Ultraviolet Spectroscopic Evidence for Decreased Motion of the Active Site Tyrosine Residue of Δ^5-3-Ketosteroid Isomerase by Steroid Binding†

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ABSTRACT: Δ^5-3-Ketosteroid isomerase (EC 5.3.3.1) from *Pseudomonas testosteroni* catalyzes the highly efficient conversion of Δ^5-3-ketosteroids to Δ^4-3-ketosteroids by a stereoselective and intramolecular transfer of the 4β-proton to the 6β-position. Tyr-14 is the critical general acid and Asp-38 is the general base involved in catalysis. The UV absorption bandwidths of Tyr-14 were much narrower than those of the other two tyrosines (Tyr-55 and Tyr-88) of isomerase or of the *N*-acetyltyrosine ethyl ester in aqueous solution, suggesting that Tyr-14 is restricted in its mobility. Further immobilization of this residue occurs upon steroid binding. Thus, 5α-estrane-3,17-dione, an A-ring saturated steroid, induces significant narrowing of the tyrosine absorption bands ($\pi \rightarrow \pi^*$) of the main peak (279.5 nm) and the shoulder (285.5 nm) of Tyr-14, with no significant changes in λ_{max}. No effects of steroid binding were found on the absorption bandwidths of Tyr-55, Tyr-88, or the phenylalanine residues. The ratio of the absorbance (A_{max}) at the absorption maximum (λ_{max}) to that at λ_{max} plus 4 nm (A_{max+4}) was used as a measure of peak sharpness. Specifically, the ratios of $A_{279.5}/A_{283.5}$ (main peak) and $A_{285.5}/A_{289.5}$ (shoulder) of Tyr-14 of the free enzyme at 25 °C were 1.25 and 1.89, respectively, and they increased to 1.41 and 2.70, respectively, in the complex. A more precise measurement of the band narrowing from 4.2 to 3.1 nm between the inflection points was obtained from the derivative spectra. The absorption bands of free and steroid-bound isomerase were narrowed significantly by lowering the temperature and were broadened by denaturation, suggesting that the unusual peak-sharpening effects induced by steroid binding arise from the restricted motion of Tyr-14, as well as from more directional hydrogen bonding resulting from the displacement of water molecules from the active site and decreased flexibility of the protein. Larger enthalpy of the sharpening effects was observed for the steroid-bound enzyme (−0.527 ± 0.016 kcal/mol) than for the free enzyme (−0.250 ± 0.018 kcal/mol) by lowering the temperature, indicating that interactions of Tyr-14 with its environment, which restrain its motion, are stronger in the steroid-bound enzyme than in the free enzyme. Hydrogen-bonding modes of Tyr-14, mobility of the active site, and protein flexibility are the environmental factors determining the absorption bandwidths of the critical Tyr-14 residue.

The Δ^5-3-ketosteroid isomerase (EC 5.3.3.1, isomerase)[1] of *Pseudomonas testosteroni* promotes the highly efficient isomerization of a variety of Δ^5-3-ketosteroids to Δ^4-3-ketosteroids by stereoselective and conservative intramolecular transfer of the 4β-proton to the 6β-position. The mechanistic details of this reaction have been intensively investigated in several laboratories, and the findings have been reviewed (Pollack et al., 1989; Schwab & Henderson, 1990). For more recent information, see Kuliopulos et al. (1991), Xue et al. (1990, 1991), Li et al. (1993a), Brooks and Benisek (1994), Holman and Benisek (1994), Hawkinson et al. (1994), and Zhao et al. (1995). This extremely efficient enzyme, with a k_{cat}/K_M value of 1.6×10^8 M^{-1} s^{-1}, is a tightly associated homodimer containing 125 amino acids per subunit, including three tyrosine residues (Tyr-14, Tyr-55, and Tyr-88) and lacking both cysteine and tryptophan. A variety of spectroscopic techniques, in conjunction with site-directed mutagenesis, have established that two amino acid residues, Tyr-14 (acting as a general acid) and Asp-38 (functioning as a general base), are critically involved in the catalytic process and that Tyr-14 is also an important participant in the binding of the dienolic intermediate to the enzyme (Kuliopolus et al., 1989, 1991; Xue et al., 1990, 1991). The ultraviolet absorption and fluorescence spectra of steroidal inhibitors of isomerase are profoundly perturbed upon binding to the active site of the enzyme (Wang et al., 1963; Kuliopulos et al., 1989; Li et al., 1993a; Zhao et al., 1994). Thus, the ultraviolet absorption spectra of Δ^4-3-ketosteroids [e.g., 19-nortestosterone (**1**)] and ring A phenolic steroids (e.g., 17β-estradiol, 17β-dihydroequilenin) are shifted to longer wavelengths upon binding to the enzyme, and those of the phenolic steroids are intensified. While these changes resemble the effects of protonation of the 3-carbonyl group

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[1] Abbreviations: isomerase, Δ^5-3-ketosteroid isomerase (EC 5.3.3.1); 19-nortestosterone, 17β-hydroxy-4-estren-3-one; Tris, tris(hydroxymethyl)aminomethane; Y/F mixture, a mixture of the ethyl esters of *N*-acetyltyrosine (Y) and *N*-acetylphenylalanine (F) in a molar ratio of 1:10, which represents the chromophoric composition of the Y55F/Y88F mutant and other mutants of isomerase in which two tyrosine residues have been mutated to phenylalanine.

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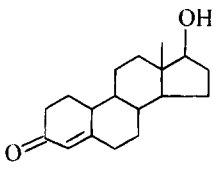
and ionization of the phenolic hydroxyl group of the steroids in aqueous solutions, respectively, the origins of these spectral changes have been reinterpreted recently and are attributed to the formation of strong hydrogen bonds between the phenolic hydroxyl group of Tyr-14 and the 3-oxygen functions of the bound steroids in a nonpolar environment (Zhao et al., 1995).

Perturbations in the optical spectra of bound substrates, coenzymes, and inhibitors have provided a wealth of information on the nature of the interactions of these ligands with their cognate enzymes. Much less information is available on how ligand binding affects the optical spectra of protein chromophores, because most proteins contain a number of tryptophan and tyrosine residues that obscure the effects of specific interactions and complicate the interpretation of the absorption spectra of these residues. Isomerase provides a uniquely favorable situation for examining the effects of ligands on a protein chromophore. The double mutant of isomerase (Y55F/Y88F) containing only a single tyrosine residue (Tyr-14), located at the active site, is nearly fully active with a k_{cat} value 4.5-fold lower and a K_M value 3.6-fold lower than those of the wild-type enzyme (Kuliopulos et al., 1989, 1991; Li et al., 1993a).[2] The value of k_{cat}/K_M for the double mutant is 80% of that for the wild-type enzyme. From studies of the Y14F mutant, it has been estimated that Tyr-14 contributes a factor of $10^{4.7}$ to catalysis (Kuliopulos et al., 1989) and at least 7.6 kcal/mol to the free energy of binding of the dienolic intermediate (Xue et al., 1990, 1991). Furthermore, the 3-carbonyl group of 19-nortestosterone (**1**) is in close proximity to Tyr-14 in the complex, as shown by nuclear Overhauser effects (Kuliopulos et al., 1991). The perturbations in UV spectra provide insight into the dynamic behavior of Tyr-14 when steroid binding occurs.



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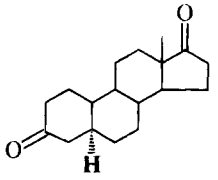

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We report here that the binding of 5α-estrane-3,17-dione (**2**), a steroid that is a potent inhibitor of isomerase and has relatively low ultraviolet absorption (λ_{max} = 285 nm, ϵ_{285} = 74 M^{-1} cm^{-1}), evokes specific changes in the absorption spectrum of Tyr-14, involving a sharpening of the absorption bands. This effect has been demonstrated with the Y55F/Y88F mutant in which Tyr-14 at the active site is the only tyrosine residue present. On the basis of similar narrowing effects on the absorption bands of Tyr-14 obtained by lowering the temperature, and the broadening effects observed upon denaturation of the enzyme, we have concluded that the motion of Tyr-14 is more restricted and the hydrogen-bonding interactions are more directional in the presence of the steroid ligand than in the free enzyme. Since the hydrogen-bonding donation of phenolic compounds induces red shifts in their absorption maxima (Zhao et al., 1995), the absence of changes in the absorption maxima of Tyr-14 suggests that strong hydrogen bond donation from Tyr-14 to the 3-ketosteroid ligand is compensated by other hydrogen bond donors, such as amide NH and/or bound water molecules. This paper demonstrates that information on the environment, mobility, and ligand interactions of a protein tyrosine residue can be obtained by UV absorption spectroscopy. To our knowledge, this work presents the first UV spectroscopic study of the dynamic behavior of a tyrosine residue of free and ligand-bound proteins.

[2] Wild-type isomerase contains three tyrosine (Tyr-14, Tyr-55, and Tyr-88) and eight phenylalanine residues per subunit of 125 amino acids. The properties of the six mutants in which one or two tyrosine residues (Y) have been replaced by phenylalanine (F) have been described (Kuliopulos et al., 1989; Li et al., 1993a). The double mutant Y55F/Y88F contains Tyr-14 as the sole tyrosine residue and 10 phenylalanine residues.

