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Ultraviolet Resonance Raman Spectroscopy of Δ^5-3-Ketosteroid Isomerase Revisited: Substrate Polarization by Active-Site Residues[†]

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ABSTRACT: The Δ^5-3-ketosteroid isomerase (EC 5.3.3.1) of *Pseudomonas testosteroni* promotes extremely rapid conversion of Δ^5- to Δ^4-3-ketosteroids by a conservative intramolecular proton transfer via an enolic intermediate. The competitive inhibitor 19-nortestosterone displays marked spectroscopic changes upon binding to the enzyme, but the mechanisms responsible for these changes have not been unequivocally established. Ultraviolet resonance Raman (UVRR) spectra are reported for 19-nortestosterone in acid solutions and for this ligand when bound to Δ^5-3-ketosteroid isomerase, as well as to its D38N and Y14F/D38N mutants. The frequencies of UVRR bands associated with C═O and C═C stretching can be used to monitor the state of polarization of the enone fragment of the steroid and the effects of the catalytic side chains, Tyr-14 and Asp-38, on these polarizations. Strong polarization is indicated by marked frequency downshifts of the C═O and C═C bands in the native protein; the downshifts are diminished by the mutations of these catalytic residues. The lower polarizing effects of the Y14F and D38N single mutants and the Y14F/D38N double mutant indicate that most of the polarization of the conjugated ketone is attributable to hydrogen-bond donation by the hydroxyl group of Tyr-14. A smaller contribution of Asp-38 is detected which is, in part, cooperative with that of Tyr-14. Reference spectra of hydrogen-bonded and protonated forms of 19-nortestosterone are reassigned, on the basis of the species identification of D. C. Hawkinson and R. M. Pollack [(1993) *Biochemistry 32*, 694−698]. On the basis of this reassignment, the strength of the 19-nortestosterone polarization produced by the native enzyme is intermediate between complete protonation and the hydrogen-bonding environment of 10 M hydrochloric acid. Since the UVRR spectrum of Tyr-14 is unperturbed upon binding of the steroid, the hydrogen bond to the carbonyl group of 19-nortestosterone may be compensated by a second hydrogen bond to Tyr-14 from another donor, possibly a backbone NH or a bound water molecule. This compensating hydrogen bond could lower the free energy of the enzyme transition state, in which the hydroxyl proton of Tyr-14 helps to dissipate the negative charge that accumulates on the steroid carbonyl group.

There is continuing interest in the mechanism whereby the enzyme Δ^5-3-ketosteroid isomerase (EC 5.3.3.1) from *Pseudomonas testosteroni* catalyzes the isomerization of Δ^5- to Δ^4-3-ketosteroids via a stereospecific and conservative transfer of the 4β-proton to the 6β-position (Pollack et al., 1989; Schwab & Henderson, 1990; Holman & Benisek, 1994; Brooks & Benisek, 1994). Two residues, Tyr-14 and Asp-38, have been determined to be critical to the mechanism by site-directed mutagenesis and various spectroscopic techniques (Kuliopulos et al., 1989). As illustrated in Figure 1, Asp-38 removes the 4β-proton from the steroid while, in the same step, Tyr-14 either protonates or donates a low-barrier hydrogen bond to the 3-carbonyl group, leading to enolization (Cleland, 1992; Cleland & Kreevoy, 1994; Li et al., 1993a; Zeng et al., 1992). Reketonization of this intermediate and proton transfer to the 6β-position complete the process.

The importance of Tyr-14 in stabilization of the transition state is indicated by the $10^{4.7}$-fold reduction of k_{cat} and 2.8-fold lowering of K_M when this residue is replaced by phenylalanine (Kuliopulos et al., 1989). Cholesterol oxidase, which places a water molecule rather than a tyrosine residue at this position (Li et al., 1993b) catalyzes a $10^{3.3}$-fold slower ketosteroid isomerase reaction (Smith & Brooks, 1977). The extent of the proton transfer and/or hydrogen bonding from Tyr-14 to the steroid carbonyl along the reaction coordinate of ketosteroid isomerase has been discussed extensively (Xue et al., 1990, 1991; Li et al., 1993a; Brooks & Benisek, 1994; Gerlt & Gassman, 1993; Cleland & Kreevoy, 1994). Additional insight into this issue may be gained from the strength of the Tyr-14−steroid interaction in the ground state. This interaction can be assessed by vibrational spectroscopy, since the internal vibrations of steroid and of the tyrosine are sensitive to the strength of the hydrogen bond. Austin et al. (1992) applied UVRR[1] spectroscopy to isomerase containing bound 19-NT, a competitive inhibitor resembling the enzyme product (Figure 2). From the downshifted C═O and C═C stretching frequencies of the inhibitor, relative to the frequencies seen in a series of solvents, they concluded that the α,β-unsaturated carbonyl group is strongly polarized

