Articles

Hydrogen Bonding at the Active Site of Δ^5-3-Ketosteroid Isomerase†

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ABSTRACT: The solution secondary structure of the highly active Y55F/Y88F "Tyr-14-only" mutant of Δ^5-3-ketosteroid isomerase complexed with 19-nortestosterone hemisuccinate has been shown to consist of three helices, a six-stranded mixed β-sheet, and five turns. The steroid binds near the general acid, Tyr-14, on helix 1, near the general base, Asp-38, on the first strand of the β-sheet, and on the hydrophobic face of the β-sheet [Zhao, Q., Abeygunawardana, C., & Mildvan, A. S. (1997) *Biochemistry 36*, 3458−3472]. On this hydrophobic face, Asp-99 is the only polar residue. Free isomerase shows a deshielded exchangeable proton resonance at 13.1 ppm assigned to the NϵH of neutral His-100. Its fractionation factor ($\phi = 0.79$) and slow exchange with solvent suggest it to be buried or involved in an H-bond. The binding of dihydroequilenin or estradiol to isomerase induces the appearance of two additional deshielded proton resonances, one at 18.2 ppm assigned to the γ-carboxyl proton of Asp-99, and the other, at 11.6 ppm, assigned to the ζ-OH proton of Tyr-14. While mutation of Asp-99 to Ala results in the disappearance of only the resonance near 18 ppm [Wu, R. W., Ebrahemian, S., Zawrotny, M. E., Thornberg, L. D., Perez-Alverado, G. C., Brothers, P., Pollack, R. M., & Summers, M. F. (1997) *Science 276*, 415−418], both of these resonances disappear in mutants lacking Tyr-14, suggesting an H-bonded catalytic diad, Asp-99-COOH---Tyr14-OH---O-steroid enolate. The catalytic diad is further supported by NOEs from the β1 and β2 protons of Asp-99 to the ϵ protons of Tyr-14, and from the ζ-OH proton of Tyr-14 to the γ-carboxyl proton of Asp-99, indicating close proximity of these two residues, and by other data from the literature. A strong, low-barrier H-bond between Asp-99 and Tyr-14 is indicated by the 6.2 ppm deshielding, low fractionation factor ($\phi = 0.34$) and slow exchange of the resonance at 18.2 ppm. A normal H-bond between Tyr-14 and the steroid is indicated by the 1.8 ppm deshielding, fractionation factor of 0.97 and the slow exchange of the resonance at 11.6 ppm. It is suggested that the $10^{4.7}$-fold contribution of Tyr-14 to catalysis is made possible by strong H-bonding from Asp-99 in the catalytic diad which strengthens general acid catalysis by Tyr-14. It is also noted that highly deshielded proton resonances on enzymes between 15 and 20 ppm, assigned to low-barrier H-bonds, generally involve carboxyl groups.

Δ^5-3-Ketosteroid isomerase (EC 5.3.3.1), a homodimeric enzyme with 125 amino acids per subunit, catalyzes the tautomeric conversion of unconjugated Δ^5- to conjugated Δ^4-3-ketosteroids via a dienolic intermediate (Xue et al., 1991; Eames et al. 1990; Holman & Benisek, 1994; Zhao et al., 1996b) accelerating this process by a factor of $\sim 10^{10}$ (Kuliopulos et al., 1990). Essential catalytic residues have been shown by single and double mutants to be Tyr-14 and Asp-38 (Kuliopulos et al., 1989, 1990) which function respectively as acid and base catalysts in the concerted enolization reaction. Thus, the Y14F and D38N mutants are $10^{4.7}$- and $10^{5.6}$-fold less active, and the Y14F/D38N double mutant is $\sim 10^{10}$-fold less active than the wild-type enzyme. Tyr-14 interacts directly with the bound steroid as shown by transferred NOE's from Tyr-14 Hϵ to the 2α and 2β protons of 19-NTHS[1] (Kuliopulos et al., 1991).

The solution secondary structure of isomerase complexed with 19-NTHS (Zhao et al., 1997) and the solution tertiary structure of the free enzyme (Wu et al., 1997) have been determined by heteronuclear NMR methods. As found by intermolecular NOEs (Kuliopulos et al., 1991; Zhao et al., 1997) and by paramagnetic effects of a spin-labeled steroid on proton resonances of the enzyme (Kuliopulos et al., 1987; Zhao et al., 1997) the steroid binds between helix 1 and the

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[1] Abbreviations: DHE, dihydroequilenin; D38N, mutant isomerase in which Asp-38 was replaced by asparagine; DMSO-d_6, perdeuterated dimethyl sulfoxide; FID, free induction decay; H-bond, hydrogen bond; HSQC, heteronuclear single quantum correlation; isomerase (KSI), Δ^5-3-ketosteroid isomerase (EC 5.3.3.1); NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; 19-NTHS, 19-nortestosterone hemisuccinate; S/N, signal to noise ratio; SS-NOESY, symmetrically shifted NOESY; Y55F/Y88F, mutant isomerase with Tyr-55 to Phe-55 and Tyr-88 to Phe-88, all isomerase in this paper refers to this mutant unless otherwise stated.

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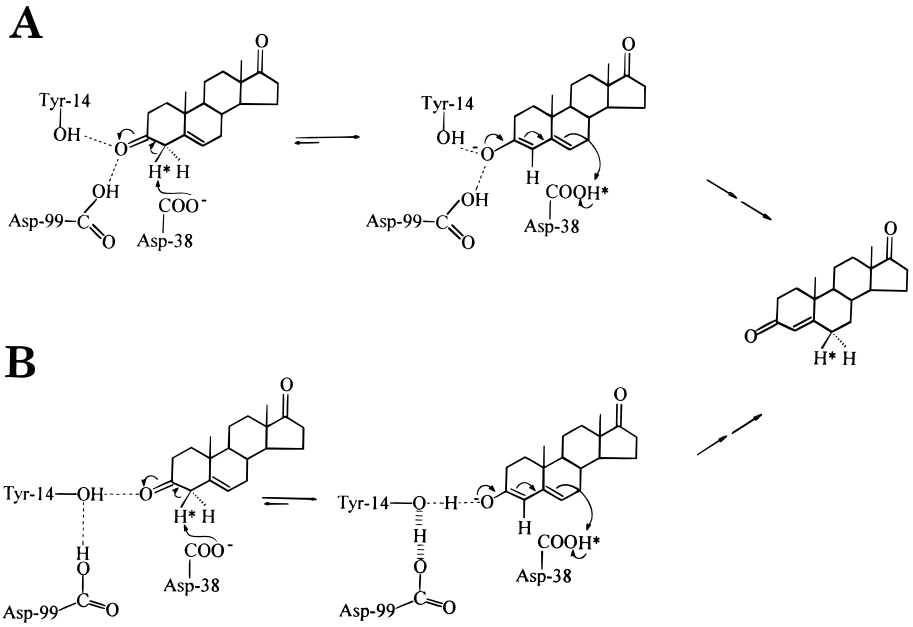

FIGURE 1: Alternative mechanisms of the Δ^5-3-ketosteroid isomerase reaction. (A) The intermediate is stabilized by two normal hydrogen bonds from Tyr-14 and Asp-99 (Wu et al., 1997). (B) The intermediate is stabilized by a catalytic diad involving a strong low barrier H-bond from Asp-99 to Tyr-14 which strengthens the H-bond from Tyr-14 to the intermediate (Austin et al., 1992, 1995; Zhao et al., 1995a,b, 1996a,b).

hydrophobic face of a six-stranded mixed β-sheet (Zhao et al., 1997). The only polar residue on this otherwise hydrophobic face of the β-sheet is Asp-99. By modeling the substrate into the active site of the free enzyme, placing it near Tyr-14 and Asp-38, Wu et al. (1997) found Asp-99 to be near the 3-keto group of the bound substrate and also near Tyr-14 and Asp-38. These authors showed the D99A mutant to have a $10^{3.7}$-fold decrease in k_{cat} below that of the wild-type enzyme, and proposed the mechanism shown in Figure 1A in which both Tyr-14 and the protonated carboxyl group of Asp-99 function as acid catalysts, both donating hydrogen bonds, normal in strength, to the lone electron pairs on the C-3 oxygen of the steroid.

An alternative role for Asp-99 (Figure 1B) is provided in a mechanism proposed by Austin et al. (1992) on the basis of UV resonance Raman spectra which indicated Tyr-14 to both donate and receive hydrogen bonds, both in the free enzyme and in the enzyme−19-NTHS complex. The red-shifted UV absorption spectrum of Tyr-14, which is not further red-shifted on steroid binding, supports such a H-bonded network (Zhao et al., 1995a,b). In the mechanism of Figure 1B, Asp-99, by donating a hydrogen bond to Tyr-14, would form a catalytic diad, strengthening the interaction of Tyr-14 with the substrate and with the enolic intermediate.

