Characterization of the Three Tyrosine Residues of Δ^5-3-Ketosteroid Isomerase by Time-Resolved Fluorescence and Circular Dichroism†

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ABSTRACT: Δ^5-3-Ketosteroid isomerase (EC 5.3.3.1) of *Pseudomonas testosteroni* converts Δ^5-3-ketosteroids to Δ^4-3-ketosteroids via an enolic intermediate. Site-specific mutagenesis has identified Tyr-14 and Asp-38 as the catalytically essential general acid and base, respectively. Three tyrosine residues (Tyr-14, Tyr-55, and Tyr-88) are the only significant fluorophores in the wild-type isomerase. Recent studies of the steady-state fluorescence of the wild-type enzyme and all six mutant enzymes in which one or two tyrosine residues have been mutated to phenylalanine show that the fluorescence intensity of Tyr-14 is very high, that of Tyr-88 is very low, and that of Tyr-55 is intermediate and comparable to that of *N*-acetyltyrosine amide in solution (Li, Y.-K., Kuliopulos, A., Mildvan, A. S., & Talalay, P. (1993) *Biochemistry 32*, 1816–1824). Extension of these experiments by time-resolved fluorescence and fluorescence anisotropy measurements demonstrates that Tyr-14, which is in a hydrophobic environment, has an unusually long fluorescence lifetime (4.6 ns) as compared to Tyr-55 (2.0 ns) or Tyr-88 (0.8 ns) and to most protein tyrosine residues (0.2–2 ns). The Förster distances obtained from the absorption and emission of these tyrosines predict that total quenching of Tyr-14 fluorescence by Tyr-55, and to a lesser degree by Tyr-88, would occur if their orientations were favorable. The lack of efficient quenching of Tyr-14 fluorescence by Tyr-55 implies that Tyr-14 and Tyr-55 are oriented unfavorably for efficient resonance energy transfer and that this orientation is rigid on the time scale of picoseconds to nanoseconds. The rigidity of Tyr-14 and Tyr-55 with respect to one another is also confirmed by time-resolved fluorescence anisotropy at 20 and 40 °C, where only one correlation time corresponding to the global motion of the protein is resolved. Circular dichroism (CD) measurements on isomerase denatured by heat or guanidine hydrochloride have also confirmed that changes of Tyr-14 fluorescence in an isolated environment are tightly coupled to the changes in the protein structure and dynamics.

Most proteins contain tryptophan and/or tyrosine residues that exhibit unique fluorescence intensity or decay times (for review, see Beechem and Brand (1985) and Ross et al. (1991)). Fluorescence of these chromophores provides information about their local environments and is often used to monitor structural changes in proteins and the interaction of proteins with other molecules. Fluorescence spectroscopy can be utilized to analyze both local and global motions of proteins. Knowledge regarding the behavior of the excited states of the aromatic amino acids will make fluorescence a more useful tool for studies of proteins. Although important information has been obtained from studies of simple model compounds such as di- or tetrapeptides, it is clear that proteins provide unique environments for understanding the photophysics of tyrosine or tryptophan, and that additional work with proteins will be required before the photophysics of the aromatic amino acids in proteins can be understood.

The presence of multiple chromophores in most proteins has limited their usefulness as reporter groups. This is especially true for tyrosine residues, which are usually only weakly fluorescent in proteins and frequently overshadowed by tryptophan (Cowgill, 1976). Site-directed mutagenesis allows tyrosine or tryptophan residues to be inserted into or deleted from a desired site in a protein. This provides a powerful means to study the luminescence properties of these fluorophores at specific positions in proteins. Several groups have recently used this approach to study tryptophan fluorescence in proteins (Harris & Hudson, 1990; Royer et al., 1990; Smith et al., 1991; Axelsen et al., 1991; Kuipers et al., 1991; Hutnik et al., 1991; Royer, 1992; Locke et al., 1992; also see review by Hart et al. (1993)). The studies by Hutnik et al. (1991) deal with tyrosine substitution as well as tryptophan.

The Δ^5-3-ketosteroid isomerase (EC 5.3.3.1) of *Pseudomonas testosteroi* is a well-studied enzyme that catalyzes the isomerization of Δ^5-3-ketosteroids to Δ^4-3-ketosteroids (see reviews by Pollack et al. (1989) and Schwab and Henderson (1990)).[1] The active enzyme is a dimer. Each subunit contains three tyrosines and lacks tryptophan. The wild-type enzyme exhibits unusually strong tyrosine fluorescence (Wang et al., 1963; Li et al., 1993). Site-directed mutagenesis has provided mutant enzymes in which one or two tyrosines have been replaced by phenylalanine (Kuliopulos et al., 1989; Li et al., 1993). The kinetic properties of these mutant enzymes and

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[1] Abbreviations and definitions: isomerase, Δ^5-3-ketosteroid isomerase (EC 5.3.3.1); Gu-HCl, guanidine hydrochloride; WT, wild-type. The six mutants of isomerase in which one or two tyrosine (Y) residues have been replaced by phenylalanine (F) are designated as follows: single mutants, Y14F, Y55F, and Y88F, each of which contains two tyrosine residues; double mutants, Y14F/Y55F, Y14F/Y88F, and Y55F/Y88F, each of which contains a single tyrosine residue.

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the interactions of the individual tyrosine residues have been described. Recent steady-state fluorescence measurements (Li et al., 1993) showed that the fluorescence emission intensities of the three tyrosines differed by more than 10-fold and that there are interactions between Tyr-14 and Tyr-55 but not between Tyr-88 and the other tyrosine residues. Isomerase therefore provides an ideal experimental system with which to examine the environments of each tyrosine residue.

In the present study we examined in more detail the environments and interactions of these tyrosine residues, and the motions of these residues with respect to one another and the protein as a whole. The results are presented in the following order. First, we examine the time-resolved fluorescene parameters of each mutant enzyme and deduce the fluorescence properties of the individual tyrosine residues. Second, we analyze the interactions between the tyrosine residues. Third, we analyze these properties as a function of temperature and guanidine hydrochloride. Fourth, we monitor the motions of individual tyrosines. The aim is to obtain a comprehensive description of the fluorescence and dynamics of each tyrosine, especially those of Tyr-14, in the hope of obtaining a better understanding of the very efficient catalysis achieved by this enzyme.

