Combined Effects of Two Mutations of Catalytic Residues on the Ketosteroid Isomerase Reaction†

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ABSTRACT: Δ^5-3-Ketosteroid isomerase (EC 5.3.3.1) catalyzes the isomerization of Δ^5-3-ketosteroids to Δ^4-3-ketosteroids by a conservative tautomeric transfer of the 4β-proton to the 6β-position with Tyr-14 as a general acid and Asp-38 as a general base [Kuliopulos, A., Mildvan, A. S., Shortle, D., & Talalay, P. (1989) *Biochemistry 28*, 149–159]. Primary, secondary, and combined deuterium kinetic isotope effects establish concerted substrate enolization to be the rate-limiting step with the wild-type enzyme [Xue, L., Talalay, P., & Mildvan, A. S. (1990) *Biochemistry 29*, 7491–7500]. The product of the fractional k_{cat} values resulting from the Y14F mutation ($10^{-4.7}$) and the D38N mutation ($10^{-5.6}$) is comparable ($10^{-10.3}$) to that of the double mutant Y14F + D38N ($\leq 10^{-10.4}$) which is completely inactive. Hence, the combined effects are either additive or synergistic. Quantitatively, similar effects of the two mutations on k_{cat}/K_M are found in the double mutant. Despite its inactivity, the Y14F + D38N double mutant forms crystals indistinguishable in form from those of the wild-type enzyme, tightly binds steroid substrates and substrate analogues, and immobilizes a spin-labeled steroid in an orientation indistinguishable from that found in the wild-type enzyme, indicating that the double mutant is otherwise largely intact. It is concluded that the total enzymatic activity of ketosteroid isomerase probably results from the independent and concerted functioning of Tyr-14 and Asp-38 in the rate-limiting enolization step, in accord with the perpendicular or antarafacial orientation of these two residues with respect to the enzyme-bound substrate. Synergistic effects of mutating two residues on k_{cat} and on k_{cat}/K_M of enzyme-catalyzed multistep reactions are shown, theoretically, to occur when both residues act independently in the same step, and simple additivity occurs when this step is rate-limiting. Other conditions for additivity of the effects of mutations of k_{cat} and k_{cat}/K_M are theoretically explored.

The enzyme Δ^5-3-ketosteroid isomerase (EC 5.3.3.1) of *Pseudomonas testosteroni* catalyzes the isomerization of Δ^5-3-ketosteroids to the conjugated Δ^4-3-ketosteroids by a stereospecific and conservative transfer of the 4β-proton to the 6β-position by way of an enolic intermediate (Batzold et al., 1976; Bantia & Pollack, 1986) (Figure 1).

Previous mechanistic studies (Kuliopulos et al., 1989) have established the importance of both Tyr-14 and Asp-38 in the isomerase reaction with the former serving as the proton donor (AH) and the latter as the proton acceptor (B) in the rate-limiting enolization step of the reaction (Xue et al., 1990). Model building studies based on X-ray and NMR data indicate these residues to be positioned in either a perpendicular or, less likely, antarafacial geometry with respect to the bound substrate, arrangements which are stereoelectronically appropriate for a concerted enolization of the substrate (Kuliopulos et al., 1989; Xue et al., 1990; Hand & Jencks, 1975; Rebek, 1988). Primary, secondary, and combined deuterium kinetic isotope effects have established the concerted enolization of the substrate as a major rate-limiting step in the overall reaction (Xue et al., 1990).

Profound decreases in k_{cat} by factors of $10^{4.7}$ and $10^{5.6}$ were reported for the Y14F and D38N mutant enzymes, respectively. Indeed, the product of these factors ($10^{10.3}$) is comparable to the overall rate acceleration ($\approx 10^{9.5}$) produced by the wild-type enzyme, as compared with the spontaneous rate of isomerization at the same temperature and pH, suggesting that the entire catalytic power of this enzyme might be explained by the concerted action of these two residues in the hydrophobic site of the enzyme (Kuliopulos et al., 1989).

As previously pointed out, however, before such quantitative conclusions can be drawn from the kinetic effects of mutations, it must be established that (1) the catalytic and structural effects of each mutation are limited to the residue altered and that (2) the contribution of each catalytic residue to the rate enhancement is independent from that of other catalytic residues and counted only once (Serpersu et al., 1987; Knowles, 1987; Carter et al., 1984; Fersht, 1987). The tight binding of substrates and substrate analogues by the Y14F and D38N single mutants (Kuliopulos et al., 1989) argues against major structural alterations beyond the residues mutated. However, these observations do not address whether the catalytic contributions of Tyr-14 and Asp-38 are independent and additive. The most direct way of testing this point is to determine how the effects of the individual mutations of these catalytic residues interact in a double mutant in which both catalytic residues have been altered (Ackers & Smith, 1985; Knowles, 1987; Carter et al., 1984; Weber et al., 1990). Accordingly, the double mutant Y14F + D38N was prepared, and its kinetic and substrate binding properties were studied. A preliminary report of this work has been published (Kuliopulos et al., 1990).