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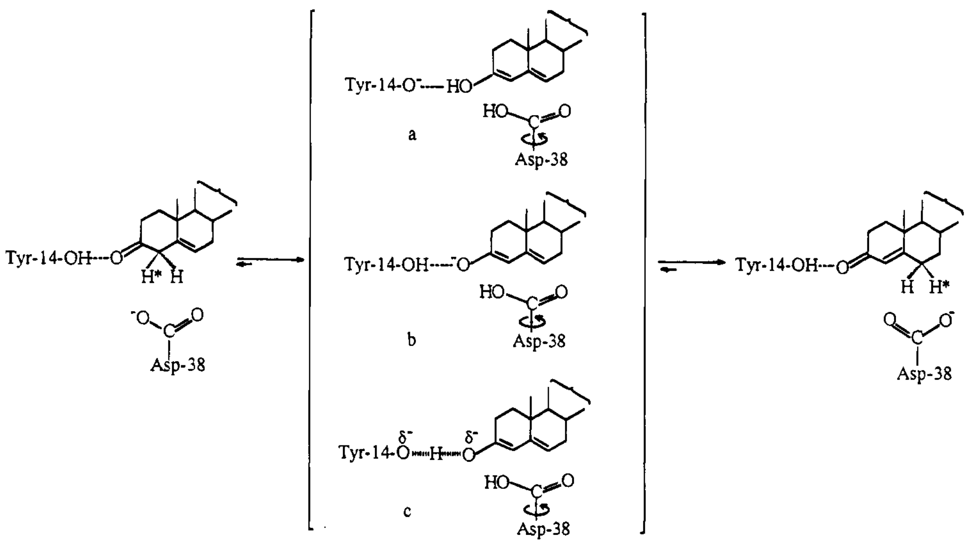
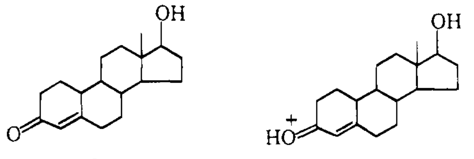


FIGURE 1: Isomerase-catalyzed conversion of Δ^5-ketosteroids to Δ^4-ketosteroids via an enolic intermediate. Dienol (a), dienolate (b), and a low-barrier hydrogen-bonded species (c) in the intermediate have been postulated in the catalysis (Cleland & Kreevoy, 1994; Gerlt & Gassman, 1993; Holman & Benisek, 1994; Li et al., 1993a; Xue et al. 1990, 1991; Zeng et al., 1992).



I II

FIGURE 2: 19-Nortestosterone (I) and its protonated form (II).

by the enzyme. UVRR bands of the Tyr-14 residue were unperturbed, however, including the Y8b band, which is known to be sensitive to hydrogen bonding (Hildebrandt et al., 1988; Rodgers et al., 1992). Austin et al. (1992) suggested that the hydrogen bond from Tyr-14 to the bound 19-NT might be compensated by another hydrogen bond to Tyr-14 from a second donor, forming a proton relay.

The reference UVRR spectra for this study included spectra of 19-NT (Figure 2, structure I) in 3.5 M and 6 M H_2SO_4, the UV absorption spectral shifts of which had been suggested (Kuliopulos et al., 1989) to reflect protonation of the steroid carbonyl in the first instance and a protonated homoannular dienol in 6 M H_2SO_4. Because the enone frequencies on the enzyme were lower than those observed in 3.5 M H_2SO_4, it was inferred that the polarization of the enone actually exceeds that produced by protonation. Subsequently, Hawkinson and Pollack (1993) concluded that the protonated homoannular dienol cannot be the species in concentrated H_2SO_4 because they found no deuterium incorporation upon quenching in D_2O and because other enones, in which dienol formation was blocked by methyl substituents, showed the same UV absorbance change in concentrated H_2SO_4. They concluded that this change reflected carbonyl protonation (Figure 2, structure II), whereas the smaller change seen in less strong acid was due to environmental effects and/or hydrogen bonding. This reassignment of the reference species led Hawkinson and Pollack (1993) to question the evidence for polarization in the enzyme and the need for a proton relay.

We have reinvestigated the UVRR spectra in the light of these new findings and have examined the mutants D38N and D38N/Y14F in addition to the Y55F/Y88F (which contains Tyr-14 as the single tyrosine residue) and Y14F mutants studied previously. While protonation does perturb the UVRR spectrum of 19-NT more than was previously thought, strong polarization is nevertheless evident when this steroid is bound to the enzyme. It is stronger, for example, than the hydrogen-bonding environment of 10 M HCl or of neat trifluoroacetic acid. The mutant data confirm a pronounced interaction of Tyr-14 with 19-NT, consistent with the large extent of proton transfer in the enzyme transition state (Xue et al., 1990, 1991; Brooks & Benisek, 1994). A compensating hydrogen bond to the Tyr-14 is still required to explain its unperturbed UVRR spectrum.

EXPERIMENTAL PROCEDURES

Materials. 19-Nortestosterone (19-NT) was a gift from Searle Chemical Co. (Chicago, IL) and was recrystallized from hexane/acetone prior to use. The sodium salt of 19-nortestosterone, obtained from Steraloids (Wilton, NH), showed a single spot on TLC ($R_f = 0.24$, developed with acetonitrile). D_2O (99.9 atom % D) was from Aldrich (Milwaukee, WI). LiCl (reagent grade) was purchased from Sigma. *Escherichia coli* strain JM 101 containing the pUC-19 plasmid with an insert at *Eco*RI and *Hin*dIII sites containing isomerase or its mutant gene were obtained as described (Kuliopulos et al., 1987a, 1989, 1990).