MATERIALS AND METHODS

Construction and purification of tyrosine mutants of Δ^5-3-ketosteroid isomerase have been described (Kuliopulos et al., 1989; Li et., 1993). Pure enzymes were obtained by chromatography and several crystallizations. All measurements were made in 100 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, at pH 7.0. Protein solutions had absorbances of less than 0.1 at 280 nm. Protein concentrations were determined as described (Li et al., 1993).

Steady-state fluorescence was measured on an SLM 8000 photon counting fluorimeter with the excitation wavelength set at 280 nm. The quantum yield of each isomerase mutant was measured with tryptophan in water as a reference, which has a quantum yield of 0.14 at 25 °C (Eisinger, 1969). For the Gu-HCl titration, the emission wavelength was at 305 nm with a slit width of 8 nm. Fluorescence was corrected for buffer background and Gu-HCl contributions. The cuvette temperature was at 25 °C unless otherwise indicated. For thermal melting experiments, the solution temperature was monitored by a platinum probe and a transmitter (Omega Engineering) was used to transfer the temperature reading to a personal computer. Circular dichroism was measured on a Jasco J-710 spectropolarimeter at room temperature (25 °C). The CD signal was monitored at 220 nm as a function of Gu-HCl concentration. In both fluorescence and CD titrations, adequate time intervals (3–10 min) were allowed for the enzyme solution to reach a nearly steady-state signal after each Gu-HCl addition. For thermal melting experiments, the temperature in the cell holder was monitored by a thermocouple. The scan rate was 0.5–1 °C/min in both thermal melting and the CD experiments.

Time-resolved fluorescence was measured by a picosecond synch-pump dye laser system and a time-correlated single-photon counting apparatus (Badea & Brand, 1979; Wu et al., 1991). The overall system response is about 60 picoseconds. The excitation wavelength was at 285 nm except for the Y14F/Y55F mutant, for which both 285- and 295-nm excitations were used. For fluorescence decay measurements, the emission wavelength was at 307 nm with a polarizer oriented at the magic angle to eliminate any contribution from the motions of the enzyme. For fluorescence anisotropy measurements, vertical and horizontal film polarizers were mounted in two separate cuvette holders. Vertically and horizontally polarized fluorescence data were collected every 10 s in an alternating manner. A Ludox scattering solution was used to collect the system response. Duplicate or triplicate measurements were made.

The fluorescence decay of tyrosines was analyzed by a sum of exponentials as

$$I(t) = \sum \alpha_i \exp(-t/\tau_i) \tag{1}$$

where α_i and τ_i are the amplitude and lifetime, respectively, of the ith component. $I(t)$ was convoluted with the measured instrument response and then compared with the experimental data (about 2000 data points) by nonlinear least-squares methods. For all the decay data presented here, the reduced χ^2 of the analysis was between 1.0 and 1.2, with no systematic deviation either in the weighted residuals or in the autocorrelation of the residuals. The motion of each tyrosine as reflected in its fluorescence anisotropy was modeled as

$$r(t) = \sum \beta_j \exp(-t/\phi_j) \tag{2}$$

where β_j is the amplitude (or the initial anisotropy) associated with the motion having a correlation time ϕ_j. $r(t)$ is related to the difference data $D(t)$ by

$$D(t) \equiv I_{\parallel}(t) - I_{\perp}(t) = I(t)\, r(t) \tag{3}$$

where $I_{\parallel}$ and $I_{\perp}$ are fluorescence intensities with polarizations parallel and perpendicular, respectively, to the polarization of excitation beam. Similar to the case of $I(t)$ in eq 1, $D(t)$ was convoluted with the system response and the parameters in eq 2 were optimized by nonlinear least-square analysis.

The Förster distance, R_0, of a pair of tyrosines is calculated from (Förster, 1965)

$$R_0^6 = (8.785 \times 10^{-5}) \frac{\kappa^2 \Phi_D J}{n^4} \tag{4}$$

where Φ_D is the quantum yield of donor in the absence of acceptor; n, the index of refraction of solution (1.4); κ^2, the orientation factor between donor and acceptor; and J, the overlap integral. J is related to the normalized emission spectrum of donor, $F_D(\lambda)$, and the absorption coefficient of acceptor, $\epsilon_A(\lambda)$, by $J = \int F_D(\lambda)\, \epsilon_A(\lambda)\, \lambda^4\, d\lambda$. Energy-transfer efficiency, E, is related to the donor–acceptor distance r by $E = R_0^6/(R_0^6 + r^6)$.

RESULTS

Fluorescence Properties of Single and Double Tyrosine Mutants of Isomerase. Wild-type Δ^5-3-ketosteroid isomerase exhibits tyrosine fluorescence that is much less quenched than that of tyrosines in most proteins (Wang et al., 1963; Li et al., 1993). In the present study we compared fluorescence properties of the wild-type enzyme (which contains three tyrosines) with those of the tyrosine mutants that contain one (Y55F/Y88F, Y14F/Y88F, and Y14F/Y55F) or two (Y14F, Y55F, and Y88F) tyrosine residues (Li et al., 1993).