Consistent with an unusually strong or low-barrier H-bond in the isomerase-intermediate complex, the binding of analogs of this intermediate such as DHE or estradiol (Figure 2) to the enzyme induced the appearance of two deshielded, exchangeable proton resonances, one at 18.15 ppm, originally assigned to the Tyr-14-OH group, and the other at 11.60 ppm which was not assigned (Zhao et al., 1996b). The downfield chemical shift, slow exchange with solvent, and low fractionation factor of the resonance at 18.15 ppm are consistent with a low-barrier H-bond (Cleland & Kreevoy, 1994; Zhao et al., 1996b, and references therein). Both of these deshielded resonances disappeared in mutants lacking Tyr-14 (Zhao et al., 1996b), while only the more downfield resonance disappeared in the D99A mutant (Wu et al., 1997),

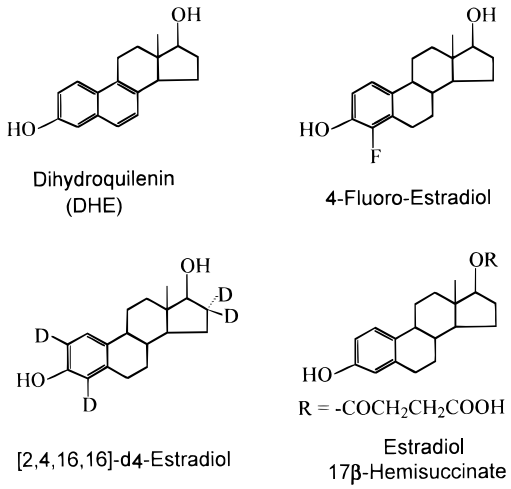

FIGURE 2: Analogs of the Δ^5-3-ketosteroid isomerase reaction intermediate used in the present NMR studies.

suggesting that the 11.60 ppm resonance rather than the 18.15 ppm resonance might be that of Tyr-14 and that the 18.15 ppm resonance might be that of the carboxyl group of Asp-99.

These possibilities require a redetermination of the assignments of the resonances at 18.15 and 11.60 ppm and measurement of the exchange properties, and fractionation factor of the resonance at 11.60 ppm. The present paper re-examines the assignments and characterizes the properties of all of the deshielded proton resonances of isomerase and detects a direct interaction of Asp-99 with Tyr-14 clarifying the roles of these residues in catalysis. A preliminary abstract of this work has been published (Abeygunawardana et al., 1997).

EXPERIMENTAL PROCEDURES

Materials. DHE, estradiol 17β-hemisuccinate, 4-fluoroestradiol, and 19-nortestosterone hemisuccinate obtained from Steraloids (Wilton, NH) all showed a single spot on

thin layer chromatography and were used without further purification. [2,4,16,16]-d_4-Estrone (≥95 atom % D) was from Isotec (Miamisburg, OH). Ammonium sulfate, buffer salts, sodium chloride, ampicillin, and DMSO-d_6 were from Sigma (St. Louis, MO).

Expression and Purification of Isomerase Mutants. Bacteria *Escherichia coli* BL21 DE3 (Novagen, Madison, WI) transformed with the pET-25b(+) expression vector carrying the Y55F/Y88F mutant or isomerase gene was used to overexpress the Y55F/Y88F mutant (Zhao et al., 1996a, 1997). The unlabeled, ^{13}C,^{15}N-uniformly-labeled, and $^{13}C_\epsilon$-Tyr-14-specifically labeled Y55F/Y88F mutants were purified to homogeneity according to the published procedures (Zhao et al., 1996a, 1997; Kuliopulos et al., 1989, 1991). D38N, Y14F/D38N, Y14F/Y88F mutants were purified to homogeneity as reported (Kuliopulos et al., 1989, 1990; Li et al., 1993). Purified isomerase mutants were stored as crystalline suspensions in 30% saturated and neutralized ammonium sulfate solution at 4 °C and were found to be stable for at least two years under these conditions. The intactness of the structure of isomerase mutants was also monitored by their specific activity and/or by the presence of upfield methyl signals at 0 to −0.5 ppm (Zhao et al., 1997).

Preparation of NMR Samples. For observation of low-field exchangeable proton resonances at −3.3 °C, NMR samples were prepared in 10 mM sodium phosphate, 20 mM sodium chloride, 9% (v/v) DMSO-d_6. The pH values of the samples were measured at 20 °C to be pH 7.6, unless otherwise noted. The enzyme concentrations, which ranged from 100 to 750 μM, were determined by using the absorbance at 280 nm ($\epsilon = 1890$ M^{-1} cm^{-1}) for the native Y55F/Y88F mutant (Li et al., 1993). For all isomerase mutants, the concentration was also determined by the absorbance at 293 nm of the ionized tyrosinate at pH 13.0 [$\epsilon = 2390$ M^{-1} cm^{-1} per tyrosinate, Goodwin and Morton (1946)]. For heteronuclear NMR experiments, the enzyme concentration was 750 μM in subunits of this dimeric enzyme, and the pH was 7.2. All other components and buffers are as described above and by Zhao et al. (1997).

Preparation of [2,4,16,16]-d_4-Estradiol. A 10.0 mg amount of [2,4,16,16]-d_4-estrone was dissolved in a mixture of 6 mL of methanol, 1 mL of benzene, and 1 mL of pyridine. After the mixture was cooled to 0 °C over ice, a total of 7 mg of sodium borohydride (98%, Aldrich) was added in two batches of 3.5 mg each with stirring (Zhao & Li, 1993). The reaction was monitored by thin-layer chromatography, developed with ethyl acetate/hexane (1:1). The R_f value for estrone was 0.77, whereas the R_f for estradiol was 0.56. Quantitative conversion to estradiol was observed after 5 h. The entire mixture was poured into16 mL of water. The pH was then adjusted to 2.0 by dropwise addition of 12 N HCl. After extraction with chloroform and evaporation of the solvent, [2,4,16,16]-d_4-estradiol was dissolved in 1.0 mL of DMSO-d_6. The purity of the [2,4,16,16]-d_4-estradiol was indicated by a single spot on thin-layer chromatography and by the identity of its UV spectrum with that of an authentic sample of estradiol. The yield was ∼97%.

NMR Spectroscopy. All NMR experiments were performed with a Varian Unityplus 600 spectrometer. ^1H, ^{13}C, and ^{15}N resonance assignments of the Y55/Y88F "Tyr-14 only" mutant of isomerase, complexed with 19-NTHS at 37 °C and pH 7.2 were made as described by Zhao et al. (1997). The NOE measurements involving non-exchangeable protons of the active site residues were obtained from 3D $^1H-^{13}C$ NOESY-HSQC spectra with a mixing time of 100 ms, as described (Zhao et al., 1997).

NMR experiments involving low-field exchangeable protons were carried out at −3.3 °C. 1D proton NMR spectra of isomerase complexes were acquired with the 1331 pulse sequence (Turner, 1983) with the excitation maximum at 18.15 ppm to avoid water excitation as described by Zhao et al. (1996b). Additional experimental parameters are given in the figure legends. Exchange rates with solvent were determined by nonlinear least squares fitting of the temperature dependences of $1/T_2$ of the low field resonances using the program GraFit (Leatherbarrow, 1992) as previously described (Zhao et al., 1996b). Fractionation factors were determined by the method of Loh and Markley (1994) as previously described (Zhao et al., 1996b) by nonlinear least squares fitting of plots of the mole fraction of H_2O in H_2O/D_2O mixtures against the normalized intensity of the proton resonance (see Figure 10).

1D difference NOE spectra were acquired for the D38N−DHE complex using low power irradiation (∼50 Hz) for 200 ms followed by the 1331 detection pulse. Two sets of spectra were collected by irradiating at 18.15, 12.89, 11.60, ppm and at −6.00 ppm as a control. A total of 4096 transients were acquired for each spectrum.

2D NOESY spectra of isomerase complexes were acquired with 50 msec mixing times with the SS-NOESY pulse sequence (Smallcomb, 1993) to avoid excitation of the water resonance by the detection pulse. The maximum excitation of the SS pulse (140 μs) was adjusted to be 7143 Hz (11.9 ppm) from water. Two NOESY data sets were acquired for the D38N−DHE sample with 96 complex points in t_1. Two different sweep widths, 16 320 and 10 800 Hz were used with 1024 and 928 scans per FID, respectively. In the data set acquired with the smaller sweep width, the diagonal peak at 18.15 ppm was allowed to fold in order to increase the digital resolution in the t_1 dimension. An equivalent experiment with 96 complex points, 1024 scans per FID, and a sweep width of 10 800 Hz in the t_1 dimension was collected for the D38N−[2,4,16,16]-d_4-estradiol complex in which the indicated aromatic protons in ring A (and methylene protons in ring D) of the steroid were deuterated.