Protein Purification. Recombinant wild-type isomerase and the D38N mutant were prepared and purified to electrophoretic homogeneity as described (Kuliopulos et al., 1987a, 1989). Proteins were recrystallized 2 or 3 times and stored as crystalline suspensions at 4 °C in 30% saturated ammonium sulfate solutions neutralized with NH_4OH. The Y14F/D38N mutant was prepared according to described procedures (Kuliopulos et al., 1987), except that the bacteria were grown at 30 °C instead of 37 °C to give a slightly higher yield of the double mutant. There was no detectable isomerase activity for the purified Y14F/D38N mutant at

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[1] Abbreviations: UVRR, ultraviolet resonance Raman; FTIR, Fourier transform infrared; isomerase, Δ^5-3-ketosteroid isomerase (EC 5.3.3.1); 19-NT, 19-nortestosterone; 19-NTS, 19-nortestosterone 17-sulfate; Y55F/Y88F, mutant isomerase in which both Tyr-55 and Tyr-88 have been mutated to phenylalanine, so that Tyr-14 is the only tyrosine present; Y14F/D38N mutant isomerase in which Tyr-14 and Asp-38 have been mutated to phenylalanine and asparagine, respectively.

concentrations up to 3.0 mg/mL, although tight steroid bonding was preserved (Kuliopulos et al., 1990). For UVRR spectroscopic studies the crystalline enzymes were dissolved in 100 mM NaCl/50 mM sodium phosphate buffer at pH 8.5. At this pH, slightly higher solubility was achieved for the D38N mutant.

Protein Concentration and Purity. The crystalline suspensions in 30% saturated neutral ammonium sulfate solution were desalted and exchanged into 50 mM sodium phosphate buffer, pH 8.5 or 7.5, by passing through a Sephadex G-25M column (Pharmacia PD-10) or repetitive concentrating and diluting using a Centricon-10 microconcentrator (Amicon, Danvers, MA). UV spectra of the enzyme solutions were taken against the same buffer. Protein concentrations were calculated using the absorbance at 280 nm, assuming A_{280} values of 0.336, 0.343 and 0.226 for 1 mg/mL samples of wild-type, D38N, and Y14F/D38N enzyme, respectively (Benson et al., 1975; Kuliopulos et al., 1989). These concentration measurements were in good agreement with measurements of tyrosinate absorbance at pH 13 using $\epsilon_m = 2390\ \mathrm{M}^{-1}\ \mathrm{cm}^{-1}$ at 293 nm (Goodwin & Morton, 1946). All concentrations refer to the subunit concentration of the dimeric enzyme. The purity of wild-type isomerase and mutants was assessed by SDS−polyacrylamide gel electrophoresis and staining with Coomassie Blue R-250. In all cases a single major band (≥95% purity) migrated appropriately on the gel. The activity of isomerase was assayed in 50 mM Tris-HCl buffer, pH 7.5, with 58.2 μM 5-androstene-3,17-dione [1.7% (v/v) methanol] by observing A_{248} using 5−10 pM wild-type isomerase and 100−300 nM D38N mutant. Specific activity was assayed before and after the UVRR experiments, and in all cases at least 92% of the enzymatic activity was retained. UV spectra and activity assays were obtained on a Cary 1E spectrophotometer or a Hewlett-Packard 8451A diode array spectrophotometer.

Measurement of the Binding Constant between 19-Nortestosterone and the Y14F/D38N Mutant. The fluorescence of Tyr-55 and Tyr-88 can be efficiently quenched by 19-NT binding, as demonstrated in single and double tyrosine mutants of isomerase (Li et al., 1993a), and therefore the fluorescence quenching of these two tyrosines by 19-NT was used to determine the K_D of the steroid for the Y14F/D38N mutant. Fluorescence was measured on a Perkin-Elmer LS50 luminescence spectrometer interfaced to a personal computer using the data managing software provided by the manufacturer. Aliquots of 1 μL of 1.5 mM 19-NT in methanol were added to 1.0 μM Y14F/D38N mutant in 2.0 mL of 10 mM sodium phosphate buffer, pH 7.5, at 25 °C. After mixing, the fluorescence intensity was measured with an excitation wavelength of 277 nm and an emission wavelength of 305 nm. The titration was stopped when a plateau of the fluorescence was achieved. Since both free and bound enzyme contribute to the fluorescence, the following hyperbolic function was used to fit the fluorescence intensity as a function of 19-NT concentration (Li et al., 1993a):

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

where F is the fluorescence intensity, K_D is the dissociation constant, E is the enzyme concentration, NT is the concentration of 19-NT, and the subscripts f, b, and t refer to the free, bound, and total species, respectively. The nonlinear least-squares fit was carried out using GraFit (Leatherbarrow, 1992). The experiments were performed in triplicate.