The emission spectra of various simple tyrosine-containing compounds are known to be similar in shape with only small variations in peak wavelengths (Cowgill, 1976). The spectral characteristics of individual tyrosines in proteins have not been compared, primarily because of difficulties in dissecting the contribution of each tyrosine residue. Figure 1 shows the

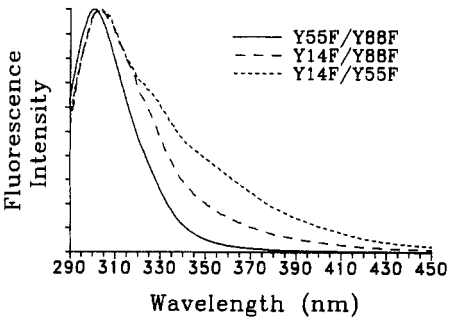

FIGURE 1: Comparison of (peak) normalized fluorescence emission spectra of the three double mutants containing single tyrosine residues: Tyr-14 in Y55F/Y88F (solid line); Tyr-55 in Y14F/Y88F (long dashed line); and Tyr-88 in Y14F/Y55F (short dashed line). Excitation was at 280 nm. The spectral shape of Tyr-14 is similar to that of free tyrosine in solution.

normalized (to peak intensity) emission spectra of the three isomerase mutants containing single tyrosine residues. The emission spectrum of Tyr-14 is homogeneous and is almost identical to that of free tyrosine in aqueous solution; that of Tyr-55 is broader; and that of Tyr-88 is much broader. Therefore Tyr 14, Tyr-55, and Tyr-88 are in different microenvironments in the protein. This finding is also consistent with differences in the UV adsorption spectra of these tyrosine residues (Li et al., 1993).

The steady-state and time-resolved fluorescence parameters of the wild-type isomerase and the six tyrosine mutants are summarized in Table 1. The quantum yields (Φ) of different tyrosine mutants differ by a factor of 5, in agreement with the results of Li et al. (1993). In the double mutants in which only one tyrosine is present, the quantum yield of Tyr-14 is the highest and that of Tyr-88 is the lowest. The quantum yields of the wild-type enzyme and three single mutants containing two tyrosine residues are intermediate between those of Tyr-14 and Tyr-88. These quantum yields are all linearly related to the average fluorescence lifetime (correlation coefficient $r = 0.988$). The average radiative lifetime of tyrosine obtained from the slope of this relation is 29 ± 2 ns. A linear correlation ($r = 0.994$; radiative lifetime 29 ± 3 ns) was also observed for the three mutant enzymes containing single tyrosine residues.

To evaluate the individual contribution of each tyrosine residue to the overall fluorescence of the wild-type enzyme and single mutants, we examined the decay characteristics of all mutants. The time-resolved fluorescence decays of the six tyrosine mutant forms were significantly different. While all mutant forms exhibited multiexponential decays, the degree of their heterogeneity (in terms of the number of exponentials) differed (Table 1). The photophysical heterogeneity is minimal when there is only one exponential decay. We use the standard deviation of the average lifetime of each mutant to examine its decay qualitatively. Among the double tyrosine mutants, the decay of Tyr-14 is the least heterogeneous. In the Y55F/Y88F mutant, Tyr-14 has a very long average lifetime, probably the longest among tyrosine residues in proteins (Ross et al., 1991). This lifetime is constant throughout the emission spectrum from 300 to 330 nm. The decay can be described by two exponentials, with one dominant component contributing 91% of the amplitude. The decay of Tyr-14 in the protein closely resembles that of *N*-acetyltyrosine amide in 2-propanol. The increase of tyrosine fluorescence decay in 2-propanol is consistent with previous steady-state fluorescence measurements (Cowgill, 1976; Li et al., 1993). Thus the hydrophobic environment at the active site is likely to contribute to the very high fluorescence of Tyr-14. It is not the only factor, however, since the fluorescence of tyrosine residues in the majority of proteins (Cowgill, 1976; Ross et al., 1991) is quenched although they may reside in hydrophobic environments similar to that surrounding Tyr-14 in isomerase. We are aware of only one other protein, parvalbumin, in which tyrosine fluorescence is greatly enhanced (Permyakov et al., 1985).

The physical significance of the two components in Tyr-14 fluorescence is difficult to interpret. It could be due to the rotameric distribution (Gauduchon & Wahl, 1978; Laws et al., 1986). The fluorescence decay of Tyr-14 can also be analyzed by a lifetime distribution model, either a Gaussian (mean lifetime 4.60 ns ± 0.53 ns), or a Lorentzian (mean lifetime 4.59 ns ± 0.10 ns). The standard deviation of the lifetime calculated from the results of multiexponential analysis (Table 1) is similar to that from a Gaussian lifetime distribution, and the heterogeneity of Tyr-14 fluorescence decay by either model seems to be small. Since tyrosine fluorescence is very sensitive to local environment, only minor variation in the milieu surrounding Tyr-14 is likely.

Tyr-55 also has a longer decay time than *N*-acetyltyrosine amide in water solution. Since Tyr-55 is located near the active site of the enzyme (Kuliopulos et al., 1987), the hydrophobic environment may also be responsible for the fluorescence enhancement of Tyr-55, similar to that of Tyr-14. The average lifetime is about the same across the emission spectrum. The decay of Tyr-55 fluorescence was also analyzed by a model of two exponentials and is more heterogeneous than that of Tyr-14 fluorescence (Table 1).

The quantum yield of Tyr-88 is very low, and its fluorescence decay is very heterogeneous, with a short average lifetime. In addition, it exhibits emission at longer wavelengths. Figure 2 shows the steady-state spectra and average lifetime as a function of emission wavelength. Upon excitation at 290 nm, the emission at longer wavelengths is enhanced relative to that at 305 nm. Similarly the excitation spectrum is red-shifted when monitored at 360 nm. These are normalized (to peak) spectra, and the relative fraction of the red-shifted species is difficult to estimate. Since there is no detectable energy transfer from Tyr-14 to Tyr-88 in Y55F and the absorption spectrum of Tyr-88 is not red-shifted (Li et al., 1993), the fraction of red-shifted species in Tyr-88 in all mutants is unlikely to be very large. Nonetheless it has some interesting features. There is only a minor shoulder around 350 nm, indicating that although there might be a distinctive emitting species, its spectrum is very broad. Furthermore, upon excitation at 295 nm, the average lifetime increases at longer emission wavelengths. Three exponentials were required to fit the decay curve, with the longest lifetime increasing from 4.8 ns at 320 nm to 5.8 ns at 440 nm. Upon excitation at 285 nm, the average lifetime increases by a factor of 3. Between 300 and 320 the average lifetime was quite short, but at longer emission wavelengths it increased, and it finally exceeded that observed with 295-nm excitation. Similar to 295-nm excitation, the longest lifetime component increased from 4.0 ns at 320 nm to 5.6 ns at 420 nm. Time-resolved fluorescence anistropy measurements (described in the following section) do not change across the emission spectra with excitation at any wavelength. This finding eliminates the possibility of impurities as major factors contributing to the heterogeneity of Tyr-88 fluorescence.