SS-NOESY data were also collected for the estradiol hemisuccinate complex of the "Tyr-14-only" mutant, in which the Cϵ carbon of Tyr-14 was selectively labeled with ^{13}C (Zhao et al., 1996a). Two data sets were acquired, with and without ^{13}C-decoupling during the t_1 period. ^{13}C-decoupling was achieved by a WALTZ-16 pulse sequence using a 3.6 kHz rf field centered at 120.0 ppm. The spectra include 48 complex points in t_1 with a sweep width of 6000 Hz and 768 scans per FID. The total time for each experiment was 42 h.

The arrangement of catalytic residues and steroid at the active site of isomerase was modeled by molecular dynamics and energy minimization with the program XPLOR 3.1 (Brünger, 1992) on a Silicon Graphics Power Challenge Computer using the interproton distances in Table 1 together with six intermolecular NOEs from the enzyme to 19-NTHS previously reported (Kuliopulos et al., 1991; Zhao et al., 1997) as well as 2064 intramolecular NOEs within the enzyme. Five structures were obtained with no NOE viola-

Table 1: Interproton NOEs and Estimated Interproton Distances at the Active Site of Δ^5-3-Ketosteroid Isomerase

complex	interaction	δ (ppm)[a]	S/N	distance (Å)[b]
Y55F/Y88F−19-NTHS[c]	D99Hα → D99Hβ1	5.79, 1.92	3.3	2.50 [f]
Y55F/Y88F−19-NTHS[c]	D99Hα → D99Hβ2	5.79, 1.32	3.4	2.5 ± 0.2
Y55F/Y88F−19-NTHS[c]	D99Hβ1 ↔ D99Hβ2	1.92, 1.32	10.3	2.1 ± 0.2
Y55F/Y88F−19-NTHS[c]	Y14Hϵ → D99Hβ1	6.75, 1.92	2.0	2.8 ± 0.2
Y55F/Y88F−19-NTHS[c]	Y14Hϵ → D99Hβ2	6.75, 1.32	2.2	2.8 ± 0.2
D38N−DHE[d]	Y14Hϵ → H_c	6.79 ± 0.04, 11.60	13.2	2.25[g]
D38N−DHE[d]	Y14Hϵ → H_a	6.79 ± 0.04, 18.05	3.0	3.0 ± 0.2
D38N−[2,4,16,16]-d_4-estradiol	Y14Hϵ → H_c	6.81 ± 0.05, 11.76	9.7	2.25[g]
D38N−[2,4,16,16]-d_4-estradiol	Y14Hϵ → H_a	6.76 ± 0.05, 18.16	2.3	3.0 ± 0.2
D38N−DHE[e]	Y14Hϵ → H_c	6.80, 11.60	3.5	2.25[g]
D38N−DHE[e]	H_a → H_c	18.04, 11.60	1.4	2.6 ± 0.2

[a] Unless otherwise indicated, the errors in δ are ≤0.02 ppm. [b] Distances were estimated from the intensity of the NOE cross peak in the optimal slice, making the two-spin approximation, assuming uniform correlation times, and using the indicated known interproton distance as a reference (Rosevear & Mildvan, 1989). The degeneracy of Y14Hϵ contributes an error of ±6% to the distances to this proton. [c] From columns taken through Hβ1 and Hβ2 of D99 in the ^{13}C plane of D99Cβ (δ = 40.3 ppm). 3D ^{1}H−^{13}C NOESY HSQC spectrum (Figure 3). [d] From columns of a 2D SS-NOESY spectrum (Figure 6). [e] From rows of a 2D SS-NOESY spectrum. [f] Reference distance obtained by model building of the staggered conformation of D99 with D99Hα equidistant from D99Hβ1 and D99Hβ2. [g] Reference distance to the nearer Y14Hϵ in the X-ray structure of *N*-acetyl-L-tyrosine (Koszelak & van der Helm, 1981).

tions. A detailed description of the solution structure of the enzyme−steroid complex will be published elsewhere.

RESULTS AND DISCUSSION

Proximity of Asp-99 to Tyr-14 at the Active Site of Isomerase

From HCCH-TOCSY spectra of the 19-NTHS complex of the Y55F/Y88F mutant of isomerase, the Hα proton of Asp-99 resonates at 5.79 ppm and is directly bonded to Cα, which resonates at 50.39 ppm. The chemical shifts of the Hβ protons of Asp-99 are 1.92 ppm (Hβ1) and 1.32 ppm (Hβ2), both of which are directly bonded to Cβ at 40.3 ppm (Zhao et al., 1997). The chemical shift of Tyr-14Hϵ is 6.75 ppm, and that of Cϵ is 118.19 ppm.

As shown in Figure 3 and summarized in Table 1, 3D ^{1}H−^{13}C NOESY-HSQC spectra of the same complex at a mixing time of 100 ms reveal equal NOEs from Asp-99 Hα to its own Hβ_1 and Hβ_2 protons (S/N = 3.3, 3.4) as monitored by the intensities of the cross peaks in optimal slices. Slightly weaker NOE's are found from Tyr-14 Hϵ to Hβ_1 (S/N = 2.0) and Hβ_2 (S/N = 2.2) of Asp-99. The approximately equal NOE's to Asp-99 Hβ_1 and Hβ_2 from its own Hα indicate a conformation of Asp-99 in which the α and β protons are staggered. Assuming this to be the case, interproton distances of 2.5 ± 0.1 Å from Hα to Hβ_1 and to Hβ_2 of Asp-99 are determined from model building. Using these NOE's and distances as internal standards, somewhat greater distances of 2.8 Å are estimated from Tyr-14 Hϵ to Hβ_1 and Hβ_2 of Asp-99 (Table 1). These proximities place the side chain of Asp-99 adjacent to the aromatic ring of Tyr-14 (Figure 4). The catalytic diad mechanism of Figure 1B requires proximity of the side chain of Asp-99 to that of Tyr-14, as is observed, while the mechanism of Figure 1A does not.

Wu et al. (1997), have made the reasonable proposal that Asp-99 is the group responsible for the pK_a of 9.5 which is found in the pH rate profile of isomerase (Weintraub et al., 1970). As found by Li et al. (1993), deprotonation of a group with this pK_a halves the fluoresence of Tyr-14, while the pK_a of Tyr-14 itself is 11.6, consistent with the proximity of Asp-99 to Tyr-14.

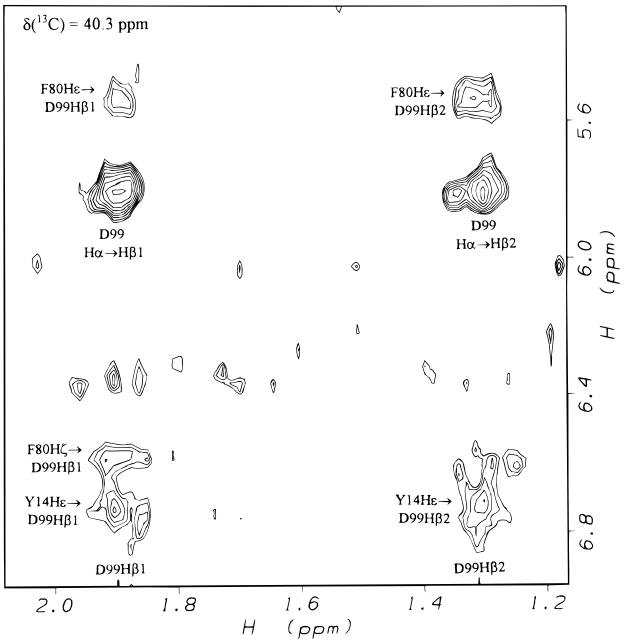

FIGURE 3: ^{13}C plane through Cβ of Asp-99 (δ = 40.3 ppm) in a 3D ^{1}H−^{13}C NOESY-HSQC spectrum of the Y55F/Y88F "Tyr-14-only" mutant of isomerase complexed with 19-NTHS in D_2O containing 9% (v/v) DMSO-d_6. Temperature, 37 °C; mixing time, 100 ms; conditions are otherwise given in Experimental Procedures and in Zhao et al. (1997). The apparent splitting of the Hα → Hβ2 cross peak results from a noise pulse as detected in the optimal 1D slice through Hα which also reveals approximately equal widths of the Hβ1 and Hβ2 resonances.