UVRR Spectroscopy. Samples of 19-NT were prepared as 150 μM solutions from a stock solution of 10 mM steroid in methanol. All acidic solutions were made by dilution of the appropriate concentrated acids with H_2O or D_2O. UV absorption spectra of the 19-NT samples were obtained using a Hewlett-Packard 8451A diode array spectrophotometer with a resolution of 2 nm. 19-NT was added to enzyme solutions from a 10 mM stock solution in methanol to the desired concentration. For the D38N mutant, spectra with 19-NT/enzyme ratios of 0.25, 0.50, 1.0, and 1.5 were used to monitor the bound and free species. Whereas binding of 1 steroid molecule/subunit was established by UV and fluoresence studies (Kuliopulos et al., 1989), the present UVRR experiments also support this conclusion.

UVRR spectra were obtained using the output of an excimer-pumped dye laser (Lambda Physik LPX130/FL3002, Göttingen, Germany) operating at 300 Hz (Su et al., 1990). The 260- and 300-nm wavelengths were generated by frequency-doubling the output of coumarin 561 and rhodamine 610 dye, respectively, using a β-barium borate nonlinear optical crystal. The UV radiation was focused onto the surface of the sample using an approximately 135° backscattering geometry. 19-NT samples in various solutions other than the enzyme-bound complex were contained in a quartz cuvette equipped with a magnetic stirrer. Spectra for 19-NT bound to the wild-type and D38N mutant isomerases were obtained from flowing samples guided between two wires and circulated using a peristaltic pump. Because of limited sample availability, the spectrum for the Y14F/D38N mutant was obtained from an 0.5-mL sample in a spinning 5-mm quartz NMR tube. The buffer solutions containing bound 19-NT were 50 mM sodium phosphate at pH 8.5 for wild type isomerase and the D38N mutant, and pH 7.5 for the Y14F/D38N mutant.

The scattered light was collected by a Cassegrain mirror, dispersed using a 1.25-m monochromator (Spex, Metuchen, NJ) equipped with a 3600 grooves/mm holographic grating and analyzed by a diode array detector (Princeton Instruments). Raman spectra were collected using 0.3−0.5 mW average power with an accumulation time of 20−30 min. The spectrometer response was determined by measuring the throughput of a standard deuterium lamp and was used to correct the spectral intensities. At the end of the experiments, the catalytically inactive double-mutant enzyme remained intact as judged by the unchanged UV spectra of the complex.

Spectra were processed using Labcalc software (Galactic Industries Corp., Salem, NH). Wavenumber calibration of the 19-NT resonance Raman spectra was achieved using the known wavenumber values for the spectra of ethanol, dimethylformamide, toluene, and acetone. On the basis of the previously determined locations of the Raman peaks of 19-NT in ethanol (Austin et al., 1992), such spectra measured twice daily were also used to calibrate the spectra of the enzyme−steroid complexes in aqueous solution. Wavenumber shifts achieved with the same calibration have an accuracy of ±1 cm^{-1}, but the absolute wavenumber accuracy is estimated at ±2 cm^{-1}. Curve-fitting of the 19-NT Raman resonance spectra was accomplished using a minimal number of 50% Lorentzian/Gaussian components with constrained bandwidths, and the fitting routine converged on very similar fits despite varying initial inputs. The estimated curve-fitting accuracy is ±0.5 cm^{-1} for the intense C=C stretching band and ±1 cm^{-1} for the weaker C=O stretching band.

FTIR Spectroscopy. FTIR spectroscopy was performed on 19-NTS, a more soluble analog of 19-NT, using a 29 mM solution in D_2O. Substitution at the 17-position will have no effect on the vibrational properties of the enone group. The FTIR cell (Aldrich, Milwaukee, WI) consisted of two calcium fluoride windows separated by a 50-μm tin spacer. Data were collected using a 10-min acquisition time and the solvent spectrum was digitally subtracted using the D_2O band at ca. 1550 cm^{-1} as a reference. The spectrum was subsequently processed using Labcalc software.

RESULTS AND DISCUSSION

Tight Binding of 19-Nortestosterone to the Y14F/D38N Mutant. The fluorescence quenching titration of the Y14F/D38N mutant by 19-NT showed the binding of one steroid per subunit as reported for other mutants (Kuliopulos et al., 1989; Li et al., 1993a). Values of K_D = 0.92 ± 0.05 μM and 62% quenching at saturation were obtained from the titration data. Under the experimental conditions used for UVRR studies, 98% of 19-NT (100 μM enzyme + 50 μM 19-NT) and 91% of 19-NT (100 μM enzyme + 100 μM 19-NT) was bound to the active site of the double mutant.

Although the catalytic activity was completely abolished by mutating these two general acid and general base groups, the capacity for binding a steroid ligand at the active site was found to be well-preserved as demonstrated by EPR studies of the spin-labeled doxyl-dihydrotestosterone (Kuliopulos et al., 1990). The present study of the binding of 19-NT to the Y14F/D38N mutant confirms that the active site of the double mutant is intact. Interestingly, the K_D of 19-NT for the Y14F/D38N mutant is about 5-fold lower than that for wild-type isomerase. The tighter binding for 19-NT to the double mutant may be attributed to the greater hydrophobicity of the active site resulting from removal of two polar groups. In addition, the competing lattice of water molecules in the active site of free isomerase, as found for most enzymes, would be more loosely bound to the active site in the absence of two candidate groups capable of hydrogen bonding.