It is of interest to compare the apparent quantum yield of each individual tyrosine in the wild-type and in the mutant forms of the enzyme containing two tyrosines. Li et al. (1993) have shown that both Tyr-14 and Tyr-55 are partially quenched

Table 1: Fluorescence Parameters of Various Tyrosine Mutants of Isomerase[a]

enzyme	tyrosine residues present	quantum yield: Φ[b]	normalized amplitude: a_i[c]	lifetime: τ_i[c] (ns)	av lifetime: $\langle\tau\rangle$[c] (ns)	std dev: σ[d] (ns)	ratio: $\sigma/\langle\tau\rangle$
WT	14 + 55 + 88	0.08	0.21	0.59	2.4	0.79	0.33
			0.28	2.07			
			0.50	3.43			
Y14F	55 + 88	0.04	0.27	0.48	1.6	0.57	0.36
			0.36	1.43			
			0.37	2.50			
Y55F	14 + 88	0.12	0.22	0.88	3.7	1.53	0.41
			0.78	4.58			
Y88F	14 + 55	0.09	0.28	1.77	2.9	0.72	0.25
			0.72	3.38			
Y55F/Y88F	14	0.16	0.09	2.84	4.6	0.55	0.12
			0.91	4.75			
Y14F/Y88F	55	0.06	0.31	1.24	2.0	0.53	0.27
			0.69	2.38			
Y14F/Y55F	88	0.03	0.55	0.43	0.72	0.37	0.51
			0.43	0.91			
			0.022	3.83			

[a] All measurements were at 25 °C unless otherwise noted. See Materials and Methods. [b] Uncertainty in Φ is about 0.01. [c] Results of multiexponential analysis using eq 1. Average lifetime was calculated from $\langle\tau\rangle = \sum\alpha_i\tau_i$. [d] Standard deviation of the multiexponential lifetimes calculated from $\sigma^2 = \sum\alpha_i(\tau_i - \langle\tau\rangle)^2/(N-1)$, where N is the number of exponential terms.

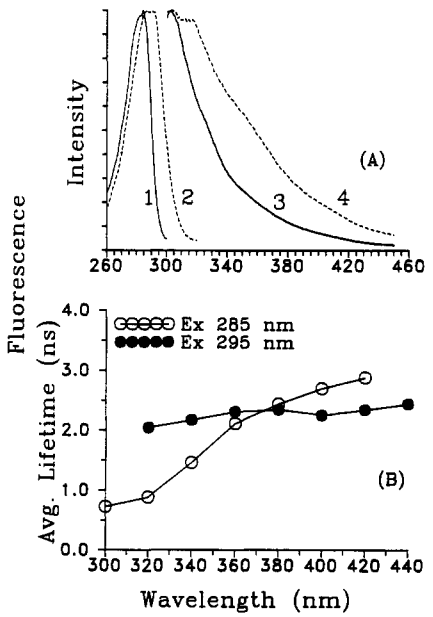

FIGURE 2: Steady-state excitation and emission spectra (A) and average emission lifetime of Tyr-88 in the Y14F/Y55F mutant as a function of wavelength (B). (A) Tyr-88 excitation spectra monitored at 310 nm (curve 1) and 350 nm (curve 2) and Tyr-88 emission spectra excited at 280 nm (curve 3) and 290 nm (curve 4). (B) Average lifetime of Tyr-88 with excitation wavelengths at 285 and 295 nm. Both A and B indicate heterogeneity in the Tyr-88 environment.

in the wild-type enzyme. Although the quantum yield of each tyrosine in the double mutants is in the order Tyr-14 > Tyr-55 > Tyr-88, their contributions are not always additive when two or more tyrosines are present (Li et al., 1993). For example, Y55F contains Tyr-14 and Tyr-88, and Y88F contains Tyr-14 and Tyr-55. Yet the quantum yield as well as the average lifetime of Y88F is lower than that of Y55F. The quantum yields of the tyrosines were calculated from a combination of the time-resolved and the steady-state data and are shown in Table 2. Within experimental uncertainty, the quantum yields of Tyr-55 and Tyr-88 remain the same in the wild type and all mutant enzymes. The quantum yield of Tyr-14 is reduced by about 26% at 20 °C in the presence Tyr-55 (in the wild type and Y88F mutant).

Possible Interactions between Tyrosine Residues. We now provide evidence that there is very little energy transfer between

Table 2: Fluorescence Quantum Yield of Each Tyrosine Residue in Isomerase

enzyme	quantum yield[a] Tyr-14	Tyr-55	Tyr-88
Y55F/Y88F	0.16		
Y14F/Y88F		0.06	
Y14F/Y55F			0.03
WT	0.12 ± 0.01	0.04 ± 0.02	0.04 ± 0.03
Y88F	0.12 ± 0.01	0.04 ± 0.02	
Y14F		0.04 ± 0.02	0.00 ± 0.03
Y55F	0.17 ± 0.02		0.05 ± 0.02

[a] The apparent quantum yields of Tyr-14, Tyr-55, and Tyr-88 in the wild type and single tyrosine mutants were calculated from the steady-state results by using, for example, $(\epsilon\Phi)_{Tyr55} = (\epsilon\Phi)_{Y8*F} - (\epsilon\Phi)_{Tyr14}$, in the mutant Y88F where only Tyr-14 and Tyr-55 are present. The following molar absorption coefficients (M^{-1} cm^{-1}) at 280 nm were used: WT, 4316, Y14F, 2815, Y55F, 2977; Y88F, 3293; Y55F/Y88F, 1859; Y14F/Y88F, 1736; and Y14F/Y55F, 1232 (taken from the absorption spectra of Li et al. (1993)).

tyrosines in isomerases in spite of their proximity of some of these residues. Failure of energy transfer is probably due to unfavorable orientation factors (close to zero in some cases). This also indicates that there is no orientational motion of the tyrosines on the nanosecond time scale.