EXPERIMENTAL PROCEDURES

Materials. Tris base, Tris-HCl, mono- and dibasic potassium phosphate, sodium dodecyl sulfate, Coomassie Blue R250, sodium acetate, poly(ethylene glycol) (PEG-8000), ammonium sulfate, bovine serum albumin, and the ethyl esters of *N*-acetylphenylalanine and *N*-acetyltyrosine were purchased from Sigma (St. Louis, MO). Ultrapure guanidine hydrochloride was from Schwarz/Mann (Spring Valley, NY). The highest grade solvents were from either J. T. Baker (Phillipsburg, NJ) or Aldrich (Milwaukee, WI). 5α-Estrane-3,17-dione and 17β-hydroxyandrosta-1,4,6-trien-3-one, purchased from Steraloids (Wilton, NH), gave a single spot on thin-layer chromatography developed with ethyl acetate when visualized with iodine vapor. 17β-Hydroxyestra-4,9-dien-3-one was a gift from J. A. Katzenellenbogen (University of Illinois, Urbana, IL). 17β-Hydroxyestra-4,6-dien-3-one was a gift from Upjohn (Kalamazoo, MI). Δ^5-Androstene-3,17-dione was synthesized from dehydroepiandrosterone (Kawahara et al., 1962).

Enzyme Preparations. *Escherichia coli* strain JM 101 carrying pUC-19 plasmids with inserts of the genes encoding wild-type isomerase, the Y55F/Y88F mutant, or other mutant genes from *Pseudomonas testosteroni* has been described previously (Kuliopulos et al., 1987, 1989). Recombinant isomerase and its tyrosine mutants, Y14F, Y55F, Y88F, Y14F/Y55F, Y55F/Y88F, and Y14F/Y88F, were prepared by growing these bacteria in LB medium and purifying the overexpressed enzymes to electrophoretic homogeneity (Kuliopulos et al., 1987, 1989). Following ion exchange chromatography by fast-flow Q-Sepharose using FPLC (Pharmacia, Piscataway, NJ), the purified enzymes were recrystallized two or three times and stored as crystalline suspensions at 4 °C in 30% saturated ammonium sulfate solution neutralized with NH_4OH. The protein concentrations of the enzymes were based on absorbances at 280 nm, assuming that solutions containing 1 mg/mL wild-type isomerase and the Y55F/Y88F mutant have A_{280} values of 0.336 and 0.157 cm^{-1} at pH 7.50, respectively (Benson et al., 1975; Kuliopulos et al., 1989; Li et al., 1993a). All molar concentrations refer to the subunit concentration of the dimeric enzyme. These extinction coefficients are in good agreement with measurements of tyrosinate absorbance at pH 13, assuming ϵ = 2390 M^{-1} cm^{-1} per tyrosinate at 293 nm (Goodwin & Morton, 1946). The purity of the isomerases was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and staining with Coomassie Blue R250.

Activities of isomerase and its mutants were measured with the use of Δ^5-androstene-3,17-dione as substrate by observing the increase in absorbance at 248 nm and 25 °C (Kuliopulos et al., 1989). Isomerase was diluted with neutralized 0.1% (w/v) bovine serum albumin solution for the activity assays.

Kinetic studies of the inhibition of the Y55F/Y88F mutant by 5α-estrane-3,17-dione were carried out with substrate concentrations of 37.5, 75.0, 112.5, and 150.0 μM and inhibitor concentrations of 0, 5.0, 10.0 and 15.0 μM at 25 °C in the presence of 1.5% (v/v) methanol.

Ultraviolet Spectroscopy. The UV spectra of isomerase and enzyme activity assays were obtained on Cary 1E UV−visible and Beckman DU-7 spectrophotometers. The UV spectra of isomerase were obtained at 25 °C (unless stated otherwise) in a quartz cuvette of 1.0- or 5.0-cm light path in a total volume of 1.0 or 4.5 mL, respectively. The solutions contained 50 mM Tris-HCl and 100 mM NaCl at pH 7.50. The slit width was set at 1.0 nm. For the determination of protein concentration, the absorbances of the appropriately diluted enzyme solutions at 280 nm were 0.1 or less with a 1.0-cm light path. For observations of the changes in the tyrosine absorption fine structure upon binding of a steroid ligand, higher concentrations of isomerase and in some cases 5.0-cm light path cuvettes were used. The absorbances are expressed as molar absorption coefficients (ϵ).

A solution containing 29 mM *N*-acetyltyrosine ethyl ester and 290 mM *N*-acetylphenylalanine ethyl ester was made in anhydrous acetonitrile. A small volume (2 μL) of this stock solution (designated the Y/F mixture) was diluted to a total volume of 1.0 mL with different solvents or water (pH 7.5, 100 mM NaCl and 50 mM Tris-HCl). The spectra of appropriate blank solutions were subtracted from those of the mixtures to yield the spectra of the model compounds.

Various concentrations of guanidine hydrochloride (0−3.25 M) in 100 mM NaCl and 50 mM Tris-HCl at pH 7.50 were used to study the dependence of the tyrosine absorption bandwidths on the concentration of denaturant at 25 °C. The Y55F/Y88F mutant (27 μM final concentration) was added to buffer solutions containing different concentrations of guanidine hydrochloride. The solutions were allowed to equilibrate for ~1 min at 25 °C before the spectra of the proteins were obtained. The spectra of appropriate blanks were subtracted.

UV Spectral Titration of the Y55F/Y88F Mutant with 5α-Estrane-3,17-dione. Titrations measuring the specific binding of 5α-estrane-3,17-dione to the Y55F/Y88F mutant were carried out by adding successive small volumes of steroid solutions in methanol to the enzyme solutions in 50 mM Tris-HCl, 100 mM NaCl, and 0.01% bovine serum albumin at pH 7.50 and to the blank solutions. Alternatively, the spectra of the buffer solutions containing the appropriate amount of 5α-estrane-3,17-dione were subtracted from the spectra of the final mixtures containing the enzyme−steroid complex. The sharpness of the tyrosine absorption peaks was quantitated using the ratio A_{max}/A_{max+4}.[3] On the basis of the assumptions that free and bound enzyme have tyrosine absorption peaks of different sharpness, i.e., different A_{max}/A_{max+4} values, and that simple additivity of the two spectra occurs with isosbestic points (as observed experimentally), the ratios obtained during the titration (designated R) were fitted to the following hyperbolic function (see the Appendix):

$$R = R_f + [(R_b - R_f)/2E_t]\{E_t + K_D + I_t - [(E_t + K_D + I_t)^2 - (4I_tE_t)]^{1/2}\}$$

where K_D is the dissociation constant, E is the concentration of the Y55F/Y88F mutant, I is the concentration of 5α-estrane-3,17-dione, and the subscripts f, b, and t refer to the free, bound, and total species, respectively. Nonlinear least-squares fitting of the titration data was carried out using the program GraFit (Leatherbarrow, 1992).

The narrowing effects of ligand binding on the bandwidth of Tyr-14 were also quantitated by plotting the first and second derivatives using the digitized spectral data at 0.25-nm intervals and the GraFit program. The distance (nanometers) between inflection points of the absorption peak or shoulder was used as a measure of the bandwidth.

Fluorescence Quenching of Isomerase by Steroids. Fluorescence spectra of isomerase were obtained at 25 °C with a Perkin-Elmer Model LS50 luminescence spectrometer. A slit width of 5.0 nm was used for both the excitation and emission beams. The spectra of blank buffers, 10 mM potassium phosphate (pH 7.50), or buffers containing various concentrations of steroids were obtained before isomerase was added at a final concentration of 1.0 μM. Steroids were added to the mixture in methanol solutions of appropriate concentrations. The final content of methanol in the mixture was 1.0% (v/v). These control spectra were subtracted from the spectra of the final mixtures to yield the fluorescence spectra of isomerase. Procedures and data fitting for titrations of fluorescence quenching of isomerase by steroid ligands have been described (Li et al., 1993a).

Temperature Dependence of the Absorption Bandwidths of Tyr-14 in Free and Steroid-Bound Y55F/Y88F Mutant. A Cary 1E UV−visible spectrophotometer equipped with a thermostated cell compartment was used to obtain the UV spectra of the mutant isomerase at different temperatures. A slit width of 1.0 nm and a data interval of 0.5 nm were used for the measurements. The cuvettes were equilibrated at each temperature (5−45 °C) for 15−20 min before the spectra were obtained. The temperature was controlled to ± 0.2 °C of the desired value. The buffer solutions containing 20 μg/mL (0.29 μM) bovine serum albumin, which does not interfere with the measurements, 100 mM NaCl, and 50 mM Tris-HCl at pH 7.50, with or without 200 μM 5α-estrane-3,17-dione, were placed in the reference beam against the complete mixtures to yield the spectra of free or steroid-bound isomerase. The final concentration of the Y55F/Y88F mutant isomerase was 62 μM in a total 2.0-mL volume. The mixtures of the steroid-bound isomerase contained a final concentration of 2.0% (v/v) methanol. The A_{max}/A_{max+4} values were correlated linearly with temperatures in the tested temperature range. Linear least-squares fits of the data were obtained with GraFit (Leatherbarrow, 1992). Narrowing of Tyr-14 bands by lowering the temperature was confirmed by plotting the derivative spectra.