Deshielded, Exchangeable Proton Resonances of Isomerase

Figure 5A shows the downfield NMR spectrum of the Y55F/Y88F "Tyr-14 only" mutant of isomerase at −3.3 °C. In the free enzyme, a single resonance at 13.10 ppm is detected, hereafter referred to for brevity as H_b. The addition of estradiol 17β-hemisuccinate, a soluble analog of the dienolic intermediate, shifts H_b slightly upfield to 12.82 ppm and induces the appearance of two additional deshielded resonances, one at 18.15 ppm (H_a), and the other at 11.60 ppm (H_c) (Figure 5B). Essentially identical H_a, H_b, and H_c signals were obtained with the D38N mutant of isomerase complexed with DHE, another analog of the dienolic intermediate (Figure 5C) (Zhao et al., 1996b). The Y14F/

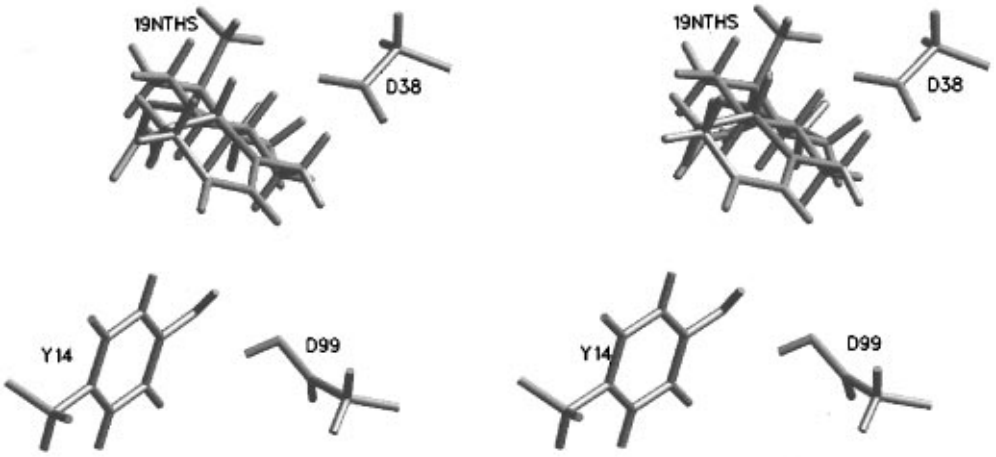

FIGURE 4: Stereopair showing the arrangement of catalytic residues and a steroid at the active site of Δ^5-3-ketosteroid isomerase. The lowest energy structure is shown, computed as described in Experimental Procedures using the distances in Table 1, together with distances from Tyr-14 Hϵ to the steroid (Kuliopulos et al., 1991), and 2064 intramolecular NOEs within the enzyme.

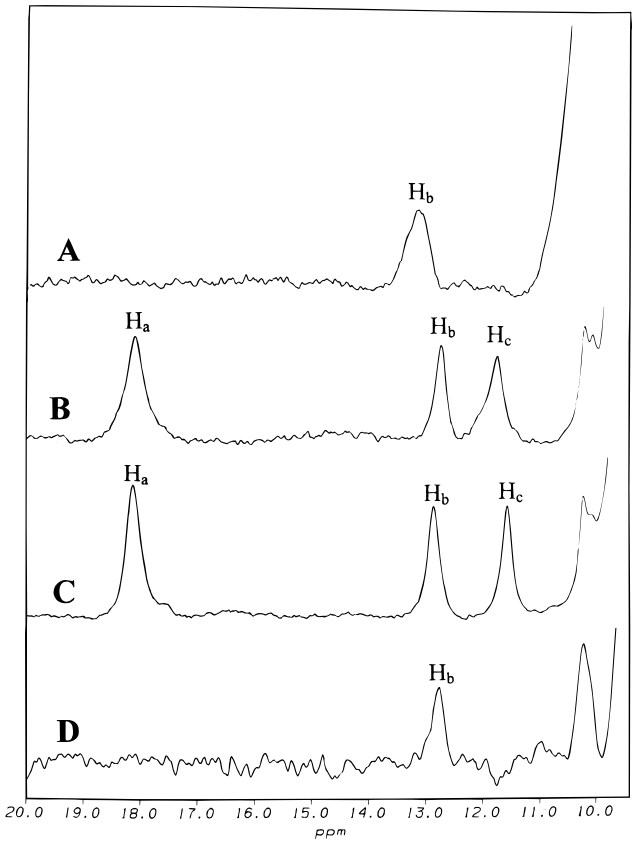

FIGURE 5: Downfield region of the ^{1}H NMR spectra of Δ^5-3-ketosteroid isomerase mutants and their complexes in H_2O with analogs of the intermediate. (A) The free Y55F/Y88F "Tyr-14-only" mutant of isomerase (0.50 mM) and (B) complexed with estradiol 17β-hemisuccinate (1.0 mM). (C) The D38N mutant of isomerase (0.50 mM) complexed with DHE (0.50 mM). (D) The Y14F/D38N mutant of isomerase (0.15 mM) complexed with DHE (0.15mM). Other components and concentrations are given in Experimental Procedures. Spectra were recorded with the 1331 pulse sequence (Turner, 1983) at -3.3 °C with a relaxation delay of 1.5 s, 512 ms acquisition time, and a 26 ms 90° pulse width. Spectra were processed with 30 Hz line broadening (A$-$C) or 50 Hz line broadening (D) and were zero-filled to 16K before Fourier transformation (Zhao et al., 1996b). For spectrum D, 64 000 transients were accumulated.

D38N double mutant, lacking Tyr-14, shows only the H_b resonance in the presence of DHE (Figure 5D) and also in its absence (data not shown). Similarly, the Y14F/Y88F double mutant, also lacking Tyr-14, shows only the H_b resonance both in the absence and presence of estradiol 17β-hemisuccinate (data not shown). Under the conditions of the NMR experiments, both of these mutants are almost completely saturated by the steroids (Kuliopulos et al., 1989).

Our finding that *both* the H_a and H_c resonances disappear in steroid complexes of mutants of isomerase lacking Tyr-14, and the finding by Wu et al. (1997) that only the H_a resonance (at 17.4 ppm under their conditions) disappears in the DHE complex of the D99A mutant, are most simply explained by reassigning H_a to the carboxyl proton of Asp-99 and H_c to the ζ-OH proton of Tyr-14.

The effects of these mutations are consistent with mechanism 1B (rather than 1A) in which Asp-99 donates a strong, low-barrier H-bond ($\delta = 17.4-18.2$ ppm) to Tyr-14 which in turn donates a normal H-bond ($\delta = 11.6$ ppm) to the intermediate, forming a catalytic diad. Mutation of Tyr-14 disrupts both H-bonds of the catalytic diad, while mutation of Asp-99 disrupts *only* the strong H-bond from Asp-99 to Tyr-14.

The H_b resonance (at 12.8 ppm) is likely that of an imidazole NH of one of the three histidines of isomerase since at -3.3 °C it selectively broadens at pH values below 7.0 and re-narrows at pH 4.3, while H_a and H_b are insensitive to pH over the range 4.3$-$9.0 (Zhao et al., 1996b). H_b is also broadened in the ^{15}N-labeled enzyme and narrows by $\sim$54 Hz on ^{15}N-decoupling (data not shown). H_b is assigned to the Nϵ-H proton of neutral His-100 based on the sequence specific assignments of the three histidines of isomerase,[2]

[2] The aromatic resonances of the three histidines of isomerase were sequentially assigned by 3D ^{1}H$-^{15}$N NOESY-HSQC spectra, 3D ^{1}H$-^{13}$C NOESY-HSQC spectra, both with mixing times of 100 ms, and by 2D NOESY spectra in D_2O with a mixing time of 50 ms. His-6 (pK_a = 5.83) was assigned on the basis of NOE's from its backbone NH to its Hδ, and from its Hδ and Hϵ to Val-10γ Me$_2$. His-122 (pK_a = 7.80) was assigned on the basis of NOE's from its backbone NH to its Hδ and from its Hδ and Hϵ to Asp-120 Hα. His-100 (pK_a = 4.3) was assigned by elimination, supported by a weak NOE from its Hδ to Ile-98γMe.

[3] In these distance estimates, no corrections were made for the exchange of the H_a and H_c protons with solvent. Differences in the exchange rates are indicated by the decreased intensities of the cross peaks to water seen in the estradiol complex (Figures 6D and E) in comparison with the DHE complex (Figures 6B and C). However, the ratios of cross peaks used to estimate the distances are insensitive to these differences in exchange rates.