The quantum yields of the individual tyrosines in the isomerase differ at least 5-fold in the double tyrosine mutants (Table 2). Long-range interaction such as resonance energy transfer would change the apparent quantum yield of tyrosines. The Förster distances between all tyrosine residues were calculated (Table 3) and found to be comparable to those obtained by Eisinger et al. (1969) for tyrosine pairs in proteins. These parameters define the distances at which the rate of resonance energy transfer equals that of fluorescence decay (for two tyrosines of the same type, e.g., Tyr-14 to Tyr-14, or of different types, e.g., Tyr-14 to Tyr-88). The distances from X-ray and NMR (Kuliopulos et al., 1987, 1991; Li et al., 1993) are, respectively, 6 Å between Tyr-14 and Tyr-55, 13.8 Å between Tyr-55 and Tyr-88, and 14.4 Å between Tyr-14 and Tyr-88. (The distance between Tyr-14 from one subunit to Tyr-14 from another in the dimer is much longer so that no interaction is expected, while the possible interaction between the two Tyr-88 cannot be resolved due to their low intensities.) If the tyrosines were free to move on the time scale of nanoseconds or less, the energy-transfer efficiencies

Table 3: Förster Distances[a] between Tyrosine Residues in Isomerase

tyrosine residues		overlap integral:	Förster distance:
donor	acceptor	$J^b \times 10^{-12}$	R_0 (Å)
Tyr-14	Tyr-14	2.10	13.1
Tyr-14	Tyr-55	1.59	12.5
Tyr-14	Tyr-88	0.77	11.1
Tyr-55	Tyr-14	1.40	10.4
Tyr-55	Tyr-55	1.07	10.0
Tyr-55	Tyr-88	0.52	8.8
Tyr-88	Tyr-14	2.14	10.0
Tyr-88	Tyr-55	1.70	9.6
Tyr-88	Tyr-88	0.88	8.6

[a] Calculated using $\langle\kappa^2\rangle = {}^2/_3$, $n = 1.4$, and the quantum yield at 25 °C. [b] In units of M^{-1} cm^{-1} nm^4. See Materials and Methods.

would be 99% from Tyr-14 to Tyr-55, 30% from Tyr-14 to Tyr-88, and 6% from Tyr-55 to Tyr-88, which would suggest strong, moderate, and weak interactions between these pairs, respectively. Experimentally, the fluorescene of Tyr-14 in the wild type and the Y88F mutant is quenched by about 26%, which is far from total quenching even if it is attributed entirely to energy transfer (which is not the case, as shown later). Since efficiency of energy transfer depends both on the distance between donor and acceptor and on their mutual orientation, the lack of efficient quenching of Tyr-14 fluorescence by Tyr-55 in the wild-type enzyme and the Y88F mutant implies that Tyr-14 and Tyr-55 are oriented unfavorably for energy transfer. The effective orientation factor can be back-calculated from the transfer efficiency and the approximate distance derived from X-ray and NMR structures (Kuliopulos et al., 1987, 1991; Li et al., 1993) to be $\langle\kappa^2\rangle$ = 0.0029 (instead of a dynamic average ${}^2/_3$). The above estimation is based on a simple calculation. If we consider the two state kinetics (forward and backward energy transfer) as well as the individual decays of each tyrosine, for example, Tyr-14 and Tyr-55, the orientation factor between the two tyrosines is about $\langle\kappa^2\rangle = 0.0043$ instead of 0.0029. By either calculation the orientation factor is very small, and it represents an upper limit since we assigned all the decreases in intensity to energy transfer. Therefore the motions of Tyr-14 and Tyr-55 are very restricted on the time scale of nanoseconds or less (otherwise a sufficient number of orientations would have been sampled to produce a larger orientation factor than the observed near-zero value).

The energy transfer between Tyr-14 and Tyr-88 is negligible (Table 1), thus setting the upper limit of E at 5%. Two factors may be responsible for the low transfer observed compared to that calculated. Similar to the energy transfer from Tyr-14 to Tyr-55, we may have an effective orientation factor smaller than ${}^2/_3$ in the enzyme, $\langle\kappa^2\rangle = 0.167$. Also, the index of refraction inside the protein may differ from that in solution since both probes are buried. For example, with a refractive index of 1.65 estimated from light scattering, the calculated transfer efficiency between Tyr-14 and Tyr-88 is reduced from 30% to 10% (the increase in n change less than 1% in the calculated transfer efficiency between the Tyr-14 and Tyr-55 pair). With a slightly nonrandom orientation factor, no energy transfer from Tyr-14 to Tyr-88 should be detected.

Stability of Ketosteroid Isomerase Mutants against Guanidinium Denaturation. The intrinsic fluorescence of proteins provides a signal commonly used to monitor conformational changes and unfolding. For this reason we determined how each tyrosine substitution influences protein denaturation. Unfolding is measured by changes in circular dichroism and also by changes in tyrosine fluorescence. In this way both the influence of the mutation on unfolding behavior and also the sensitivity of each tyrosine residue to unfolding can be evaluated.