RESULTS

Differences in UV Spectral Behavior between Tyr-14 and Tyr-55 and Tyr-88 of Isomerase. In addition to the principal absorption maximum (274−280 nm) that is present in wild-type isomerase and in all tyrosine mutants studied, there is a distinct shoulder (285.5 nm) in the wild-type isomerase. This shoulder *disappeared* when Tyr-14 was mutated to phenylalanine (see Y14F in Figure 1), whereas the mutations of the other tyrosine residue(s) to phenylalanine made this small shoulder of the wild-type enzyme significantly *more prominent,* as demonstrated by the spectra of the Y55F,

[3] A_{max} is the absorbance at the tyrosine absorption maximum (λ_{max}), and A_{max+4} is the absorbance at λ_{max} plus 4 nm. The ratio (R) A_{max}/A_{max+4} is used to define the sharpness of the absorption peaks.

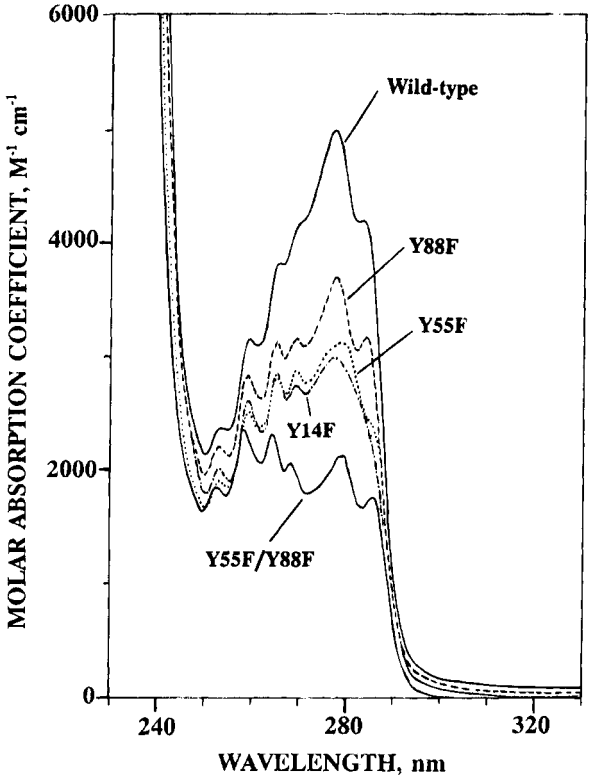


FIGURE 1: UV spectra of wild-type and the Y14F, Y55F, Y88F, and Y55F/Y88F mutants of isomerase at pH 7.50 in 100 mM NaCl and 50 mM Tris-HCl at 25 °C [see also Kuliopulos et al. (1989)]. Note that the distinct shoulder in the UV absorption spectra of isomerase depends solely on the presence of the active site Tyr-14.

Y88F, and Y55F/Y88F mutants (Figure 1). The Y88F mutant had a more prominent shoulder than the Y55F mutant (Figures 1 and 2A). Although the precise reasons for these differing spectral features are not clear, they confirm conclusions from earlier fluorescence studies indicating that the three tyrosine residues reside in different environments in the protein (Li et al., 1993a; Wu et al., 1994). Therefore, the broader absorption bands of Tyr-88 probably arise from exposure to and hydrogen-bonding interactions with the bulk solvent, whereas those of Tyr-55 are less broad because this residue is at least partially buried in the interior of the protein. The sharp shoulder of the tyrosine absorption of wild-type isomerase is due entirely to Tyr-14, which is located in the hydrophobic environment of the active site and has restricted motion (see the following).

Environments and Mobility of Tyrosine Residues in Isomerase Reflected by Absorption Peak Sharpness. Since the sharpness of the absorption bands of Tyr-14 is markedly increased by steroid binding (see the following), the peak widths of the absorption bands of Tyr-55 and Tyr-88 were also investigated. Figure 2A shows the spectra of Tyr-88 in the Y14F/Y55F mutant, Tyr-55 in the Y14F/Y88F mutant, and Tyr-14 in the Y55F/Y88F mutant. The absorption bands of Tyr-55 and Tyr-88, as measured in the respective double mutants, were broader than those of Tyr-14, which has been shown by NMR docking, absorption enhancement, and time-resolved fluorescence spectroscopy to be immobilized deep in the hydrophobic active site cavity (Kuliopulos et al., 1987b, 1991; Li et al., 1993a; Wu et al., 1994). The elevated pK_a value of 11.6 for Tyr-14 compared to 10.2 for that of *N*-acetyltyrosine amide in water (Li et al., 1993a) also indicates the inaccessibility of Tyr-14 to the bulk solvent.

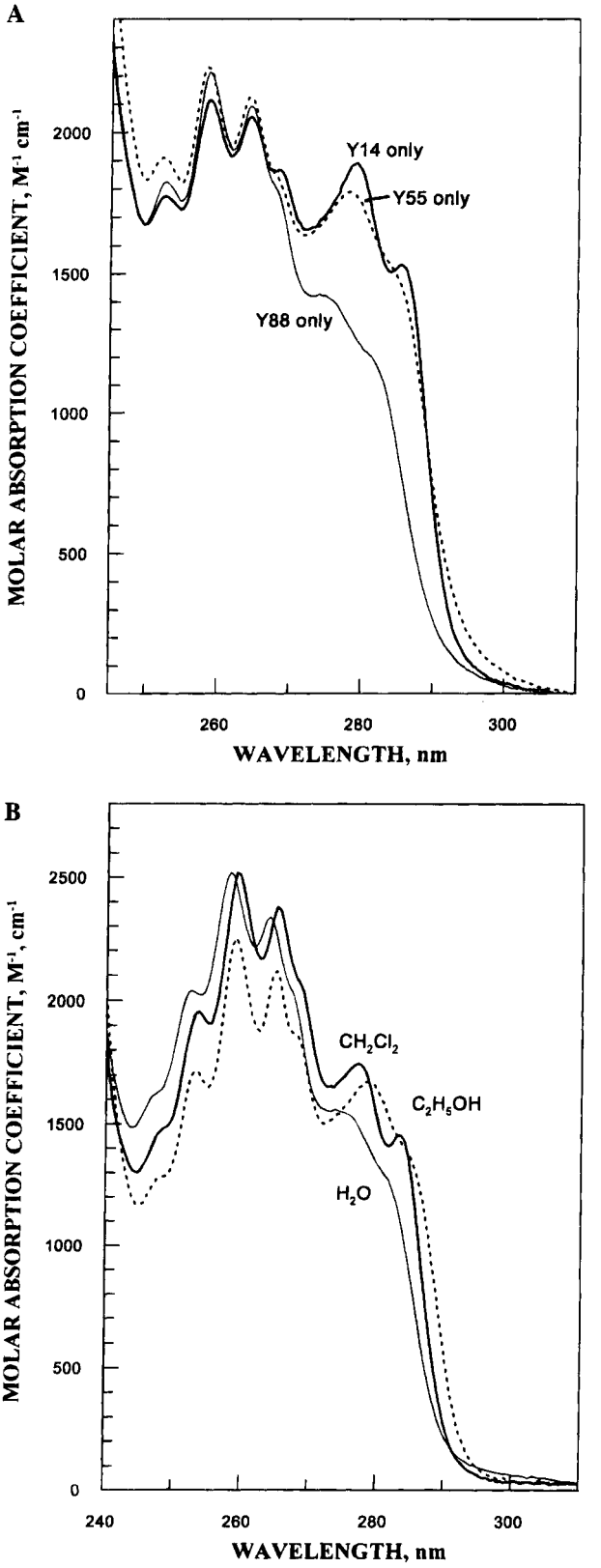


FIGURE 2: Comparison of the spectra of double mutants of isomerase and the Y/F mixture in different solvents reflected by the similar sharpness of tyrosine absorption peaks. (A) Spectra of Y55F/Y88F (Y14 only), Y14F/Y88F (Y55 only) and Y14F/Y55F (Y88 only) at pH 7.50 in 100 mM NaCl and 50 mM Tris-HCl at 25 °C. (B) Spectra of a mixture of the ethyl esters of *N*-acetyltyrosine and *N*-acetylphenylalanine in a molar ratio of 1:10 in methylene chloride, ethanol, and water (pH 7.50, 100 mM NaCl, and 50 mM Tris-HCl) at 25 °C. The spectra of the model mixture were obtained in the presence of 0.2% acetonitrile. Note the similarities of the spectral behavior of different tyrosine residues in isomerase with the exposure of tyrosine to different media.