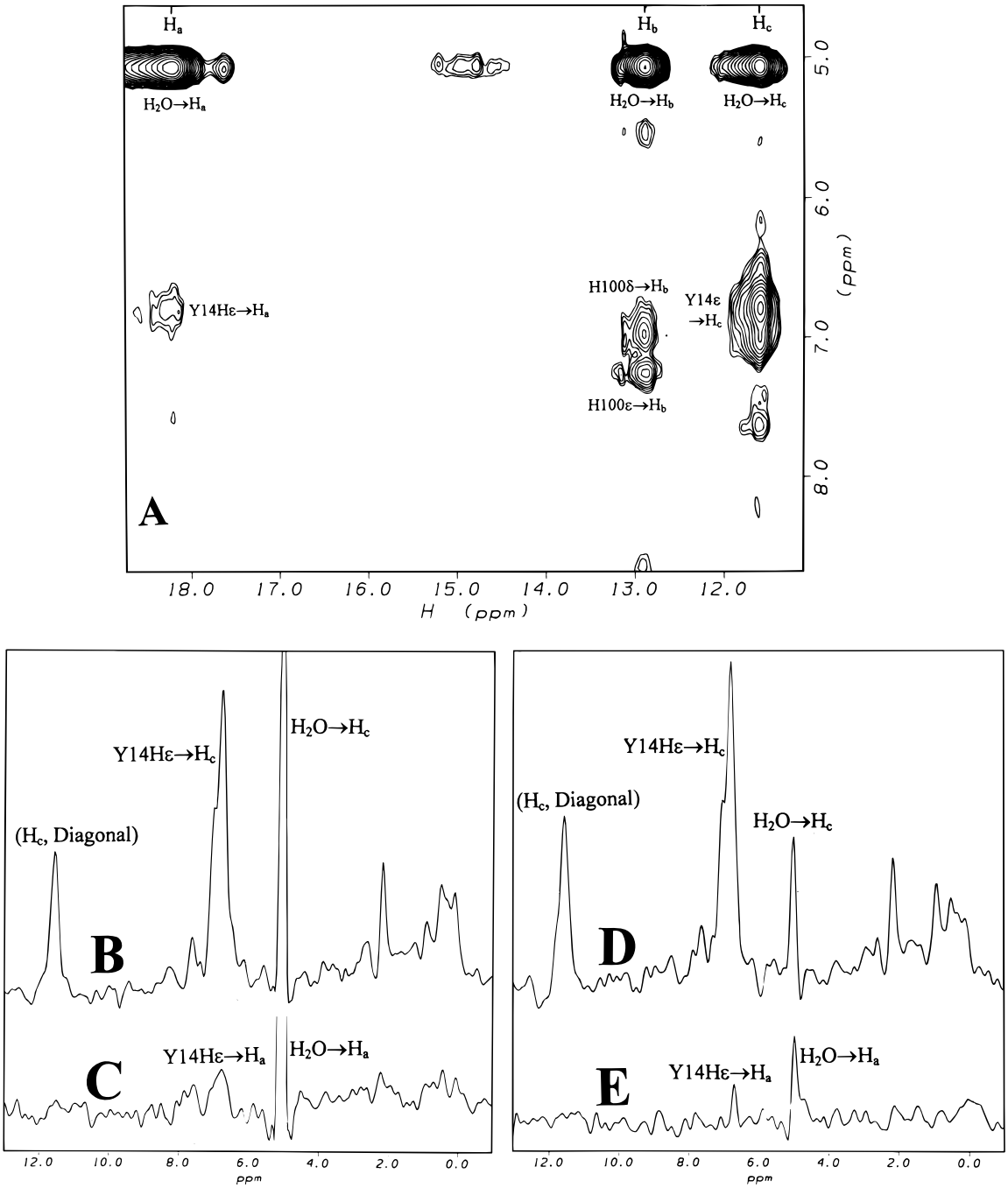

FIGURE 6: 2D SS-NOESY spectra in H_2O of the D38N mutant of Δ^5-3-ketosteroid isomerase at −3.3 °C complexed with intermediate analogs. (A) 2D display of NOE's to the deshielded signals H_a, H_b, and H_c in the DHE complex. (B) Column through H_c at 11.60 ppm and (C) through H_a at 18.15 ppm in the DHE complex. (D) Column through H_c at 11.60 ppm, and (E) through H_a at 18.15 ppm in the complex with [2,4,16,16]-d_4-estradiol. Conditions are given in Experimental Procedures. Unlabeled cross peaks have not been assigned.

all of which are remote from the active site (Kuliopulos et al., 1987; Zhao et al., 1997; Wu et al., 1997). The tautomeric form of His-100 was established by the strong and equal NOEs from H_b to both the δCH and ϵCH of His-100 (Figure 6A). This histidine was previously shown to have an unusually low pK_a of ∼4.3 at 27 °C which would be expected to increase at −3.3 °C, explaining the sensitivity of its line width to pH values between 4.3 and 7.0 (Kuliopulos et al., 1991). The reason for this low pK_a, based on the secondary (Zhao et al., 1997) and tertiary structure of isomerase (Wu et al., 1997) is that this residue is partially buried at the subunit interface near Arg-102 and Arg-113, possibly donating an H-bond to Ser-111 or to Glu-77. In the solution structure of Enzyme III Glc, the Nϵ-H of His-90, with a similar chemical shift (δ = 13.1 ppm), was found to be H-bonded to a negative oxygen of phosphohistidine-70 (Pelton et al., 1993). None of the three histidines of isomerase are conserved in the enzyme from *Pseudomonas putida*, which is otherwise 34% identical in sequence to the enzyme from *Pseudomonas testosteroni* (Linden & Benisek, 1986). However, Tyr-14, Asp-38 and Asp-99 and their neighboring residues are conserved in this variant, and the importance of Tyr-14 and Asp-38 to catalysis was confirmed by mutagenesis (Kim & Choi, 1995).

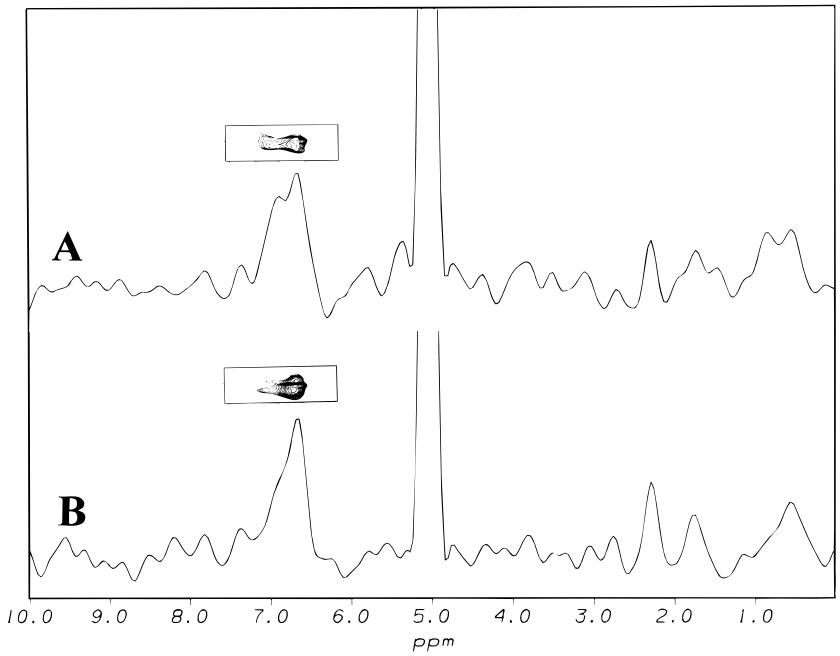

FIGURE 7: 2D SS-NOESY spectra in H_2O of the Y55F/Y88F "Tyr-14-only" mutant of isomerase with $^{13}C\epsilon$-labeling of Tyr-14, complexed with estradiol 17β-hemisuccinate. Columns through H_c are shown without (A) and with (B) ^{13}C-decoupling during t_1. Insets show 2D contour plots of the cross peaks. Significant (109 Hz) narrowing of the cross peak in the f_1 dimension establishes an NOE from Tyr-14Hϵ to H_c. The asymmetric shape of the cross peak suggests a small contribution from an overlapping unassigned signal. $T = -3.3$ °C. Conditions and other components are given in Experimental Procedures.