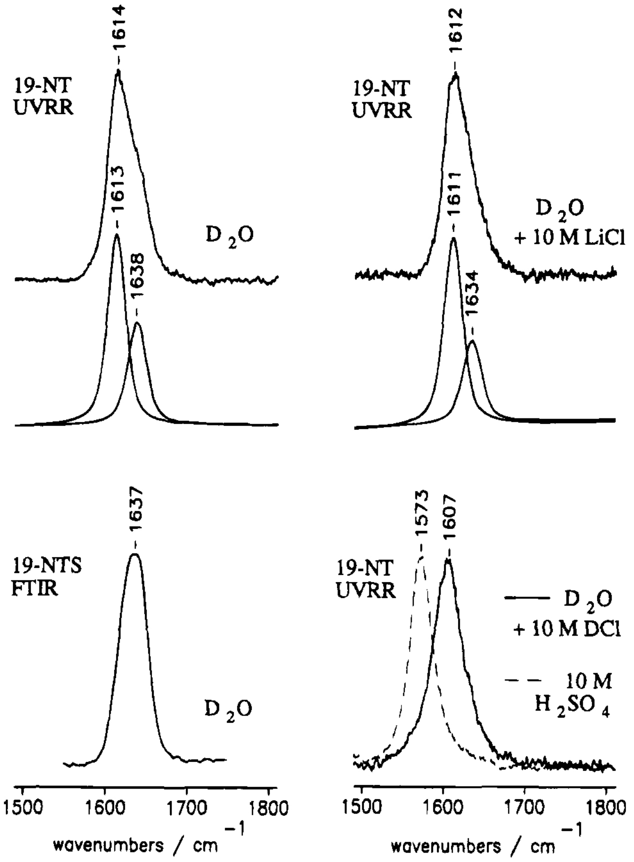

FIGURE 3: Enone vibrational bands in the UVRR spectra of 19-nortestosterone (0.15 mM) in D_2O, 10 M LiCl/D_2O, 10 M DCl/D_2O, and 10 M H_2SO_4, together with the FTIR spectrum of 19-nortestosterone 17-sulfate sodium salt (29 mM) in D_2O. The UVRR spectra were obtained with 260-nm excitation, except for the spectrum in H_2SO_4, which was obtained with 300-nm excitation. For the UVRR spectra in D_2O and 10 M LiCl/D_2O, the pair of bands assigned to C=C (stronger) and C=O (weaker) stretching were deconvoluted as shown. In 10 M DCl/D_2O, the C=O contribution could not be deconvoluted. In 10 M H_2SO_4, the band is symmetric and the C=O stretch is probably not resonance-enhanced.

Hydrogen Bonding and Protonation of 19-Nortestosterone in Aqueous Acid. Austin et al. (1992) examined UVRR spectra of 19-NT in acid solutions to assess the effect of protonation, but their assignments require revision in the light of the reassessment of the acid chemistry by Hawkinson and Pollack (1993). The UV absorption band of 19-NT has a maximum at 248 nm in H_2O, 258 nm in 10 M HCl, and 286 nm in 10 M H_2SO_4. Kuliopulos et al. (1989) ascribed the shift in 10 M HCl to protonation of the carbonyl and the larger shift in 10 M H_2SO_4 to formation of a protonated homoannular dienol. Hawkinson and Pollack (1993), however, found no deuterium incorporation into 19-NT when a solution in 12 M H_2SO_4 was rapidly diluted into D_2O. Had a protonated dienol been present, then deuterium should have been incorporated during reconversion to the parent steroid. In addition a strong red shift, from 242 to 276 nm, was also seen for a 10 M H_2SO_4 solution of 3,6,6-trimethyl-2-cyclohexen-1-one, in which the pair of methyl groups at the 6-position block dienol formation. The 286-nm absorption band of 19-NT in 10 M H_2SO_4 was therefore judged to arise from protonation of the carbonyl (Figure 2, structure II), while the shift to 258 nm in 10 M HCl was attributed to an environmental effect of the acid or to enhanced hydrogen bonding. A smaller medium effect was also seen in 10 M LiCl, which shifted the absorption maximum from 248 to 252.5 nm.

Figure 3 shows UVRR spectra in the region of the C=C and C=O stretching bands for 19-NT in D_2O, in 10 M LiCl/D_2O, in 10 M DCl and in 10 M H_2SO_4, together with the FTIR spectrum of 19-NTS in D_2O. D_2O was used for UVRR spectroscopy of the first three of these solutions to avoid interference from a broad weak band at 1640 cm^{-1} due to the H_2O bending mode. For 19-NT in D_2O, the UVRR spectrum showed the C=C stretch at 1613 cm^{-1} with the C=O stretch seen as a shoulder on the high-frequency side. Band deconvolution, illustrated in Figure 3, gave 1638 cm^{-1} as the C=O frequency (Table 1). The two frequencies are in good agreement with those reported earlier by Austin et al. (1992) in H_2O, 1613 and 1641 cm^{-1}, respectively, which conformed to a linear correlation between frequency and solvent acceptor number. Complementary information can be obtained from FTIR spectroscopy, where IR absorption