Table 1: Sharpness of Tyrosine Absorption Peaks for a Mixture of the Ethyl Esters of *N*-Acetyltyrosine and *N*-Acetylphenylalanine (Molar Ratio, 1:10) in Different Solvents and of the Single Tyrosine Residue in Free and Steroid-Bound Y55F/Y88F Mutant and in Y14F/Y88F and Y14F/Y55F Mutants

	A_{max}/A_{max+4} (λ_{max}, nm)	
	main absorption peak	absorption shoulder
Tyrosine in Different Solvents[a]		
water	1.08 (274.0)	1.31 (280.0)
methanol	1.11 (278.0)	1.31 (283.5)
ethanol	1.14 (278.5)	1.50 (284.5)
2-propanol	1.16 (279.0)	1.27 (284.5)
acetonitrile	1.21 (278.0)	1.70 (283.5)
chloroform	1.14 (278.0)	1.46 (283.0)
diethyl ether	1.25 (279.5)	2.32 (286.0)
methylene chloride	1.23 (277.0)	2.16 (283.5)
n-hexane	1.21 (278.0)	1.69 (284.0)
Tyrosine Residue in Isomerase Mutants		
Y55F/Y88F (Tyr-14 only)		
free[b]	1.25 (279.5, 1890)[d]	1.89 (285.5)
steroid-bound[b]	1.41 (279.5)	2.70 (285.5)
Y14F/Y88F (Tyr-55 only)[c]	1.11 (278.0, 1790)[d]	1.37 (284.0)
Y14F/Y55F (Tyr-88 only)[c]	1.08 (274.0, 1430)[d]	1.24 (280.0)

[a] In this model system, the Y/F mixture was used to simulate the chromophoric constituents of isomerase. The Y/F mixture (58 μM *N*-acetyltyrosine ethyl ester + 580 μM *N*-acetylphenylalanine ethyl ester) was exposed to different solvents to observe the medium effect on the sharpness of the absorption bands. All vessels contained 2.0% acetonitrile in a final volume of 1.0 or 3.0 mL. [b] Results for free and bound Y55F/Y88F mutant were from data fitting as described in Experimental Procedures. Reported values are results of the averages from three individual measurements. [c] No significant perturbation in the UV spectra of the isomerase was observed upon the binding of 5α-estrane-3,17-dione (**2**) using these mutants. [d] The second number in parentheses is the molar absorption coefficient (ϵ, M^{-1} cm^{-1}) at the corresponding absorption maximum.

The differences in sharpness of the absorption bands of Tyr-14, Tyr-55, and Tyr-88 in the respective double mutants reflect differences in both the environments (e.g., polarity, accessibility to the bulk solvent, etc.) and the dynamics of each individual tyrosine residue. These results indicate progressively decreasing mobility of these residues in the following order: Tyr-88 > Tyr-55 > Tyr-14 (see the following discussion). This conclusion is consistent with the substantial red shift and intensification of the tyrosine absorption in isomerase in the reverse order: Tyr-14 > Tyr-55 > Tyr-88.

Comparison of the spectra shown in Figure 2A with those of the Y/F mixture in water, ethanol, and methylene chloride (Figure 2B) suggests that peak sharpness correlates with solvent polarity. However, a more extensive study of the Y/F mixture in solvents ranging widely in polarity (Table 1) shows that this correlation does not extend to *n*-hexane, suggesting that hydrogen bond acceptor properties of the solvents also contribute to the absorption band sharpness of tyrosine. It is noteworthy that small red shifts in the phenylalanine absorptions were observed when the Y/F mixture was exposed to progressively less polar solvents (Figure 2B). This is in contrast to the unperturbed spectral behavior of phenylalanine residues in the respective double tyrosine mutants (Figure 2A). These observations indicate that the environments of phenylalanine residues of the double tyrosine mutants are indistinguishable from one another and from the wild-type enzyme.

Effects of Denaturation on the UV Spectra of the Three Tyrosine Residues of Isomerase. The differences in the bandwidths and absorption maxima of the three tyrosine residues of native isomerase (Table 1) are largely abolished by denaturation with either guanidine hydrochloride or urea. The distinctive shoulder of the Y55F/Y88F mutant at 285.5 nm progressively diminishes with higher denaturant concentrations. The midpoint for the unfolding of the Y55F/Y88F mutant occurs at about 1.2 M guanidine hydrochloride at pH 7.50 and 25 °C, which is consistent with the concentration of 1.3 M guanidine hydrochloride reported for half-maximal unfolding of isomerase as observed by circular dichroism and fluorescence measurements (Wu et al., 1994). Figure 3 shows the UV spectra of the Y55F/Y88F mutant in the native and denatured states at pH 7.50. When the proteins were denatured in 3.25 M guanidine hydrochloride (or 6.0 M urea) at pH 7.50, the UV spectra of the Y55F/Y88F, Y14F/Y88F, and Y14F/Y55F mutants showed the same absorption bandwidths (R = 1.10 for the main peak), with absorption maxima very similar or identical to that of tyrosine (278.0 nm for the Y55F/Y88F and Y14F/Y88F mutants and 276.0 nm for the Y14F/Y55F mutant). These results indicate that the differences in the UV absorption characteristics of buried and exposed tyrosine residues are observable only in the native conformation of isomerase.

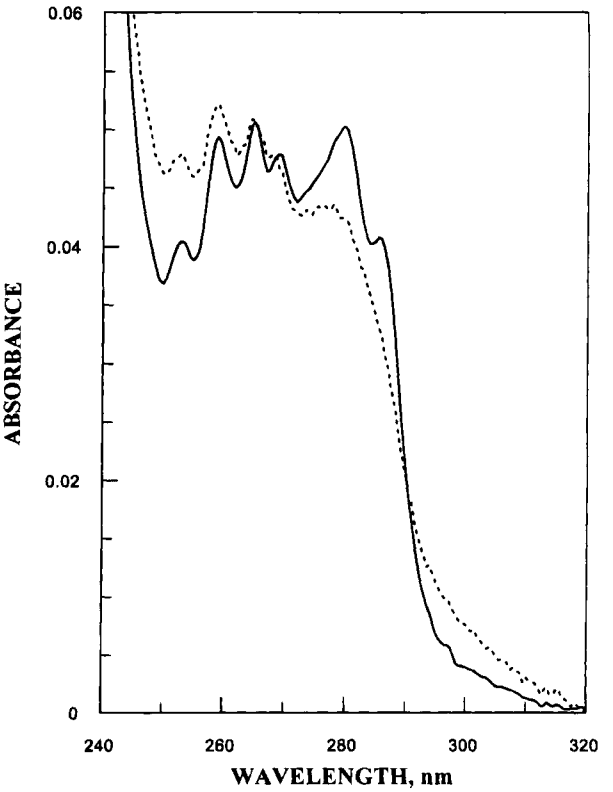


FIGURE 3: UV spectra of 27 μM Y55F/Y88F mutant isomerase in the native state (solid line) in 100 mM NaCl and 50 mM Tris-HCl at pH 7.5 and in unfolded states (dotted line) in the same buffer containing 1.5 M guanidine hydrochloride at pH 25 °C.

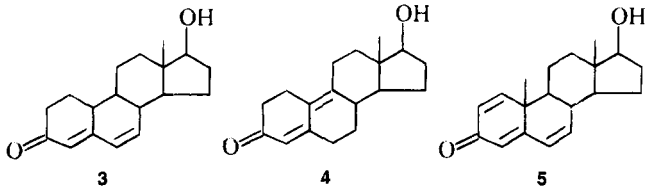
Specific Interactions of Isomerase with Various Steroids. The capacity of the functional group at C-3 of a steroid to act as a hydrogen bond acceptor or donor and the steroid skeleton are the only significant structural factors involved in the specific interaction of a steroid ligand with isomerase. Other structural factors, such as the presence of unsaturation or aromaticity in the A or B rings of steroid ligands, play only minor roles in the specific binding. For instance, 5α-estrane-3,17-dione (**2**), with saturated *trans*-A/B rings, and steroids containing extended conjugated systems, e.g., $\Delta^{4,6}$-,

Table 2: Comparison of Dissociation Constants and Fluorescence Quenching Efficiency of 5α-Estrane-3,17-dione and 19-Nortestosterone for Wild-Type Isomerase and Its Tyrosine Mutants

	5α-estrane-3,17-dione		19-nortestosterone[a]	
isomerase	K_D (μM)	quenching (%)	K_D (μM)	quenching (%)
wild type	8.9 ± 0.4	90	4.4 ± 0.4	94
Y88F	8.0 ± 0.3	86	3.3 ± 0.6	94
Y55F/Y88F	4.0 ± 0.2	97	2.3 ± 0.3	97
Y14F/Y88F	8.8 ± 0.2	88	17 ± 0.5	91
Y14F/Y55F	26 ± 1	40	71 ± 14	74

[a] From Li et al. (1993a). The % of quenching refers to the ratio of fluorescence of bound enzyme (extrapolated to infinite ligand concentration) versus free enzyme.