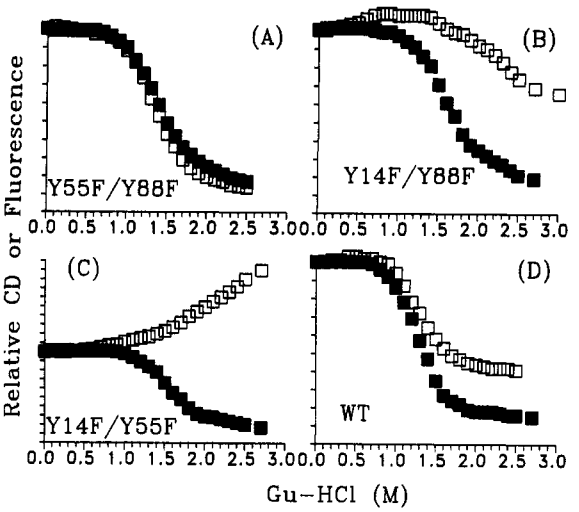

FIGURE 3: Gu-HCl denaturation of the wild-type and single tyrosine containing mutants as monitored by tyrosine fluorescence at 310 nm (open squares) and by CD (filled squares).

The guanidine denaturation of the wild type and three double tyrosine mutants is shown in Figure 3. The wild-type enzyme is denatured with a midpoint of the unfolding transition at about 1.3 M guanidine. All mutants are slightly more stable than the wild type except Y88F, which exhibits the same stability as the wild type. In the wild-type enzyme and the Y55F/Y88F mutant, the changes in tyrosine fluorescence follow the protein denaturation and the fractions of the native protein calculated from the fluorescence and from the CD values are similar at each Gu-HCl concentration. On the other hand, neither the fluorescence of Tyr-55 in Y14F/Y88F nor that of Tyr-88 in Y14F/Y55F follows the change in protein structure. Consequently the change of fluorescence in the wild-type enzyme arises mainly from Tyr-14. The denaturation of Y55F/Y88F by Gu-HCl was reversible as measured by time-resolved fluorescence, although longer times (more than 24 h) in the denaturant solution led to aggregation.

Temperature Dependence of Tyrosine Fluorescence in Isomerase. Temperature perturbation provides another means to gain insight into factors influencing tyrosine fluorescence in the isomerase. Thermal denaturation of the wild type and the Y55F/Y88F mutant was monitored by fluorescence and by CD measurements (Figure 4). The Y55F/Y88F mutant was denatured with a midpoint of transition at 58–59 °C based on CD measurement. The fluorescence intensity of Tyr-14 in this mutnat decreased linearly as function of temperature primarily due to increased thermal quenching, and then it decreased in a two-state transition with a midpoint at about the same temperature as obtained from CD (transition temperature was determined from the base lines in the native and denatured states). As for Gu-HCl denaturation, the change of Tyr-14 fluorescence was coupled to the disruption of the secondary structures of the protein. In both CD and fluorescence measurements, the protein became irreversibly aggregated after it was thermally denatured. In contrast, the change in fluorescence of the wild-type isomerase does not follow the structural breakdown of the protein (compared with CD in Figure 4B): the fluorescence intensity (primarily from Tyr-14) decreased much faster than did that of the single Tyr-14 present in the Y55F/Y88F mutant, indicating that additional quenching factors may be involved.

We also measured the thermal denaturation of other mutants (Figure 5). Y55F, like the Y55F/Y88F mutant, shows a

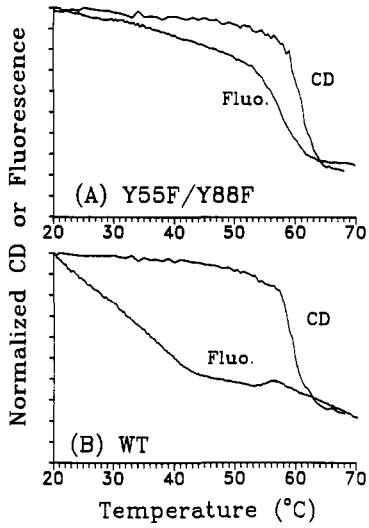

FIGURE 4: Temperature dependence of tyrosine fluorescence and CD signals of the Y55F/Y88F mutant (containing only Tyr-14) (A) and the wild-type enzyme (B). Scan rate in temperature was 0.5–1 °C/min.

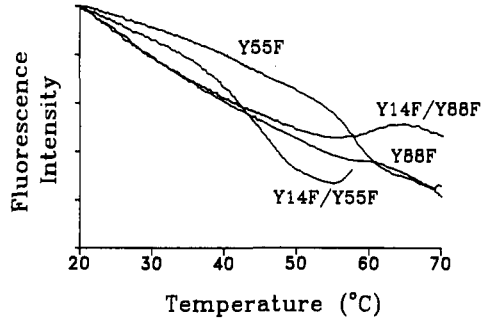

FIGURE 5: Temperature dependence of tyrosine fluorescence of the Y55F, Y88F, Y14F/Y55F, and Y14F/Y88F mutants. The apparent intensity increase near the melting points is due to increased light scattering from protein aggregation.

structural transition due to disruption of the secondary structure of the protein. The changes in fluorescence intensity of the Y88F, Y14F/Y88F, and Y14F/Y55F mutants, however, did not correlate with denaturation of the enzymes. The Y88F mutant (containing Tyr-14) showed no cooperative transition. Earlier studies (Cowgill, 1976) had shown that the slope of temperature dependence of tyrosine fluorescence in solution or in proteins varies from 0.8 to 1%. The initial slope (normalized to intensity at 20 °C) of fluorescence change as a function of temperature in the Y55F/Y88F mutant is 0.6%, and that of Y55F is 0.9%. These values agree with those of tyrosines in solution and in other proteins studied. The slopes of fluorescence dependence on temperature of the wild-type enzyme and Y88F and Y14F/Y88F mutants are higher: 2.4% in the wild type and 2.1% in both Y88F and Y14F/Y88F. In the Y14F/Y55F mutant (containing only Tyr-88) the initial slope is intermediate and, at temperatures between 40 to 50 °C, the slope increases from 1.4% to 2.6%, while the protein is still native.

Fluorescence Anisotropy. The motions of Tyr-14, Tyr-55, and Tyr-88 could be coupled to the global or internal motions of the protein. We used time-resolved fluorescence anisotropy of the individual tyrosine residues to analyze these relations. Table 4 shows the anisotropy parameters of the three mutants containing single tyrosine residues. Both Tyr-14 and Tyr-55 show only one correlation time, indicating that there is no internal motion of either of these residues within the resolution time of the instrument (less than 60 ps without convolution).