Table 1: Absorption Maxima and C=C and C=O Stretching Frequencies for 19-Nortestosterone in Solution

solution	λ_{max} (nm)	$\nu_{C=C}{}^{a}$ (cm^{1})^{a}	$\Delta\nu$ (cm^{-1})	$\nu_{C=O}{}^{a}$ (cm^{-1})^{a}	$\Delta\nu$ (cm^{-1})
D_2O	248	1613	0	1638	0
D_2O + 10 M LiCl	252	1611	−2	1634	−4
10 M DCl	258	1607	−6	*b*	
3.5 M H_2SO_4^{c}	260	1608	−5	*b*	
10 M H_2SO_4	286	1573	−40	*b*	
trifluoroacetic acid^{c}	*d*	1606	−7	*b*	

[a] Band frequencies were determined by curve-fitting the spectrum to a sum of 50% Lorentzian/Gaussian components with constrained bandwidths. The estimated frequency accuracy is ±0.5 cm^{-1} for the C=C stretch and ±1 cm^{-1} for the C=O stretch frequency. [b] The C=O stretching frequency could not be determined. [c] From Austin et al. (1992). [d] Absorption of solvent interfered with UV measurements.

will be strong for the stretching vibration of the polarized C=O but weak for the stretch of the C=C bond. In Figure 3 the FTIR spectrum of 19-NTS in D_2O shows a strong C=O stretch at 1637 cm^{-1}, which confirms the assignment from the UVRR spectrum.

When 19-NT is dissolved in nonaqueous solvents, these bands are found at higher frequencies and show a linear dependence on the solvent electron acceptor number, which is a measure of the Lewis acidity of the solvent [Figure 5 of Austin et al. (1992)]. Both frequencies decreased with increasing acceptor number, reflecting increased polarization of the enone π system, but the C=O frequency decreases faster than the C=C frequency. The correlations crossed at a frequency of 1607 cm^{-1}, when the acceptor number was 109. Trifluoroacetic acid, whose acceptor number (105.3) is close to the crossover value, shows a single Raman resonance band at 1606 cm^{-1} (Austin et al., 1992).

In 10 M LiCl/D_2O (Figure 3), 19-NT has a C=C frequency of 1611 cm^{-1}, which is 2 cm^{-1} lower than in D_2O (Table 1). The band is asymmetric on the high-frequency side, and the C=O vibration (1634 cm^{-1}) was resolved by deconvolution. In 10 M DCl solution, the band is symmetric, with a peak frequency of 1607 cm^{-1}, which is assigned to the dominant C = C mode. Essentially the same C=C frequency (1608 cm^{-1}) was reported by Austin et al. (1992) in 3.5 M H_2SO_4. A much lower frequency, 1573 cm^{-1}, is observed for 19-NT in 10 M H_2SO_4, the same frequency as reported in 6 M H_2SO_4 (Austin et al., 1992). In both studies, the mode associated with the 286-nm-absorbing species in the concentrated H_2SO_4 solutions was specifically enhanced by tuning the laser to 295 or 300 nm.

We adopt Hawkinson and Pollack's assignment of the 286-nm-absorbing species to the protonated carbonyl group of 19-NT. The 1573-cm^{-1} band, which is noticeably narrower than the bands of 19-NT in other aqueous solutions, is then the C=C stretch for this structure; the C=O stretch is probably at a still lower frequency but is not resonance-enhanced, due to the electronic effect of protonation. The 1611- and 1607-cm^{-1} bands in 10 M LiCl and 10 M DCl, respectively, or 3.5 M H_2SO_4 (1608 cm^{-1}), are the C=C stretches of unprotonated 19-NT which is subject to enhanced hydrogen bonding, relative to pure water. LiCl may alter the water structure, slightly increasing the polarization of 19-NT. Much larger polarizing effects are seen with 10 M DCl and 3.5 M H_2SO_4, indicating that the hydronium ions in the acid solutions form stronger hydrogen bonds to 19-NT than do water molecules, comparable in strength to that found in neat trifluoroacetic acid. The C=C frequency places the 10 M DCl and 3.5 M H_2SO_4 solution at the same average hydrogen-bond strength as trifluoroacetic acid (Table 1).

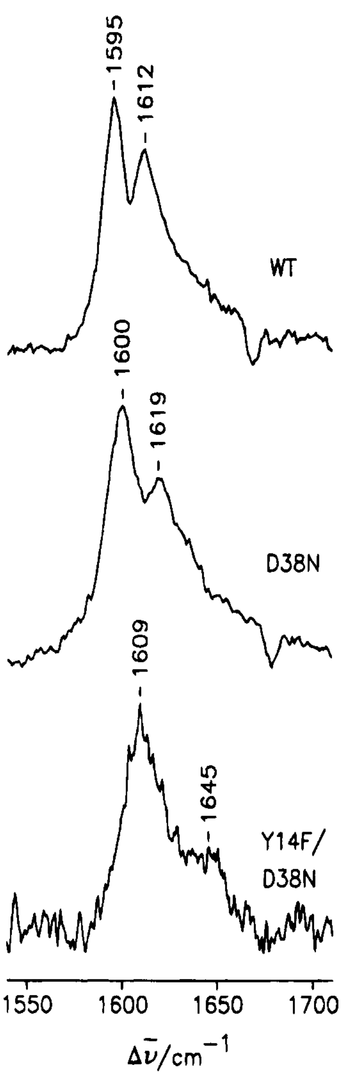

FIGURE 4: Enone vibrational bands in the UVRR spectra of 19-nortestosterone bound to wild-type isomerase (WT) and to the D38N and Y14F/D38N mutants, obtained with 260-nm excitation. The final concentrations of both enzyme and 19-NT in the mixtures were 100 μM.