$\Delta^{4,9}$-, and $\Delta^{1,4,6}$-3-ketosteroids (**3**–**5**), all have dissociation constants similar to that of 19-nortestosterone (**1**) for the Y55F/Y88F mutant. K_D values of 4.0 μM for compound **3**,



3 **4** **5**

3.5 μM for compound **4**, and 4.0 μM for compound **5** in the presence of 1.0% (v/v) methanol are consistent with K_D's of 13 μM for compound **1** and 10 μM for compound **4** in the presence of 3.3% (v/v) methanol for wild-type isomerase reported by Falcoz-Kelly et al. (1968). Steroids that are poorer hydrogen bond acceptors or donors at C-3 (e.g., 3-ethoxyandrosta-3,5-dien-17-one, 4-androstene-3β,17β-diol, 19-nortestosterone 3-oxime, and 17β-hydroxy-5(10)-estren-3-one 3-oxime) showed either weak or no binding to isomerase. The active site of isomerase also recognizes the whole steroid framework as an important participant in the isomerase–steroid interactions. Weintraub et al. (1977) showed that 2-naphthol derivatives [e.g., α,α-dimethyl-β-ethylallenolic acid, which closely resembles the A/B ring of 17β-dihydroequilenin (a potent inhibitor of isomerase)] do not bind to the active site of isomerase. The hydrophobic steroid skeleton bearing a polar group at C-3 directs the ligand into the hydrophobic active site, thereby favoring hydrogen-bonding interactions with polar residues as well as hydrophobic interactions with the active site surface. Hydrogen-bonding interactions in hydrophobic environments are very efficient because of a lack of competition with solvent (Chandra & Banerjee, 1962; Pekary, 1978; Zhao et al., 1995; Chignell & Gratzer, 1968; Gramstad, 1963). At the active site of an enzyme, the free energy of formation of a hydrogen bond could be much greater (Cleland & Kreevoy, 1994).

5α-Estrane-3,17-dione (**2**) and 19-nortestosterone (**1**) showed very similar binding constants and fluorescence quenching with wild-type and three of four mutant isomerases (Table 2). Thus, the presence of an olefinic bond in ring A contributes relatively little to the quenching of Tyr-14 fluorescence or to binding. Kinetic analysis showed that 5α-estrane-3,17-dione (**2**) is a linear competitive inhibitor with a K_i value of 6.5 ± 0.3 μM, which is consistent with the K_D values obtained by UV or fluorescence titrations (Table 2 and Figure 5).

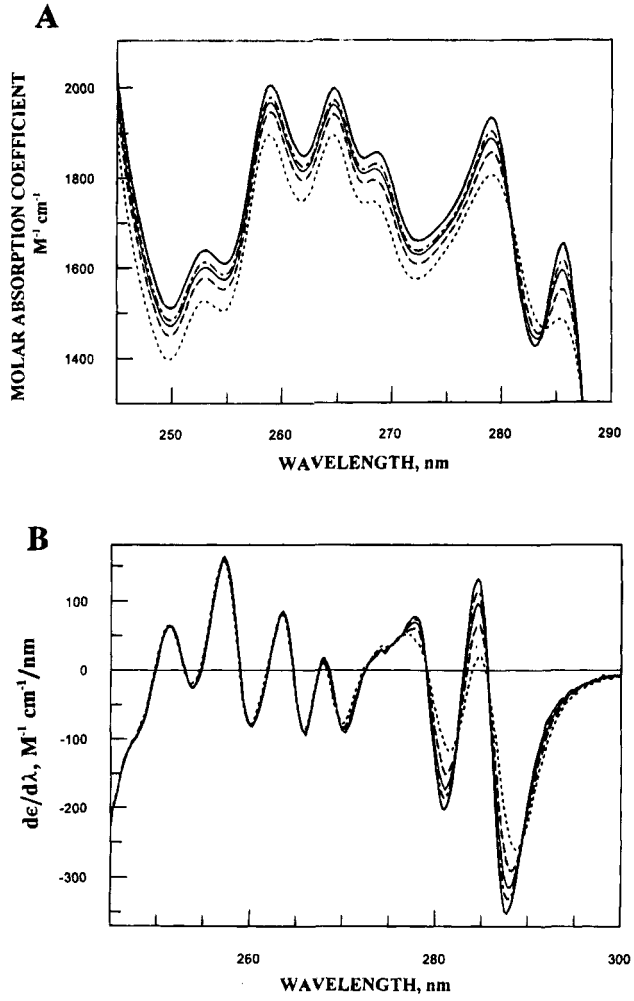


FIGURE 4: Peak-sharpening effects of increasing concentrations of 5α-estrane-3,17-dione on the UV spectra of the Y55F/Y88F mutant of isomerase which contains only a single tyrosine residue (Tyr-14). Spectra were measured in 100 mM NaCl and 50 mM Tris-HCl at pH 7.50 and 25 °C. The enzyme subunit concentration was 64.0 μM, and the concentrations of steroid were 0 (···), 22.2 (- - -), 40.0 (—), 62.2 (— · —) and 133 μM (▬) in the final mixture. (A) UV spectra of the region of phenylalanine and tyrosine absorptions. (B) First-derivative plots of the UV absorption spectra shown in panel A.

Effects of Steroid Binding on Absorption Bandwidths of Tyr-14. The main peak (at 279.5 nm) and the shoulder (at 285.5 nm) of the Tyr-14 absorption of the Y55F/Y88F mutant of isomerase were significantly narrowed and intensified upon the binding of 5α-estrane-3,17-dione (Figure 4). These spectral sharpenings and intensifications were quantitated by the use of changes in absorption ratio (R) as a function of steroid concentration: $A_{279.5}/A_{283.5}$ for the main peak and $A_{285.5}/A_{289.5}$ for the shoulder, respectively. Fitting of these observed ratios at different concentrations of 5α-estrane-3,17-dione gave K_D values of 3.7 ± 0.3 μM for the main absorption peak and 3.9 ± 0.3 μM for the shoulder. These values are in very good agreement with the K_D value of 4.0 ± 0.2 μM (Table 2) for this steroid obtained by fluorescence quenching (Figure 5). The average of the R values from the data fitting for Tyr-14 in the steroid-bound Y55F/Y88F mutant is 1.41 at 25 °C, an increase from 1.25 for free enzyme at the same temperature (Table 1). The width of the absorption bands of tyrosine, as measured from the distance between the ascending and descending inflection points (peak and valley in the first-derivative plots, Figure

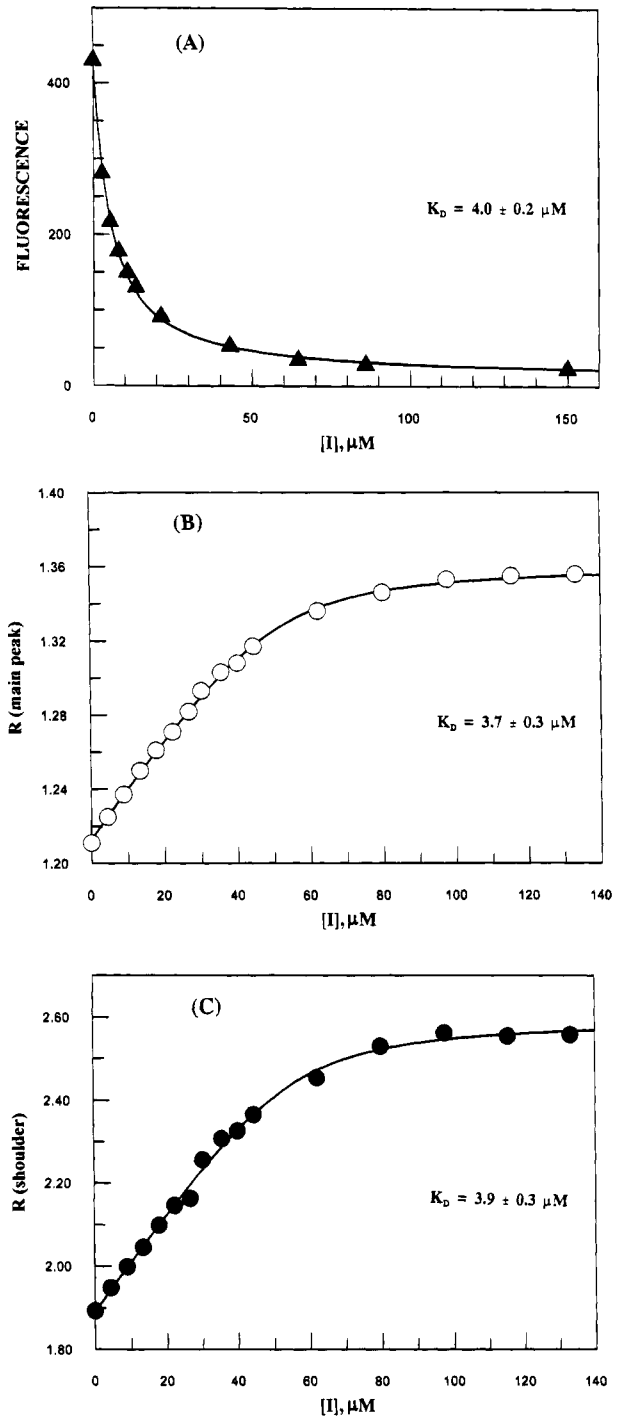


FIGURE 5: Binding of 5α-estrane-3,17-dione to the Y55F/Y88F mutant of isomerase as shown by fluorescence quenching and UV absorption peak-sharpening of Tyr-14. (A) Fluorescence quenching of the single tyrosine by increasing concentration of the steroid. Emission was measured at 307 nm with the excitation wavelength set at 278 nm. The final enzyme concentration was 1.75 μM. The experimental points are fitted to a curve with $K_D = 4.0 \pm 0.2$ μM. (B) Titration curve of the $A_{279.5}/A_{283.5}$ ratio (main peak) as a function of the concentration of the steroid ($K_D = 3.7 \pm 0.3$ μM). (C) Titration curve of the $A_{285.5}/A_{289.5}$ ratio (shoulder) as a function of the concentration of the steroid ($K_D = 3.9 \pm 0.3$ μM). In (B) and (C), the titration was carried out at an enzyme concentration of 64.0 μM. The equation for data fitting in (A) was described in Li et al. (1993a), and the equation for data fitting for (B) and (C) is described in Experimental Procedures.