Interproton Proximities to the Deshielded Protons, H_a and H_c

To test the above assignments, and the mechanism of Figure 1 by an independent method, the proximity of H_a and H_c to Tyr-14 Hϵ and to each other were studied by nuclear Overhauser effects. Figure 6A shows an SS-NOESY spectrum with a mixing time of 50 msec of the D38N mutant of isomerase complexed with DHE, collected without excitation of the water resonance by the detection pulse. A strong NOE is seen from Tyr-14 Hϵ to H_c (S/N = 13.2) (Figure 6B) while a 4.4-fold weaker NOE is seen from Tyr-14 Hϵ to H_a (S/N = 3.0) (Figure 6C), indicating H_a to be 1.28-fold farther from Tyr-14 Hϵ than H_c is (Table 1). To eliminate possible ambiguities in the assignments of the NOE's in the aromatic region, estradiol, deuterated in the 2- and 4-positions, was complexed with the D38N mutant of isomerase, and nearly identical NOESY spectra were obtained (Figure 6D,E). Again, a strong NOE is seen from Tyr-14 Hϵ to H_c (S/N = 9.7) (Figure 6D) while a 4.2-fold weaker NOE is seen from Tyr-14 Hϵ to H_a (S/N = 2.3) (Figure 6E), indicating H_a to be 1.27-fold farther from Tyr-14 Hϵ than H_c is (Table 1). These proximities are consistent with the reassignment of H_a to the -COOH proton of Asp-99 and the assignment of H_c to the ζ-OH proton of Tyr-14. Assuming H_c to be the resonance of the ζ-OH proton of Tyr-14 places it at a distance of 2.25 Å from Tyr-14 Hϵ as found in the X-ray structure of *N*-acetyl-L-tyrosine (Koszelak & van der Helm, 1981). Using this distance as an internal reference, the distance from H_a to Tyr-14 Hϵ is 3.0 Å (Table 1).

A separate NOESY experiment was carried out with $^{13}C\epsilon$-labeled Tyr-14 in the highly active Y55F/Y88F mutant of isomerase in which Tyr-14 is the only tyrosine residue. The complex of this "Tyr-14 only" mutant with estradiol hemisuccinate showed an NOE cross peak, broadened due to $^1H\epsilon-{}^{13}C\epsilon$ hyperfine coupling, from H_c to Tyr-14 Hϵ (Figure 7A). This NOE cross peak sharpened by ~109 Hz on ^{13}C-decoupling (Figure 7B) confirming a strong NOE (S/N = 4.7) between H_c and Tyr-14 Hϵ. Again a 4-fold weaker NOE (S/N = 1.3) was found between H_a and Tyr-14 Hϵ.

Since weak NOEs are expected between pairs of solvent exchangeable protons, proximity between H_a and H_c was initially established by 1D NOE studies of the D38N complex of DHE (Figure 8). Pre-irradiation of H_a for 200 ms induced a weak NOE to H_c (S/N = 2.2) (Figure 8B), and conversely, pre-irradiation of H_c induced a similar NOE to H_a (S/N = 2.0) (Figure 8C), while pre-irradiation of H_b produced no NOE's to either H_a or H_c (Figure 8D). To estimate the distance between H_a and H_c, the row through H_c in the SS-NOESY spectrum of the D38N−DHE complex was analyzed. A moderately strong NOE from Tyr-14 Hϵ to H_c was found (S/N = 3.5), and in the same row, a weak NOE was detected from H_a to H_c (S/N = 1.4). No NOE's from H_b to H_a or to H_c were detected consistent with the 1D NOE experiments (Figure 8). Assuming the distance from H_c to Tyr-14 Hϵ to be 2.25 Å (see above) a distance of 2.6 Å from H_a to H_c is calculated (Table 1).

The catalytic diad mechanism (Figure 1B) requires this distance from the γ-COOH proton of Asp-99 (H_a) to the ζ-OH proton of Tyr-14 (H_c) to be $\leq$3.0 Å, while the mechanism involving two separate and normal H-bonds to the enolate (Figure 1A) predicts an H_a to H_c distance $\geq$3.5 Å. The observed distance of 2.6 $\pm$ 0.2 Å supports the mechanism of Figure 1B. The errors in distances determined by NOEs tend to be small because of the sixth root taken in the distance calculations which truncates experimental errors by a factor of 6. Even when the generous errors in Table 1 are doubled, the distances support the catalytic diad mechanism of Figure 1B rather than that of Figure 1A.

Figure 4 exemplifies a model of the active site consistent with all of the distances measured here and previously,

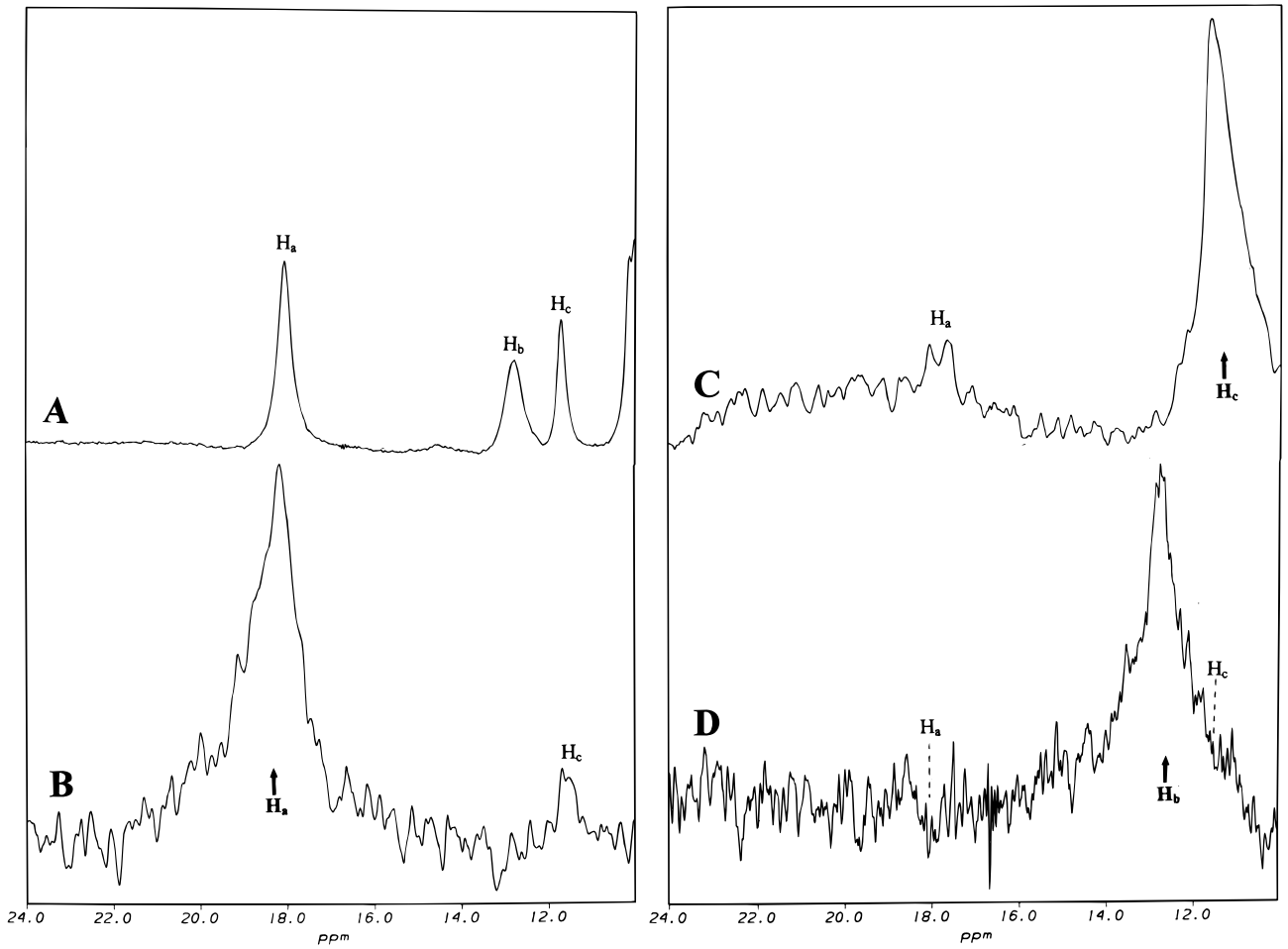

FIGURE 8: 1D Difference NOE spectra of the D38N mutant of Δ^5-3-ketosteroid isomerase complexed with DHE at −3.3 °C in H_2O. (A) Reference spectrum showing the deshielded resonances, H_a, H_b and H_c. (B) Pre-irradiation at H_a for 200 ms (δ = 18.15 ppm) results in an NOE to H_c (δ = 11.60 ppm). (C) Reciprocal effect of pre-irradiation at Hc which results in an NOE to H_a. (D) Pre-irradiation at H_b (δ = 12.89 ppm) results in no detectable NOEs to either H_a or H_c. Spectra were processed with line broadenings of 10, 70, 70, and 30 Hz for A, B, C, and D, respectively. Other conditions are given in Experimental Procedures.

computed as described in Experimental Procedures. From the modeling, distances from the phenolic oxygen of Tyr-14 to the 3-oxygen of the steroid are 2.8 ± 0.2 Å indicating H-bonding between Tyr-14 and the steroid, while distances from the carboxyl oxygens of Asp-99 to the 3-oxygen of the steroid are 3.9 ± 0.7 and 5.5 ± 1.2 Å, indicating weak or absent H-bonding between Asp-99 and the steroid. Distances from the carboxyl oxygens of Asp-99 to the phenolic oxygen of Tyr-14 are 2.6 ± 0.2 and 4.1 ± 0.5 Å, consistent with strong H-bonding between Asp-99 and Tyr-14, in accord with the catalytic diad mechanism of Figure 1B.