Steroid Polarization by Wild-Type and Mutant Isomerase. Figure 4 compares the same region of the UVRR spectrum for 19-NT to wild-type isomerase and to the D38N and Y14F/D38N mutants. In each case, two bands are observed. The lower frequency and stronger band is assigned to the C=C stretch and the higher frequency and weaker band to the C=O stretch. Because of a weaker Raman scattered signal from 19-NT bound to the Y14F/D38N mutant, the C=O stretching band is overlapped with the H_2O band at ca. 1640 cm^{-1}. The frequencies are shown in Table 2, along with those of the Y14F mutant, determined by Austin et al. (1992). These residue replacements raise the C=C and C=O frequencies, implying reduced polarization of the enone moiety of 19-NT.

Hawkinson and Pollack (1993) discounted the UVRR evidence for unusual polarization of 19-NT by isomerase because of the misassignment of the species present in the acid solution. However, the reassignment of the identity of these species in solution in no way diminishes the very large

Table 2: Spectroscopic Properties of 19-Nortestosterone Bound to Wild-Type and Active-Site Mutants of Isomerase

isomerase	K_D (μM)	λ_{max} (nm)	$\nu_{C=C}$ (cm^{-1})	$\Delta\nu$ (cm^{-1})	$\nu_{C=O}$ (cm^{-1})	$\Delta\nu$ (cm^{-1})
wild type	4.4 ± 0.4[a]	258	1595	0	1612	0
Y14F[b]	35 ± 5[a]	248	1608	13	1641[c]	29
D38N	5.5 ± 2.0[d]	258	1600	5	1619	7
Y14F/D38N	0.92 ± 0.05	248	1609	14	1645[c]	33

[a] Li et al. (1993a). [b] Spectroscopic data from Austin et al. (1992). [c] The C=O stretching band is overlapped with the H_2O bending mode at ca. 1640 cm^{-1}. [d] Kuliopulos et al. (1989).

frequency depression observed upon binding to the wild-type protein. The C=C frequency, 1595 cm^{-1}, is intermediate between the protonated 19-NT frequency (1573 cm^{-1}, the frequency observed in concentrated H_2SO_4) and the frequency observed in 10 M HCl (1607 cm^{-1}). Relative to the water frequency (1613 cm^{-1}), the C=C downshift is 2.5 times as large in isomerase as in trifluoroacetic acid (1606 cm^{-1}), implying a much larger polarization effect. Most of this effect is clearly due to Tyr-14, since its replacement by phenylalanine raises the C=C stretch by 13 cm^{-1} and the C=O stretch by 29 cm^{-1} (Table 2). Thus, the hydrogen bond from the Tyr-14 hydroxyl group to the 19-NT is very strong.[2]

The effect of the Asp-38 residue is smaller, but not insignificant; its replacement by asparagine raises the C=C stretch by 5 cm^{-1} and the C=O stretch by 7 cm^{-1} (Table 2). Additional evidence for a direct effect of Asp-38 on the enone polarization is the fact that the C=C stretches in the complexes with wild-type protein and Y14F are lower than expected if the C=O frequency is used to place the data on the acceptor number correlation, as pointed out by Austin et al. (1992). Thus, the placement of the Asp-38 carboxylate group near the C=C bond reduces its stretching frequency beyond the reduction attributable to the effect of C=O polarization alone.

The Y14F/D38N double mutant does not show additivity for the two replacements. Indeed, the frequencies are only slightly higher than those of Y14F. Such partially additive effects of two mutations on a measured parameter are most simply ascribed to cooperative effects of the two residues on that parameter, and the departure from additivity measures the amount of cooperativity (Weber et al., 1990; Mildvan et al., 1992). Thus, for the C=C bond, the increases in frequency caused by the Y14F (13 cm^{-1}) and D38N mutations (5 cm^{-1}), when summed to 18 cm^{-1}, exceed that observed in the double mutant (14 cm^{-1}) by 4 cm^{-1} (Table 2). These results suggest that 4 cm^{-1} of the 13 cm^{-1} (31%) of the effect of Tyr-14 on polarizing the C=C bond is cooperative with Asp-38, and 4 cm^{-1} of the 5 cm^{-1} (80%) of the effect of Asp-38 is cooperative with Tyr-14. Similarly, for the C=O group, 3 cm^{-1} of the 29 cm^{-1} (10%) of the polarizing effect of Tyr-14 is cooperative with Asp-38, and 3 cm^{-1} of the 7 cm^{-1} (43%) of the polarizing effect of Asp-38 is cooperative with Tyr-14. The similarity of the cooperative portions of the polarizing effects on both the C=C and C=O bonds (3−4 cm^{-1}) supports the validity of this simple analysis, since the interaction of Tyr-14 with Asp-38 responsible for the cooperativity is expected to operate through both components of the conjugated enone system.