4B), narrows progressively as the concentration of the steroid increases, from 4.2 nm in the free enzyme to 3.1 nm in the fully bound isomerase for both absorption peak and shoulder. In contrast, no significant perturbations in the UV absorption spectra were observed upon binding of 5α-estrane-3,17-dione to the Y14F/Y88F and Y14F/Y55F mutants, indicating that Tyr-55 and Tyr-88 do not interact with the steroid ligand.

Model Studies of the Narrowing of Tyrosine Absorption Bands. Since the factors responsible for the narrowing of tyrosine absorption bands are not understood, efforts were made to simulate this effect in different model systems and to correlate the peak sharpness with the environment and dynamic behavior of specific tyrosine residues. Significant peak narrowing of tyrosine absorptions was observed by exposing the Y/F mixture to diethyl ether or methylene chloride, but not to any of the other solvents tested (Table 1, Figure 2B). The extraordinarily sharp peaks of Tyr-14 in steroid-bound isomerase, however, *could not* be simulated by the Y/F mixture in any solvent at 25 °C, although one might expect sharper absorption peaks at extremely low temperatures (Chance, 1957). Nevertheless, extremely sharp peaks were observed for phenol in *n*-hexane (data not shown) or cyclohexane (Nemethy & Ray, 1973). The narrow absorption bandwidths of phenol in *n*-hexane (data not shown) approach those of the UV spectrum of *p*-cresol vapor at 80 °C (Chignell & Gratzer, 1968), indicating a lack of dimerization or hydrogen-bonding interactions among phenol molecules in this inert solvent. The peaks of phenol absorption in *n*-hexane are much sharper than those of Tyr-14 in the steroid-bound Y55F/Y88F mutant. Interestingly, these sharp, distinct absorption bands of phenol in pure *n*-hexane can be abolished by the addition of small (slightly larger than stoichiometric) amounts of methanol or 2-propanol (data not shown) presumably because of the multiple types of hydrogen-bonding interactions of phenol with these alcohols (see the following). The lack of sharp peaks of the Y/F mixture in *n*-hexane is probably due to the multiplicity of hydrogen-bonding interactions of the phenolic hydroxyl group of tyrosine and the ester or amide functional groups.

Besides the changes due to the altered solvent refractive index, hydrogen bonding seems to play a role in determining peak sharpness (Nemethy & Ray, 1973). Among all of the solvents tested, only diethyl ether and methylene chloride sharpened the tyrosine absorption bands of the Y/F mixture (Table 1). This may be due to the fact that diethyl ether and methylene chloride can function *only* as *acceptors* of hydrogen bonds. Thus, the multiple hydrogen-bonding interactions to the phenolic group of tyrosine, which account for the broadening of the absorption bands (see the following discussion), do not occur in these solvents.

Effect of Temperature on the Absorption Bandwidths of Tyr-14. The absorption bands of Tyr-14 were narrowed by lowering the temperature. Since the tertiary structure of the Y55F/Y88F mutant is intact below 60 °C, as indicated by circular dichroism spectroscopy, and the fluorescence of Tyr-14 is not significantly perturbed below 50 °C (Wu et al., 1994), the temperature dependence of the UV spectra of the free and steroid-bound Y55F/Y88F mutant was studied over the temperature range 5−45 °C (Table 3, Figure 6). Peak-sharpening effects of tyrosine absorption bands by lowering the temperature were quantitated by the absorbance ratio A_{max}/A_{max+4} and confirmed by derivative spectra. Linear correlation of the peak sharpness with respect to temperature was observed for Tyr-14 in the free Y55F/Y88F mutant (Figure 7A,B). Comparison by extrapolation of the peak sharpness at 25 °C of Tyr-55 and Tyr-88 with that of Tyr-

Table 3: Temperature Dependence of Absorption Peak Sharpness and Absorption Maxima [A_{max}/A_{max+4} (λ_{max}, nm)] of Tyr-14 of the Free and 5α-Estrane-3,17-dione-Bound Y55F/Y88F Mutant in 100 mM NaCl and 50 mM Tris-HCl Solution at pH 7.50

	main absorption peak		absorption shoulder[a]	
temperature (°C)	free enzyme	bound enzyme[b]	free enzyme	bound enzyme
5.0	1.31 (280.0)	1.51 (280.0)	2.16 (286.0)	13.4 (286.0)
10.0	1.29 (280.0)	1.49 (280.0)	2.10 (286.0)	3.26 (286.0)
15.0	1.28 (280.0)	1.47 (280.0)	2.05 (286.0)	2.93 (286.0)
20.0	1.27 (280.0)	1.47 (280.0)	2.06 (286.0)	3.14 (286.0)
25.0	1.26 (279.5)	1.43 (279.5)	1.79 (285.5)	2.74 (286.0)
30.0	1.26 (279.5)	1.40 (279.5)	1.56 (285.0)	2.60 (286.0)
35.0	1.26 (279.5)	1.38 (279.5)	1.53 (285.0)	2.55 (286.0)
40.0	1.24 (295.5)	1.35 (279.5)	1.53 (285.0)	2.47 (286.0)
45.0	1.23 (295.5)	1.34 (279.5)	1.53 (285.0)	2.12 (285.5)

[a] The ratios and maxima reported for the shoulder are not as reliable as the results for the main peak since the absorption maxima are not precisely identifiable in some cases, especially at the higher temperatures for the free enzyme. [b] For calculations of the ratios (R) of the steroid-bound mutant isomerase at 25 °C and below, $A_{max+3.5}$ was used instead of A_{max+4} as the absorption minimum appeared at 3.5 nm, rather than 4.0 nm, off the main peak under these conditions.

14 showed that the extent of immobilization of Tyr-14 is similar to that of a presumed ~90 °C cooling of Tyr-55 and ~100 °C cooling of Tyr-88 (Figure 7A). On the other hand, the peak sharpness of Tyr-14 in the bound enzyme at 25 °C correlates with the extrapolated value of Tyr-14 at −55 °C, which corresponds to about 80 °C cooling of the free enzyme (Figure 7A). A linear least-squares fit of R values for the main absorption peak of tyrosine with respect to temperature gave a slope of −0.0018 ± 0.0001 for free enzyme and −0.0044 ± 0.0003 for the steroid-bound Y55F/Y88F mutant of isomerase (Figure 7B). The enthalpy barriers for the sharpening effects on the tyrosine absorption bands calculated from plots of ln R with respect to $1/T$ were −0.250 ± 0.018 kcal/mol for the free enzyme and −0.527 ± 0.016 kcal/mol for the steroid-bound enzyme (Figure 7B, inset).

Interestingly, lowering of the temperature of the enzyme gave noticeably sharper peaks for phenylalanine absorption bands, as well as for tyrosine absorption bands. This is in contrast to the above-mentioned peak sharpening by ligand binding, in which *only* the tyrosine, not the phenylalanine, absorption bands were narrowed. This is easily discernible from Figures 4B and 6A,B. Such specific band narrowing is attributed to an altered location of Tyr-14 in the complex in which it interacts directly with the steroid (Kuliopulos et al., 1989, 1991), whereas at low temperatures the internal motion was decreased throughout the entire protein. In addition, the ligand binding has more pronounced effects than lowering the temperature on sharpening the tyrosine absorption bands of the isomerase. For instance, the difference in R between free and steroid-bound isomerase at 25 °C corresponds to an approximately 80 °C difference in temperature, assuming that the linear relationship of the temperature dependence prevails beyond the tested temperature range. Besides the sharpening of the absorption peak of tyrosine, lowering of the temperature produced red shifts of 0.5 nm for the main absorption peak and 1.0 nm for the shoulder of the absorption of Tyr-14 (Table 3). These slight, yet definite, red shifts of the absorption maxima of Tyr-14 at lower temperatures may result from a more hydrophobic active site of a more compact protein.