Exchange Properties of the Deshielded Proton Resonances of Isomerase

We have previously reported the exchange rate, activation parameters, and the fractionation factor of H_a, the most deshielded proton resonance in the D38N−DHE complex (Zhao et al., 1996b) which has here been reassigned to the carboxyl proton of Asp-99. The H_c signal at 11.6 ppm, now assigned to the ζ-OH proton of Tyr-14, is also important to catalysis. Hence its exchange properties have also been determined as have those of H_b, assigned to the Nϵ-H proton of neutral His-100, a residue which points away from the active site (Zhao et al., 1997; Wu et al., 1997) (Figure 9, Table 2).

All three of the deshielded resonances show large protection factors indicating much slower proton exchange with solvent than residues which are fully exposed (Table 2). The Nϵ-H of His-100 may be involved in a normal H-bond, as suggested by its slow exchange rate and slightly reduced fractionation factor (Figure 10, Table 2). As may be seen in Table 2, the more deshielded the proton resonance, the faster its exchange rate with solvent, due primarily to a lower enthalpy barrier, and the lower is its fractionation factor. An unusually strong, low-barrier H-bond for H_a, assigned to Asp-99, is indicated by its chemical shift (18.2 ppm) which is 6.2 ppm downfield from that of carboxyl groups in non-aqueous solvents [∼12 ppm, Bovey et al. 1988)], its low fractionation factor (ϕ = 0.34) [on the basis of the criteria of Kreevoy and Liang (1980)], and its significantly slowed exchange rate with solvent (Table 2).

In crystals of small molecules studied by both X-ray diffraction and solid state NMR, McDermott and Ridenour (1996) have found a strong correlation of OH···O H-bond distances (D) with the chemical shifts (δ) of the protons in 59 H-bonds ranging in length from 2.43 to 2.71 Å, with chemical shifts between 21.1 and 11.8 ppm. By empirical least squares fitting (Leatherbarrow, 1992), we have found their 59 data points to be well fit (χ^2 = 0.00106) by eq 1,

$$D = 5.04 - 1.16 \ln \delta + 0.0447\delta \qquad (1)$$

Table 2: Assignments and Properties of the Three Deshielded Protons in the D38N Mutant of Δ^5-3-Ketosteroid Isomerase Complexed with the Intermediate Analog, Dihydroequilenin (DHE)

chemical shift δ (ppm)[a]	$\Delta\delta$ (ppm)[b]	H-bond interaction	fractionation factor (ϕ)	k_{ex} at 25 °C (s^{-1})[c]	protection factor[d]	Activation Parameters				
						E_a (kcal/mol)[e]	$\Delta H^{\ddagger}$ (kcal/mol)[f]	$\Delta S^{\ddagger}$ (cal/mol K)[f]	$-T\Delta S^{\ddagger}$ at 25 °C (kcal/mol)[f]	$\Delta G^{\ddagger}$ at 25 °C (kcal/mol)[f]
18.15 (H_a)	6.2	D99(COO**H**)···Y14	0.34 ± 0.02	1330	$\geq 10^{1.4}$	12.9 ± 1.2	12.3	−2.9	0.9	13.2
12.89 (H_b)	−0.5	H100(ϵ-N**H**)···?	0.79 ± 0.09	260	$10^{2.3}$	15.8 ± 1.2	15.2	3.4	−1.0	14.2
11.60 (H_c)	1.8	Y14(O**H**)···O$^-$−C3	0.97 ± 0.08	34	$10^{3.3}$	19.5 ± 0.5	18.9	11.7	−3.5	15.4

[a] Chemical shifts are with respect to 3-(trimethylsilyl)propronate-2,2,3,3-d_4 at −3.3 °C in presence of 9% (v/v) DMSO-d_6. [b] The $\Delta\delta$ was calculated from the chemical shift of the corresponding proton in organic solvent. [c] The exchange rates (k_{ex}) at 25 °C were calculated from the exchange contribution to the transverse relaxation rate as a function of temperature as shown in Figure 9. [d] Protection factor is the ratio of intrinsic exchange rate and the determined rate at the same temperature. This factor was calculated at −3.3 °C for H_a and H_b, and at 4 °C for H_c. [e] Activation energy, E_a, was calculated from an Arrhenius plot of the temperature dependence of the transverse relaxation rate as shown in Figure 9. [f] The exchange rates, and the rate of crossover, k_BT/h (6.2×10^{12} s^{-1}), were used to estimate the activation free energy.

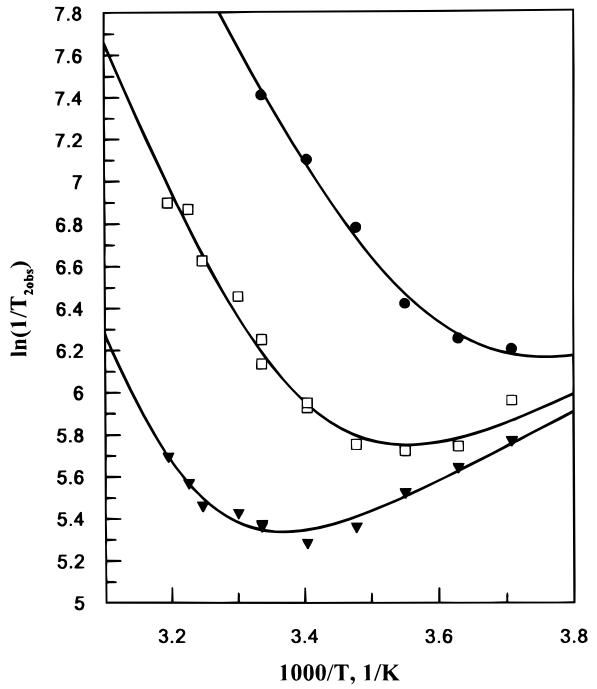

FIGURE 9: Arrhenius plots of the effect of temperature on the observed transverse relaxation rates ($1/T_2 = \pi \cdot$ line width) of H_a (18.15 ppm) (●), H_b (12.89 ppm) (□), and H_c (11.60 ppm) (▼). The average $1/T_2$ values of the two most downfield amides at 10.24 and 10.09 ppm, and the average $1/T_2$ of six upfield methyl signals (from −0.07 to −0.46 ppm) were used to derive the dipolar contribution to the line width. Lorentzian curve fitting of the spectra was used to determine the line widths of these resonances. Assuming the low-field resonances to have the same activation energy (−3.3 kcal/mol) for the dipolar contribution to $1/T_2$ as the average of the amide and methyl protons, the data for the three low field resonances were fitted to the Arrhenius form of the equation: $1/T_{2\ obs} = 1/T_{2(dipolar)} + k_{ex}$, yielding the exchange contribution from which the activation parameters and exchange rates were calculated (Zhao et al., 1996b). The curves through the data points are calculated for the composite results for both the dipolar and exchange contributions to $1/T_2$. The probe temperature was calibrated with neat methanol.