An alternative interpretation of the partial additivity is that the removal of both Tyr-14 and Asp-38 allow the bound steroid to interact with other polar residues in the vicinity of the active site. In the absence of the polarizing side chains of Tyr-14 and Asp-38, 19-NT might move into a position to optimize other polar interactions. However, three observations make this explanation less likely. The crystal structure suggests a predominantly nonpolar steroid-binding environment (Kuliopulos et al., 1987b), the local dielectric constant near Tyr-14 has been estimated to be 18 ± 2 by three independent methods (Li et al., 1993a), and the Y14F/D38N double mutant binds a spin-labeled steroid (Kuliopulos et al., 1990) in a manner indistinguishable from the wild-type enzyme, as reflected in identical paramagnetic effects on the longitudinal relaxation rate of water protons.

Hydrogen-Bond Compensation. Hydrogen-bond donation by Tyr-14 is expected to influence its own vibrational frequencies. The frequency of the Y8b band in the tyrosine UVRR spectrum is a useful monitor of the hydrogen-bond strength. A negative linear correlation is found with the enthalpy of hydrogen bonding for the tyrosine analog *p*-cresol in a series of hydrogen-bond acceptor solvents (Hildebrandt et al., 1988; Rodgers et al., 1992). Austin et al. (1992) examined the tyrosine bands in the UVRR spectrum of the Y55F/Y88F double mutant, in which Tyr-14 is the only tyrosine residue present, and found *no* downshift of Y8b upon binding 19-NT or 4-fluoro-19-nortestosterone (the UVRR frequencies of which also reveal strong polarization by the isomerase). To explain this result, Austin et al. (1992) proposed that a second hydrogen bond from a nearby donor to Tyr-14 compensates for the hydrogen bond to the steroid, thereby keeping the effective charge on the Tyr-14 oxygen atom unchanged. This compensating hydrogen bond could play a mechanistic role by lowering the free energy of the transition state as the tyrosine hydrogen bond accommodates the negative charge accumulating on the steroid carbonyl group (Gerlt & Gassman, 1993). Hawkinson and Pollack (1993) dismissed this "proton relay" proposal as being unnecessary if the substrate is not strongly polarized. Since, however, it *is* strongly polarized, the need for a compensatory effect to explain the unperturbed tyrosine spectrum remains.

Austin et al. (1992) speculated that a lysine side chain might provide the needed hydrogen-bond donor, because catalytic activity of isomerase decreases with a pK_a of 9.4−9.7 (Weintraub et al., 1970; Pollack et al., 1986) and because deprotonation of a group with a pK_a of 9.7 quenches Tyr-14 fluorescence by 50%, although the pK_a of Tyr-14 is 11.6 (Li et al., 1993a). None of the four lysines is close to Tyr-14 in the X-ray structure, but a substrate-induced conformational change is believed to occur (Kuliopulos, et al., 1989, 1991) and could bring the residues into juxtaposition. However, Li et al. (1993a) found only slight reductions in catalytic activity when the four lysines were mutated in turn to asparagine or leucine. The two tyrosines other than Tyr-14 were also found by mutagenesis to have only modest effects on catalysis. Since the three histidines all have pK_a values below 8 (Benisek & Ogez, 1982; Kuliopulos et al., 1987b, 1991), and the N-terminal amino group has a pK_a of 7.45 (Benisek & Ogez, 1982), all of the plausible candidates for an activity-determining group with pK_a 9.7 appear to have been ruled out, leaving a pH-induced conformational change

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[2] It has been estimated that Tyr-14 contributes at least 7.6 kcal/mol to the free energy of binding of the dienolate intermediate (Xue et al., 1991), which is consistent with a low-barrier hydrogen bond (Figure 1c).

as the most likely explanation (Li et al., 1993a). It is possible that a backbone amide NH or a bound water molecule provides the needed hydrogen bond to Tyr-14 and that this interaction is disrupted during the conformational change at high pH.

CONCLUSIONS

While protonation of 19-NT in solution has a larger effect on the enone vibrational frequencies than previously thought, the evidence is clear that isomerase polarizes the steroid strongly via the Tyr-14 and Asp-38 side chains, and these polarizing effects appear to be cooperative. Indeed, the net polarization at the enzyme active site can now be estimated as intermediate between outright protonation and the average hydrogen-bond environment in 10 M HCl or in neat trifluoroacetic acid. Thus, the Tyr-14 hydroxyl group hydrogen-bonds strongly to the steroid carbonyl group, even in the ground state, and is poised to interact more strongly with the developing negative charge on the 3-carbonyl oxygen in the transition state and in the intermediate of the enzymatic reaction.[2]

Despite this strong hydrogen bond, the Tyr-14 UVRR spectrum is unperturbed and does not show the expected downshift of the Y8b frequency. Thus, the tyrosine ring does not experience an increase in negative charge, implying that the donor hydrogen bond must be compensated by an acceptor hydrogen bond from another donor group. This compensation would assist catalysis by lowering the free energy cost of partially transferring the Tyr-14 proton to the steroid carbonyl group. Plausible donor candidates among the protein residues appear to have been ruled out, suggesting that the donor may be a backbone amide NH or a bound water molecule.

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