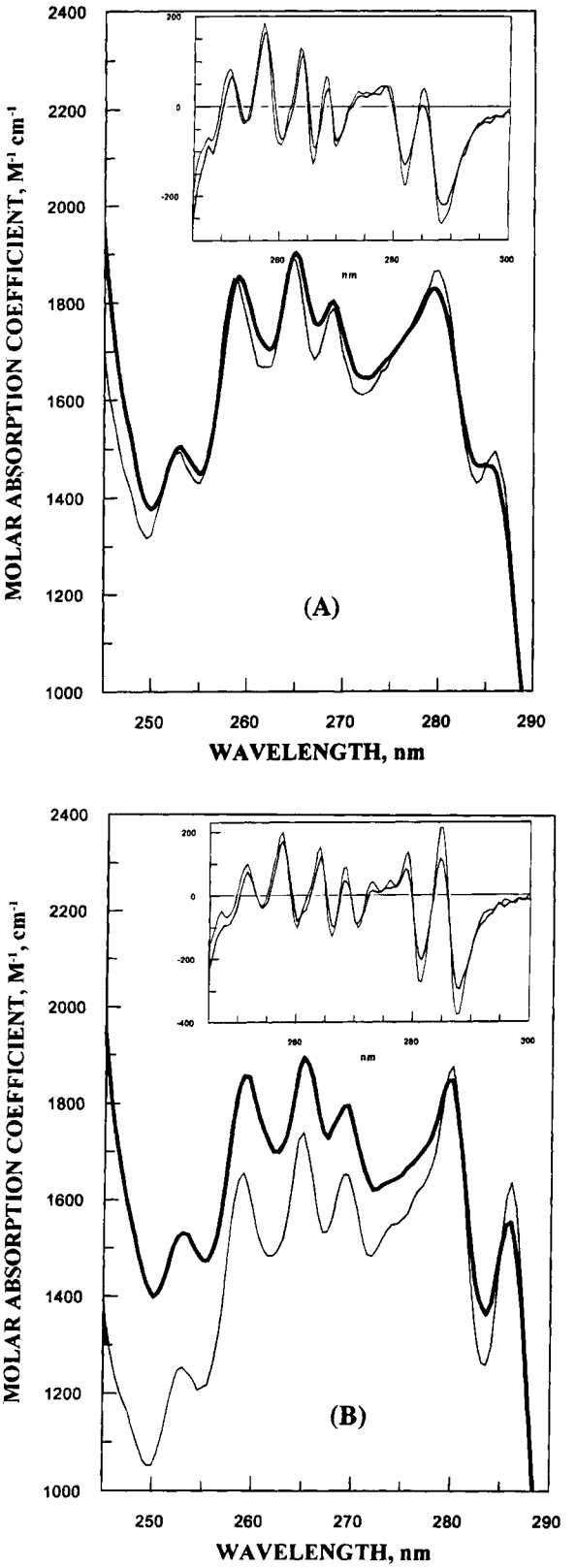


FIGURE 6: Absorption band narrowing effects of isomerase spectra by lowering the temperature. UV spectra of the free and steroid-bound Y55F/Y88F mutant in 100 mM NaCl and 50 mM Tris-HCl (pH 7.50) at 5 (thin line) and 40 °C (thick line). Insets: First-derivative spectra. (A) UV spectra of the free enzyme. (B) UV spectra of the Y55F/Y88F mutant bound with 5α-estrane-3,17-dione.

DISCUSSION

Multiple Modes of Hydrogen Bonding and Motion Account for the Broadening of Tyrosine Absorption Bands. The

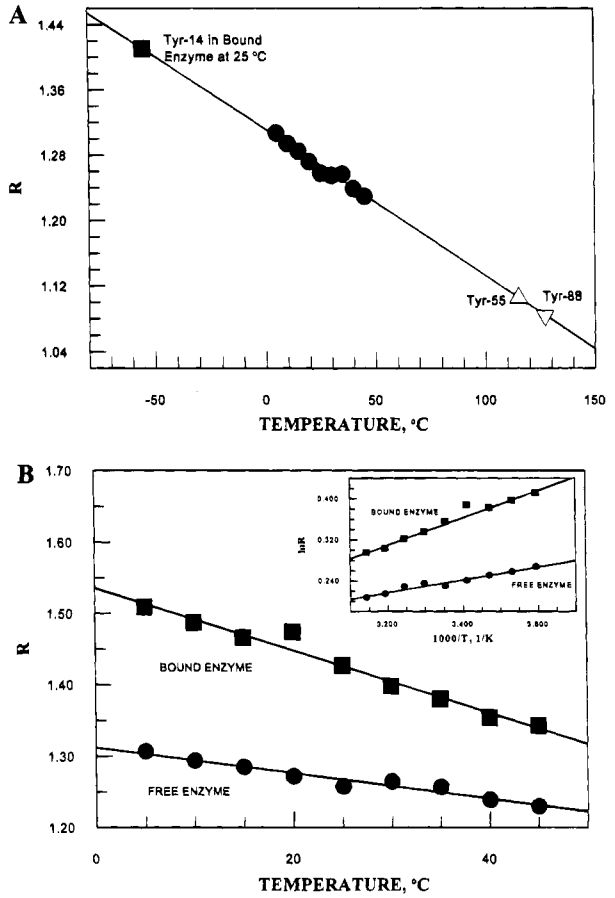


FIGURE 7: Linear correlation between absorption peak sharpness ($A_{279.5}/A_{283.5}$) of Tyr-14 in the Y55F/Y88F mutant with respect to temperature. (A) Temperature dependence of the peak sharpness of Tyr-14 in the Y55F/Y88F mutant as compared to the spectral behavior of Tyr-55 and Tyr-88 in their respective double tyrosine mutants and Tyr-14 in steroid-bound enzyme at 25 °C. (B) Temperature dependence of the peak sharpness of Tyr-14 in the free and steroid-bound Y55F/Y88F mutant. Inset: van't Hoff plot of the sharpening effects on the tyrosine absorption band (ln R with respect to $1/T$), in which the slopes correspond to the enthalpy for the peak-narrowing process.

change in polarity at the active site upon steroid binding cannot explain the significant narrowing of the Tyr-14 absorption bands, because such unusually sharp absorption bands in the steroid–enzyme complex could not be simulated by exposing a mixture of the ethyl esters of *N*-acetyltyrosine and *N*-acetylphenylalanine (molar ratio 1:10) to nonpolar solvents such as *n*-hexane (Table 1). The formation of a hydrogen bond between the phenolic group and the carbonyl group of the 3-ketosteroid *itself* is not the sole origin of the narrowing, since the hydrogen bond formation between phenol and dioxane in isooctane slightly broadens and red shifts the initially narrow absorption bands (Nemethy & Ray, 1973). Nevertheless, the broadening and sharpening of tyrosine absorption bands described earlier can be interpreted as follows. In theory, vibrational and, to a much lesser extent, rotational motions are responsible for the broadening of the electronic spectra of individual transitions. The absorption bands in the spectra of phenolic compounds in polar solvents, particularly aqueous solutions, are generally broad. In addition to the vibrational motion, this broadening effect on the absorption bands has been attributed to the multiple hydrogen-bonding interactions with solvent molecules (Nemethy & Ray, 1973; Yarwood et al., 1978). A narrowing of the broad absorption band of phenol in the 280–300-nm region was observed in aqueous solutions containing 0.25 M or higher concentrations of pyridine (Pekary, 1978). This prevention by pyridine of the broadening of phenol absorption bands was attributed to strong hydrogen bonding within a structured 1:1 complex. The nitrogen atom of pyridine can act *only* as a strong hydrogen bond acceptor from the phenol in contrast to water or alkyl alcohols. Different modes of hydrogen bonding, as shown in Figure 8A, can cause shifts of various directions and magnitudes. The formation of a hydrogen bond in which the phenolic hydroxyl group is the proton donor (Figure 8A, I) releases electron density from the O–H bond toward the oxygen and, by an inductive effect, toward the aromatic ring. This causes a red shift of the $\pi \rightarrow \pi^*$ transition (Ito, 1960; Baba & Suzuki, 1961; Chignell & Gratzer, 1968). Conversely, if a hydrogen bond is formed in which the phenolic oxygen is a proton acceptor (Figure 8A, II), electrons are withdrawn from the ring and a blue shift is observed (Ito, 1960; Grinspan et al., 1966). In hydroxylic solvents, such as water and aliphatic alcohols, both forms of hydrogen bonding may occur simultaneously (Figure 8A, III). In this case, opposing effects on the partial charge on the phenolic oxygen atom and, in turn, on the aromatic ring can occur, thus complicating the shifting effects of the $\pi \rightarrow \pi^*$ transition. These multiple species can result in significant broadening of the absorption bands of tyrosine residues in proteins.

The directionality of a hydrogen-bonding interaction can also significantly affect its strength. This is especially important in the hydrogen bonding in a protein, particularly in its interior, as the orientation of a residue is fixed by the tertiary structure of the protein. The more polar environment (i.e., exposure to bulk solvent) and multiple modes of hydrogen-bonding interaction (Figure 8A) of Tyr-55 and Tyr-88, particularly Tyr-88, are mimicked by exposing the Y/F mixture to solvents capable of progressively stronger and multiple modes of hydrogen bonding (Figure 2). Further narrowing of the tyrosine absorption bands, but *not* of the phenylalanine absorption bands, of Tyr-14 in the Y55F/Y88F mutant upon the binding of a steroidal ligand probably results from more directional hydrogen bonding, as shown by NMR (Kuliopulos et al., 1991) and decreased motion of the aromatic ring. The lattice of water molecules in the active site is displaced by the nonpolar steroidal ligand, the active site cavity is occupied by the steroid skeleton, and the motion of Tyr-14 is restricted by directional hydrogen bonding to the *trans* lone electron pair of the 3-ketosteroid (Kuliopulos et al., 1989, 1991). Exclusion of water molecules from the active site by a steroid ligand has been demonstrated with cholesterol oxidase by X-ray crystallographic studies (Li et al., 1993b). The broadening effects on tyrosine absorption caused by multiple modes of hydrogen bonding (Figure 8A) and vibrational and rotational motions of the aromatic ring (Figure 8B) are not mutually exclusive. Instead, they can be coupled to each other since a buried tyrosine, upon becoming more mobile, can participate in different forms of hydrogen bonds with other residues, amide bonds, or water molecules. In addition to the local mobility of the active site discussed earlier, the global fluctuation of the whole protein molecule could also result in the broadening of the tyrosine absorption bandwidths since the observed spectrum of the enzyme is the average of many fluctuating states (Ansari et al., 1985; Frauenfelder et al., 1991).