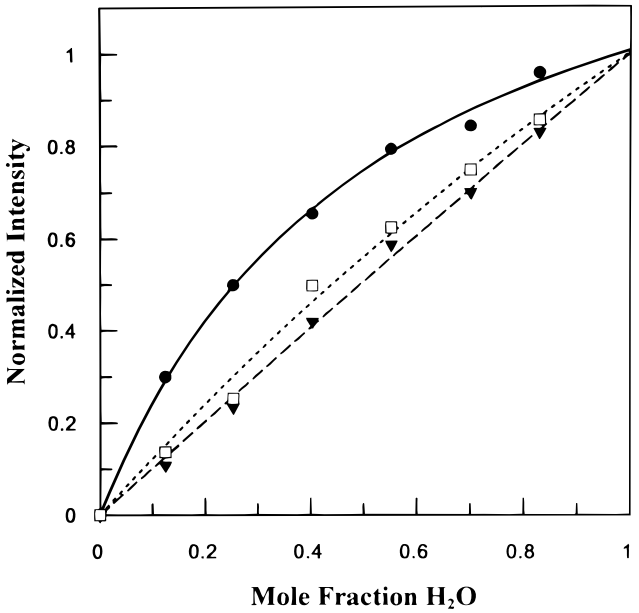

FIGURE 10: Signal intensities of deshielded protons in the D38N mutant complexed with the intermediate analog, DHE, as a function of the H_2O content in the H_2O/D_2O mixture. Intensities of H_a (●), H_b (□), and H_c (▼) are shown. Peak areas of the low-field resonances were normalized to the peak area of upfield non-exchangeable methyl signals (−0.07 to −0.46 ppm) in the same spectra. Nonlinear least squares fit of the data (Leatherbarrow, 1992) to the equation, $I_x = I_o x/[\phi(1 - x) + x]$, where x is the mole fraction of H_2O, I_x is the signal intensity in H_2O/D_2O mixture, and I_o is the intensity in H_2O, yielded the fractionation factor, ϕ = [Enz-D]-[H_2O]/[Enz-H][D_2O]. Fractionation factors for H_a, H_b, and H_c are 0.34, 0.79, and 0.97, respectively (Table 2).

where D is expressed in Å and δ is in ppm (see Figure S1 of the Supporting Information). From eq 1 the chemical shift of H_a of 18.15 ppm corresponds to an H-bond length of 2.49 ± 0.02 Å, consistent with a low barrier H-bond. The chemical shift of H_c (11.6 ppm), which is downshifted from an exposed tyrosine by 1.8 ppm, [δ = 9.8 ppm, Liepinsh et al. (1992)] corresponds to an O−H···O distance of 2.71 ± 0.04 Å, indicative of a normal H-bond between Tyr-14 and the enolate intermediate (Figure 1B). The fractionation factor of 0.97 ± 0.08 is consistent with a normal H-bond. The exchange rate of H_c with solvent is $10^{3.3}$-fold slower (at 4 °C) than those of tyrosine ζ-OH protons in unstructured peptides (Table 2), (Liepinsh et al., 1992) consistent with its participation in an H-bond and with its being buried in a hydrophobic cavity with a dielectric constant of ∼18 or lower.

Replacement of DHE (pK_a = 9.0) on the D38N mutant with 4-fluoroestradiol (pK_a = 7.4), which increases the difference in pK_a from that of Tyr-14 (pK_a = 11.6) and from that of Asp-99 (pK_a = 9.5), decreases the deshielding effects of the H-bonding on both H_a and H_c, upshifting H_a from 18.15 to 16.41 ppm and H_c from 11.60 to 10.60 ppm. From the correlation of O−H···O bond distances with chemical shifts (McDermott & Ridenour, 1996) (eq 1) these shift changes correspond to H-bond lengthenings of 0.04 Å for H_a and 0.06 Å for H_c. Thus, the H-bonds involving both H_a and H_c show comparable lengthening (or weakening), consistent with a tightly coupled catalytic diad (Figure 1B),

or less likely, with both residues interacting directly with the enolate intermediate (Figure 1A). In either case, however, the H-bond donated by Asp-99 would be much stronger than that donated by Tyr-14, contrary to the mechanism of Figure 1A.

The dielectric constant at the active site of free isomerase is ~18 by three independent measurements (Li et al., 1993). This value would be further decreased when water molecules in the active site are replaced by a nonpolar steroid molecule in the enzyme−substrate or enzyme−intermediate complex. The strength of an H-bond is substantially increased in nonpolar media (Zhao et al., 1995a; Shan et al., 1996; Shan & Herschlag, 1996). The hydrophobic active site cavity of isomerase is lined by phenylalanine and valine residues on one face of the six-stranded β-sheet which may exclude bulk solvent from the enzyme reaction center (Zhao et al., 1997; Wu et al., 1997). Mutation of one of the phenylalanine residues on this wall of the active site would be expected to increase the solvent accessibility, and increase the local dielectric constant, thereby weakening the H-bonding and lowering the catalytic power. The latter effect was detected by the progressive decrease in k_{cat} when Phe-101 was mutated to smaller residues, i.e., 30-fold with F101L and 270-fold with F101A (Brothers et al., 1995).

A recent quantum chemical calculation concluded that, in the hydrolysis of nitriles catalyzed by the Q19E mutant of papain, the differential transition state stabilization energy provided by a single H-bond is ≥8.5 kcal/mol (Zheng & Bruice, 1997), consistent with the experimental value of ≥7.6 kcal/mol which corresponds to a $\geq 10^{5.6}$-fold catalytic rate enhancement in the mutant (Dufour et al., 1995). Similarly, in the case of Δ^5-3-ketosteroid isomerase, a strong, low-barrier H-bond from Asp-99 coupled to a normal H-bond from Tyr-14 to the intermediate (Figure 1B) can account for the differential stabilization of the intermediate by ≥7.1 kcal/mol (Zhao et al., 1996b) consistent with a $10^{4.7}$-fold contribution to the overall catalytic power of this enzyme (Kuliopulos et al., 1990).

CONCLUSIONS

Four lines of evidence support the mechanism of Figure 1B involving a catalytic diad mediated by an unusually strong H-bond (Austin et al., 1992, 1995; Zhao et al., 1995a,b, 1996a,b), over that of Figure 1A involving separate H-bonds, normal in strength (Wu et al., 1997). First, H-bond donation by Tyr-14 is compensated by another H-bond donor as detected by UV resonance Raman (Austin et al., 1992, 1995) and UV absorption spectra of Tyr-14 with and without steroid binding (Zhao et al., 1995b). Second, the side chain of Asp-99 is close to the aromatic ring of Tyr-14 as indicated by NOEs from the Cβ protons of Asp-99 to Tyr-14 Hϵ (Figures 3 and 4, Table 1), and by the half quenching of the fluorescence of Tyr-14 on titrating a group in the free enzyme with a pK_a of 9.5 (Li et al., 1993), presumably Asp-99 (Wu et al., 1997). Third, the γ-COOH proton of Asp-99 is near the ζ-OH proton of Tyr-14 based on NOEs (Table 1, Figures 4 and 8). Fourth, the H-bond donated by Asp-99 is much stronger than that donated by Tyr-14 as indicated by the greater deshielding (6.2 versus 1.8 ppm) and lower fractionation factor (0.34 versus 0.97) of H_a in comparison with H_c. These two protons are linked in an H-bonded network since both of their resonances shift upfield on lowering the pK_a of the intermediate analog by 1.6 units. The shifts correspond to comparable lengthenings of the H-bonds donated by both Tyr-14 and Asp-99.

The low-barrier H-bond between Asp-99 and Tyr-14 in the catalytic diad (Figure 1B) makes Tyr-14 a better acid catalyst of the enolization reaction possibly by permitting partial protonation of the enolate intermediate, thus dispersing its negative charge over four oxygen atoms with the catalytic diad instead of over two oxygen atoms with tyrosine alone, in an otherwise hydrophobic environment. This effect is highly reminescent of the low-barrier H-bond in the catalytic triad of serine proteases, e.g., chymotrypsin, between Asp-102 and His-57, which makes His-57 a better base for deprotonating Ser-195, which in turn becomes a better nucleophile for attacking the peptide carbonyl group of the substrate (Frey et al., 1994; Cassidy et al., 1997).

Finally, as also suggested by Mollova et al. (1997), we note that highly deshielded proton resonances at 15−20 ppm assigned to strong H-bonds at the active sites of the enzymes Δ^5-3-ketosteroid isomerase (Figure 1B; Zhao et al., 1996b), triose phosphate isomerase (Harris et al., 1997), chymotrypsin (Cassidy et al., 1997), aspartate−amino transferase (Mollova et al., 1997), and 2-amino-3-ketobutyrate-CoA ligase (Tong & Davis, 1995) all involve carboxyl groups, suggesting a unique role for Asp and Glu residues in forming low-barrier H-bonds on enzymes.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

One figure (S1) showing a correlation of OH···O H-bond distances from X-ray diffraction with proton chemical shifts from solid state NMR (McDermott & Ridenour, 1996) fitted by eq 1 (1 page). Ordering information is given on any current masthead page.

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