Table 4: Anisotropy Parameters of the Three Mutant Enzymes Containing Single Tyrosine Residues

enzyme	initial anisotropy: r_0[a]	correlation time: ϕ[a] (ns)	hydrodynamic radius a[b] (Å)	a_{calc}[c] (Å)
Y55F/Y88F	0.30	18.0	26	23
Y14F/Y88F	0.29	16.8	25	23
Y14F/Y55F	0.05	0.60		
	0.14	17.9	26	23

[a] Initial anisotropy and correlation time obtained from data analysis of anisotropy decay at 20 °C. [b] Calculated from the longest correlation time of each mutant. [c] Calculated from the molecular weight, partial specific volume, and hydration of the protein.

The initial anisotropy of about 0.30 is lower than the theoretical maximum 0.4 and may be due either to picosecond fast motions in the protein or to the artificial depolarization in the film polarizers used. We can rule out picosecond motions as the source of the initial depolarization. If we assume that the initial depolarization of Tyr-14 and Tyr-55 is due to picosecond motions, then the minimum value of the orientation factor can be determined (Dale et al., 1979) from the initial anisotropy of Tyr-14 and Tyr-55 to be $\langle\kappa^2\rangle_{min} = 0.0942$, which is much larger than the maximum, 0.0043, deduced from the energy-transfer results. The lowest electronic transition dipole is oriented perpendicular to the symmetry axis of the phenol ring (Hooker & Schellman, 1970); the lack of motion implies that both Tyr-14 and Tyr-55 are rigidly held in almost all directions on the time scale of picoseconds and nanoseconds. Thus fast motions are unlikely to exist to an appreciable level in Tyr-14 and Tyr-55 in the enzyme.

The long correlation time reflects the global motion of the protein. The equivalent hydrodynamic radius, a, of the dimeric protein is $a = (3kT\phi/(4\pi\eta))^{1/3}$ (where η is the solution viscosity). With a correlation time of 18 ns, the radius is calculated to be 26 Å at 20 °C. The equivalent hydrodynamic radius can also be calculated from the hydrated protein (Cantor & Schimmel, 1980). Assuming that the specific partial volume of the protein is 0.73 cm^3/g, and the fraction of hydration is 0.4 g of water/(g of protein), the radius is 23 Å for a dimer with a molecular weight of 26 800. The difference between the radius calculated from hydration and from anisotropy is due to the asymmetric shape of the protein (Cantor & Schimmel, 1980), which is known from the X-ray structure (Westbrook et al., 1984; Kuliopulos et al., 1987).

From early studies by gel filtration and sedimentation (Tivol et al., 1975; Benson et al., 1975) is is known that the protein remains in a dimeric form at very low concentrations, indicating very tight association between the monomers. To determine whether dissociation is coupled to the protein denaturation, monomer–dimer association was monitored by time-resolved fluorescence anisotropy measurements. Figure 6 shows the correlation time of each single tyrosine-containing mutant as a function of Gu-HCl concentration. The correlation time of TYR-14 and Tyr-55 remains constant when the proteins are in the native state. It decreases only when the proteins are denatured, under such conditions that segmental or internal motions dominate the fluorescence anisotropy. The initial anisotropy of either Tyr-14 or Tyr-55 remains flat up to 1 M Gu-HCl. Thus the dimeric protein remains intact until the protein is denatured. Similar results were obtained for Tyr-88 at lower Gu-HCl concentrations; at higher Gu-HCl concentrations the protein aggregated (the concentration of Y14F/Y55F was higher, about 10–20 μM in the anisotropy measurements, because of its low quantum yield).

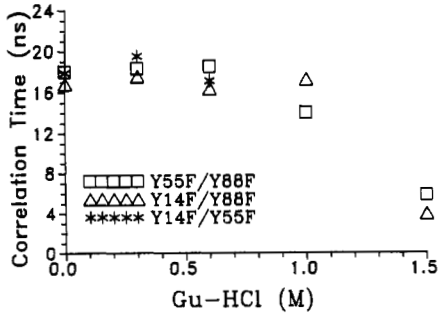

FIGURE 6: Correlation time of three single tyrosine containing mutants as a function of Gu-HCl concentration. One correlation time was obtained from 0 to 1 M Gu-HCl for the Y55F/Y88F and Y14F/Y88F mutants, and two correlation times were obtained at 1.5 M Gu-HCl. Two correlation times were obtianed for Y14F/Y55F. The correlation times refer to the global motion of the protein. Protein concentration was in the range of 5–15 μM. Solution temperature was at 20 °C.

DISCUSSION

There is a substantial difference in the fluorescence decay behaviors of the three tyrosine residues of the isomerase.

Tyrosine 14. When examined in the Y55F/Y88F mutant, this residue shows a very high quantum yield and a long lifetime. The high fluorescence of Tyr-14 (and of Tyr-55 in Y14F) in isomerase and that of a tyrosine residue of parvalbumin (Permyakov et al., 1985) are exceptions to the common observation that tyrosine fluorescence is severely quenched in most proteins (Cowgill, 1976; Ross et al., 1991). Three factors probably account for the unusually high fluorescence of Tyr-14. (a) The hydrophobic environment at the active site (Li et al., 1993) appears to be the most important factor. (b) The absence of nearby quenching groups probably contributes to the long fluorescence lifetime of Tyr-14 in the Y55F/Y88F mutant. (c) The rigidity of Tyr-14 in the protien is probably also important since it minimizes the opportunities for interactions of the phenol ring with potential quenching groups.