It is a well-recognized phenomenon that the decreased motion of chromophores at low temperatures induces sharper

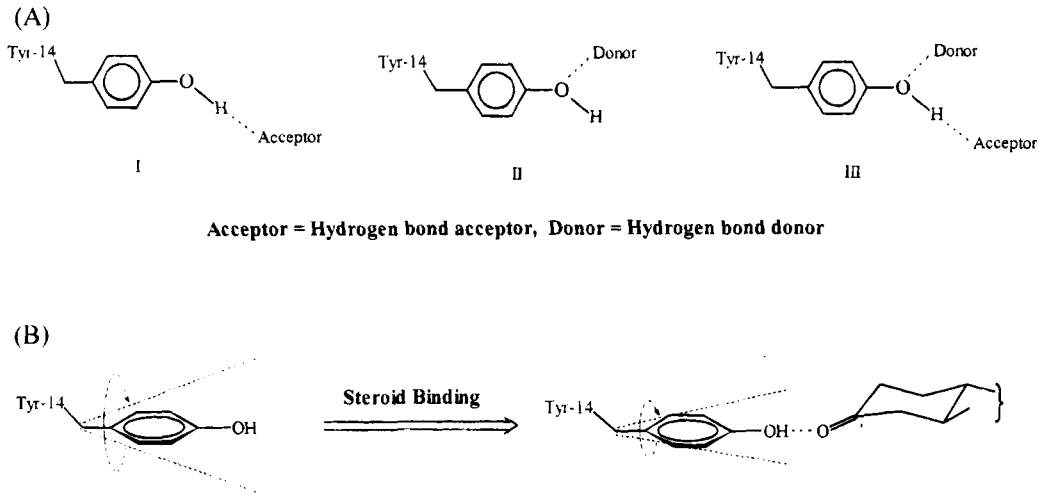


FIGURE 8: Schematic illustrations of the origins of the broadening of tyrosine absorption bands and the sharpening of the Tyr-14 absorption bands in the Y55F/Y88F mutant in the steroid-bound complex and/or at lower temperatures. (A) Multiple modes of hydrogen bonding for the phenolic hydroxyl group of tyrosine. (B) Immobilization of Tyr-14 at the active site by ligand binding. Repositioning of Tyr-14 occurs in response to steroid binding (Kuliopulos et al., 1989, 1991).

absorption bands. Sharpening of the absorption bands of ligands at lower temperatures has been demostrated by Chance (1957) and Keilin and Hartree (1949) with hemoproteins. An unusually long fluorescence lifetime (4.6 ns) for Tyr-14 as compared to Tyr-55 (2.0 ns) or Tyr-88 (0.8 ns) in the respective double tyrosine mutants and compared to most protein tyrosine residues (0.2−2 ns) was observed in the free isomerase (Wu et al., 1994). This suggests that no motion of Tyr-14 occurs on the picosecond to nanosecond time scale as detected by fluorescence at the hydrophobic active site. Unfortunately, studies of the fluorescence of the steroid-bound enzyme are not feasible since the motion of Tyr-14 is expected to be slower, and the fluorescence of Tyr-14 is quenched almost completely in the steroid-bound complex.

The significant sharpening of the tyrosine absorption bands induced by lowering of the temperature or binding of the steroid ligand may result from truncations of the numbers of vibrational and rotational states of Tyr-14 and/or equilibrium fluctuation substates of the protein in the liganded enzyme compared to the free enzyme. Immobilization or freezing at the reaction center (FARCE) (such as the restricted motion of Tyr-14 in isomerase) in the complex would promote catalysis by restricting the location of catalytic residues to functional positions [Mildvan (1974), and references therein].

Hydrogen Bond Compensation in Ligand Binding for Tyr-14 in Isomerase. Implication for an Efficient Hydrogen Bond Network during Catalysis. Strong hydrogen-bonding interactions between the active site, presumably Tyr-14, and the steroid ligand have been demonstrated (Wang et al., 1963; Kuliopulos et al., 1989, 1991; Austin et al., 1992, 1995; Zhao et al., 1995). Recent studies of the UV resonance Raman spectra of 19-nortestosterone bound to isomerase suggested the presence of an extraordinarily strong hydrogen bond to the carbonyl group of 19-nortestosterone, on the basis of the decreased C=C stretching frequency of the isomerase-bound steroid, which was intermediate between that of protonated 19-nortestosterone in 10 M H_2SO_4 and that of hydrogen-bonded 19-nortestosterone in 10 M HCl (Austin et al., 1995). Strong hydrogen bond donation by phenolic compounds has been shown to result in significant red shifts in their absorption maxima (Baba & Suzuki, 1961; Grinspan et al., 1966; Pekary, 1978; Zhao et al., 1995). In an apparent paradox, the strong hydrogen bond formation to the ketosteroid contrasts with the finding that the ligand binding did not change the net hydrogen-bonding status of Tyr-14, as reflected by unshifted absorption maxima (Figure 3). Thus, the strong hydrogen-bonding donation from Tyr-14 must have been compensated by other hydrogen bond donor(s). Compensatory hydrogen bond donation to Tyr-14 has been proposed to rationalize the unperturbed UV resonance Raman bands of Tyr-14 in the steroid-bound complex (Austin et al., 1992, 1995). Assisted hydrogen bonding by Tyr-14 contributes at least 7.6 kcal/mol toward binding and stabilizing the dienolic intermediate (Xue et al., 1991), thus lowering the overall free energy barrier of the isomerase-catalyzed reaction.

CONCLUSIONS

The present study demonstrates that the hydrophobic steroid skeleton and a polar moiety capable of efficient hydrogen-bonding interactions (i.e., carbonyl or phenolic hydroxyl group) at C-3 are two critical prerequisites for the tight binding of steroids to the active site of isomerase. The high fluorescence of the sole tyrosine residue, Tyr-14, in the Y55F/Y88F double mutant of isomerase (which retains high catalytic activity) is efficiently quenched by all steroids that bind to the active site. The directional hydrogen-bonding interactions between the ligand and the active site of isomerase, coupled with the intimate atomic contact of the steroid skeleton and the nonpolar surface (consisting mainly of phenylalanines) of the active site cavity, result in the immobilization of Tyr-14 and the steroid ligand in the active site of isomerase. This "freezing" effect of Tyr-14 is demonstrated by the sharpening of the tyrosine absorption bands when steroids bind to the active site and is supported by the analogous effect of lowering the temperature of the free and steroid-bound isomerase. Such freezing of a catalytic residue would be expected to enhance its catalytic effect by restricting its location to an optimal position [Mildvan (1974) and references therein]. The unchanged Tyr-14 absorption maxima in the steroid-bound isomerase suggest that the hydrogen bond donation of Tyr-14 to the ketosteroid is compensated by other hydrogen bond donors during ligand binding and catalysis.

ADDED IN PROOF

Consistent with Figure 8B, we have recently shown, by T_1, T_2, and NOE measurements at 125.7 and 150.9 MHz of ϵ-^{13}C-enriched Tyr-14 in the Y55F/Y88F mutant isomerase, that the ^{13}C order parameter (S^2) increases from 0.75 ± 0.04 to 0.86 ± 0.04 when 19-nortestosterone hemisuccinate binds, indicating a decreased amplitude of motion of the Tyr-14 ring in the steroid complex (Q. Zhao, C. Abeygunawardana, P. Talalay, and A. S. Mildvan, unpublished observations).

APPENDIX

5α-Estrane-3,17-dione is a competitive inhibitor (I) that binds to the active site of isomerase (E). Thus,

$$E + I \rightleftharpoons E{\cdot}I$$

The total enzyme concentration in solution is the sum of the free and bound enzyme:

$$E_t = E_b + E_f \quad \text{and} \quad I_t = I_b + I_f = E_b + I_f$$

wherein subscripts t, f, and b refer to total, free, and bound species, respectively.

Thus,

$$K_D = \frac{E_f I_f}{E_b} = \frac{(E_t - E_b)(I_t - E_b)}{E_b} \tag{1}$$

The roots for eq 1 are

$$E_b = \frac{K_D + E_t + I_t \pm \sqrt{(K_D + E_t + I_t)^2 - 4E_tI_t}}{2} \tag{2}$$

where only the root with negative sign is real.

By assuming that the observed peak sharpness (R) is the weighted average of both free and bound enzyme, the overall R can be expressed as

$$R = \frac{R_bE_b + R_fE_f}{E_t} = \frac{R_bE_b + R_f(E_t - E_b)}{E_t} = R_f + \frac{R_b - R_f}{E_t}E_b \tag{3}$$

By substituting eq 2 into eq 3, we obtain the final equation for data fitting:

$$R = R_f + \frac{R_b - R_f}{2E_t}(K_D + E_t + I_t - \sqrt{(K_D + E_t + I_t)^2 - 4E_tI_t} \tag{4}$$

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