-14 to the 2α- and 2β-protons of the bound steroid were thus estimated as 3.1 ± 1.0 and 4.2 ± 1.0 Å, respectively, and, within error, were independent of mixing time, arguing against significant effects of spin diffusion.

These distances, together with the previously detected hydrogen bonding of Tyr-14 to the 3-keto group of 19-nortestosterone, on the basis of the red shift in the UV spectrum of the steroid bound to the wild-type enzyme and the absence of this red shift on the Y14F mutant (Kuliopulos et al., 1989), were used to position Tyr-14 near the bound steroid. We make the simplest limiting assumption that the closest approach of one of the 3,5 pair of protons of Tyr-14 gives rise to the observed NOE, while the other proton is too far away to contribute (≥5.5 Å). By symmetry, a 180° ring flip of Tyr-14 reverses their respective positions. Thus for half of the time the 3-proton is near the substrate, and for half of the time the 5-proton is near the substrate. The calculated root-mean-sixth average distance represents the distance of closest approach of either proton, yielding the orthogonal arrangement shown in Figure 8. This assumption fits the observed NOE's to the 2α- and 2β-protons of the steroid and the absence of NOE's to the 4- and 6α-protons.

The opposite limiting assumption is that both the 3- and 5-protons of Tyr-14 are simultaneously near enough to the bound steroid to contribute equally to the observed NOE's. This assumption requires that the measured average distances be increased by 12% to yield the individual distances and that the ring flip interconverts but does not alter these distances. Model building based on this alternative assumption leads to structures with relative distances well beyond the measured ratio and predicts NOE's from the 3,5-protons of Tyr-14 to H4 and to H1α of the steroid that are not observed. The arrangement of Tyr-14 and the substrate shown in Figure 8 is not very sensitive to the sizable range of absolute interproton distances and places the 3,5-protons of Tyr-14 ≥5.5 Å from the 4- and 6α-protons of the steroid and places the 2,6-proton of Tyr-14 ≥5.5 Å from the 2α- and 2β-protons of the steroid, consistent with the absence of primary, intermolecular NOE's between these protons. The absence of NOE's from Tyr-14 to the 1α-, 4-, and 6α-protons of 19-nortestosterone rules out

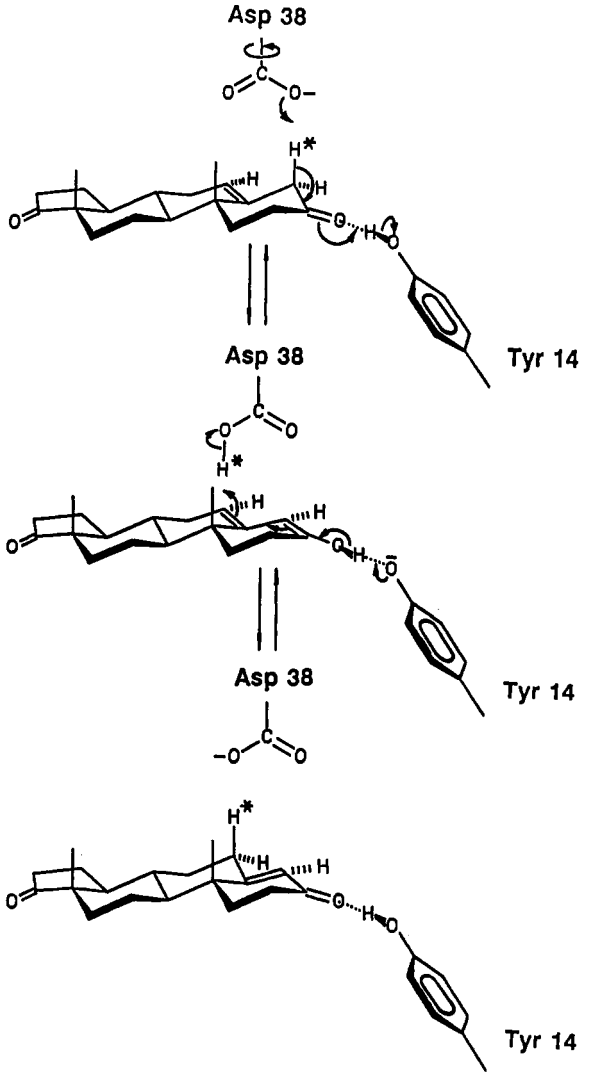

FIGURE 9: Orthogonal concerted mechanism of enolization and reketonization in the ketosteroid isomerase reaction, consistent with the geometry of Figure 8.

the suprafacial, antarafacial (Figure 2), and cis-orthogonal orientations of Tyr-14 with respect to the bound steroid and indicates a trans-orthogonal orientation (Figure 8). The hydrogen bonding of Tyr-14 to the lone pair of the keto group that is trans to the double bond of 19-nortestosterone is consistent with a survey of hydrogen bonding in crystalline cyclic enones that shows the trans lone pair to be favored for hydrogen bonding, over the cis, by a factor of ~2 (Murray-Rust & Glusker, 1984).

We conclude that the orthogonal arrangement of the proton donor and proton acceptor with respect to the bound substrate determined by this study is stereoelectronically appropriate for a concerted enolization that occurs at a very rapid rate, 54000 s^{-1}, on the enzyme ketosteroid isomerase (Kuliopulos et al., 1989; Xue et al., 1990). A mechanism consistent with this geometry is shown in Figure 9. Despite this rapid rate of enolization and the favorable geometry for hydrogen bonding, the arrangement of catalytic residues on ketosteroid isomerase may not be fully optimal since the resulting enol formed in which the OH hydrogen is trans to the double bond is a higher energy structure than that with a cis OH hydrogen, on the basis of coupling constants of stable enols in nonaqueous solvents (Capon et al., 1988). In this regard, it is of interest that the enzyme 4-oxalocrotonate tautomerase catalyzes a similar enolization (Aird & Whitman, 1990) with a k_{cat} ~50-fold greater than that of ketosteroid isomerase.[3] Al-

though the acid and base catalysts on 4-oxalocrotonate tautomerase are unknown, a trans approach of the proton donor to the 2-keto group of 4-oxalocrotonate could be hindered by the 1-carboxylate. Hence a cis enol might form on this enzyme and contribute to its greater activity.

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