The fluorescence of Tyr-14 in the wild type and Y88F mutant enzyme is partially quenched compared to that of Y55F/Y88F mutant containing only Tyr-14 (Table 2), and the degree of quenching is larger at higher temperatures (Figures 4 and 5). What factors are responsible for quenching Tyr-14 fluorescence? The temperature dependence of the fluorescence of Tyr-14 makes it unlikely that direct quenching by resonance energy transfer to Tyr-55 is a major factor in the suppressino of Tyr-14 fluorescence in the temperature range of the native state. For example, the ratio of apparent orientation factors deduced from fluorescence change between 20 and 40 °C is $\langle\kappa^2\rangle_{20}/\langle\kappa^2\rangle_{40} = 0.25$, i.e., a change of almost 4-fold. On the other hand, the initial anisotropy of Tyr-14 changed from 0.30 to 0.29 and that of Tyr-55 from 0.30 to 0.26, at 20 and 40 °C, respectively (with one correlation time). This can change the minimum orientation factor by about 20%. Hence the temperature-dependent quenching cannot be attributed entirely to increased motions of the tyrosine residues. Conceivably, other quenching group(s) may be close to Tyr-14 when Tyr-55 is present in the wild-type and Y88F mutant enzymes. Phenylalanines at the active site might produce a temperature-dependent quenching of Tyr-14 fluorescence by partial stacking interaction. Since quenching is absent in the Y55F and Y55F/Y88F mutants, the change of interaction is very small and remains to be solved.

The inefficient quenching of Tyr-14 fluorescence by Tyr-55 does not itself define precisely the geometry of the two tyrosines since a large number of orientations exist that lead to zero values of orientation factor and prohibit energy transfer (Haas et al., 1978; Dale et al., 1979; Wu & Brand, 1992). One such condition is the perpendicular orientation of the two transition dipoles of the tyrosine residues, which may be the case in isomerase (Kuliopulos et al., 1989).

The rigidity of Tyr-14 on the time scale of pico- and nanoseconds implies that any significant conformational change in this residue occurs at longer time scales. It is known that the catalysis of the isomerase is very efficient and probably involves a conformational change in the active residue Tyr-14 (Kuliopulos et al., 1991) on a time scale as short as microseconds. Thus the dynamics of Tyr-14 in the catalysis is an activated process, and it may be induced by substrate binding (Kuliopulos et al., 1991).

Tyrosine 55. Tyr-55, like Tyr-14, is in a hydrophobic environment, and its fluorescence is enhanced in the Y14F/Y88F and Y14F mutants. Its lower quantum yield (compared to that of Tyr-14) is probably due to the presence of nearby quenching groups. This may be inferred from the temperature dependence of Tyr-55 fluorescence (Figure 5). Studies of the three mutant enzymes containing only single tyrosine residues demonstrate that Tyr-55 has the highest temperature coefficient, which is clearly caused by the proximity of quenching group(s) since Tyr-14 and Tyr-88 display only general thermal quenching effects.

The changes in Tyr-55 fluorescence as a function of either temperature or Gu-HCl exposure do not correlate with the structural breakdown of the enzyme, even though the chromophore is tightly held in the protein in the native state. In addition, the absence of orientational motion of Tyr-55 demonstrates that if any motion of the phenolic ring exists, it is limited to local vibrations. The lack of efficient quenching of Tyr-14 fluorescence by Tyr-55 implies that both residues exist in a unique orientation.

Tyrosine 88. Tyr-88 is near the surface of the enzyme, and thus its fluorescence is readily subject to quenching by other groups. The heterogeneity of Tyr-88 fluorescence decay appears to be the result of local conformational heterogeneity and multiple emitting species. The species emitting at long wavelengths is likely to result from dipolar relaxation surrounding Tyr-88 on a time scale comparable with that of the fluorescence decay. The wavelength-dependent lifetime indicates a continuous relaxation process (Mazurenko & Bakhshiev, 1970). Because of the complexity of the decay and the presence of tyrosine emission, it is difficult to extend the analysis in a more quantitative way. Dipolar relaxation in proteins has been observed in the presence of bound dyes (Gafni et al., 1977). The overall lwo quantum yield of Tyr-88 implies that the fluorescence is efficiently quenched by nearby groups, including possibly Glu-87 (Li et al., 1993). The excitation-dependent lifetimes imply that there are more than one red-emitting species. Hydrogen-bonded Tyr-88 may be responsible for the emission. Although tyrosinate is essentially nonfluorescent in aqueous solution (Ross et al., 1991) with a picosecond decay (Willis et al., 1990), higher quantum yield and longer decay time are possible in a more hydrophobic environment (Eisinger et al., 1969).

CONCLUSIONS

The results of the present study can be summarized as follows. The Tyr-14 residue has the following characteristics: (a) it has a high quantum yield (0.16) and a long decay time (4.5 ns) in an isolated environment in the double mutant form of the enzyme; (b) its fluorescence is partially quenched

in the wild type and Y88F mutant when Tyr-55 is present; (c) the quenching is temperature dependent (in addition to general thermal quenching); (d) resonance energy transfer to Tyr-55 is not efficient with an orientation factor close to zero; (e) it is rigidly held inside the protein with no detectable motion on the time scale of picoseconds to nanoseconds; (f) when present alone, its fluorescence intensity change parallels the denaturation of the protein by heat and Gu-HCl; (g) its fluorescence intensity change in the wild-type enzyme parallels the protein denaturation by Gu-HCl, but not by temperature; (h) replacement of Tyr-14 by phenylalanine leads to a more stable enzyme; and (i) the dimeric form of the protein does not dissociate until denaturation occurs.

The Tyr-55 residue is characterized as follows: (a) it has a moderate quantum yield (0.06) and lifetime (2 ns) in an isolated environment in the double mutant form of the enzyme; (b) its fluorescence is about equal in the wild-type and Y88F mutant enzymes; (c) its fluorescence intensity is highly temperature dependent, suggesting the presence of other nearby quenching groups; (d) it is rigidly held inside the protein with no detectable motion on the time scale of picoseconds to nanoseconds; and (e) its fluorescence intensity change is not correlated with protein denaturation either by Gu-HCl or by temperature.

The Tyr-88 residue has the following features: (a) it has a low quantum yield (0.03) and a short decay time; (b) the decay time is wavelength dependent; and (c) there are small fractions of red-emitting species.

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