Experimental Procedures

Materials. Spiro[doxyl-2,3′-5′α-androstan]-17′β-ol (doxyl-DHT),[1] mp 172–174 °C, was obtained from Aldrich

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[1] Abbreviations: doxyl-DHT, spiro[doxyl-2,3′-5′α-androstan]-17′β-ol; BTP-Cl or bis-Tris-propane-HCl, 1,3-bis[[tris(hydroxymethyl)-methyl]amino]propane dihydrochloride.

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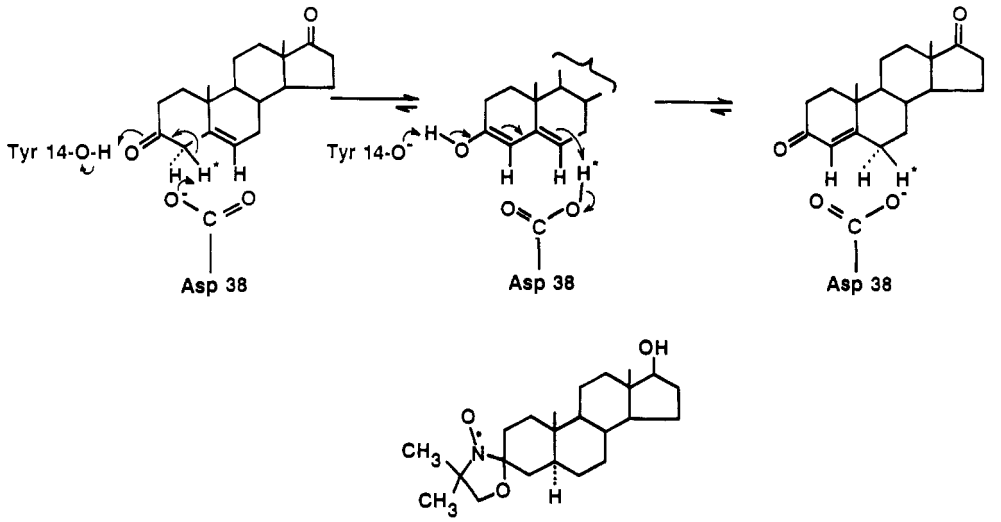

FIGURE 1: (Top) Reaction mechanism for the conversion of Δ^5-3-ketosteroids to the corresponding Δ^4-3-ketosteroids by Δ^5-3-ketosteroid isomerase of *Pseudomonas testosteroni*. The reaction proceeds via a $\Delta^{3,5}$-dienol intermediate. The general acid (Tyr-14) protonates the 3-carbonyl oxygen, and the general base (Asp-38) abstracts the 4β-proton (H*) and conservatively transfers the proton to the 6β-position. (Bottom) Structure of the linear competitive inhibitor of the enzyme: spiro[doxyl-2,3′-5′α-androstan]-17′β-ol or doxyl-DHT (doxyldihydrotestosterone).

Chemical Co. (Milwaukee, WI). All buffer solutions used in the magnetic resonance studies were treated with Chelex-100 to remove metal ions. Water was doubly distilled from glass and passed over a mixed-bed ion-exchange resin. The substrate, Δ^5-androstene-3,17-dione, was synthesized from dehydroepiandrosterone (Kawahara et al., 1962), and 19-nortestosterone, a gift of Searle Chemical Co. (Chicago, IL), was purified by recrystallization and high-vacuum sublimation. The source of the *Escherichia coli* (strain DH5αF′) competent cells and the M13mp18 plasmid was BRL (Bethesda, MD).

Site-Directed Mutagenesis. The techniques used to prepare the wild-type (WT) and the Y14F, D38N, and Y55F single-mutant isomerase genes have been described (Kuliopulos et al., 1987a, 1989). The Y14F + D38N double mutant was constructed by the method of Zoller and Smith (1983) with modifications as follows: the DNA which codes for the D38N mutation served as the uracil-containing single-stranded template constructed with the M13mp18 plasmid. A deoxyoligonucleotide 17-mer corresponding to bases 31–47 and containing the Tyr-14 → Phe mutation served as the primer (Kuliopulos et al., 1989). Five picomoles of the 5′-phosphorylated 17-mer was annealed to ≈500 ng of single-stranded template DNA by incubating this mixture (25 μL) for 2 min at 70 °C and then for 30 min at 40 °C in 20 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, and 50 mM NaCl. The annealing mixture (10 μL) was then removed, and the primer extension reaction was initiated by adding 5–8 units of Sequenase (U.S. Biochemical Corp., Cleveland, OH) and 4.5 units of T4 DNA ligase (Boehringer, Indianapolis, IN) in 1 mM ATP, 7 mM DTT, and 300 μM each dNTP (dATP, dTTP, dCTP, dGTP), in a total volume of 15 μL. The reaction was allowed to proceed for 10 min at 25 °C, then for 3 h at 30 °C, and finally for 25 min at 37 °C. The reaction was terminated by adding 90 μL of 10 mM Tris-HCl, pH 8.0–15 mM EDTA and freezing. *E. coli* (strain DH5αF′) competent cells were transformed with the M13mp18 plasmid that contained the Y14F + D38N double mutation. The desired clone was identified by sequencing the entire gene (Sanger et al., 1977). This method yielded the correct double mutant with a very high frequency of 90–100%. Similarly high frequencies were obtained by this method in preparing other mutants of ketosteroid isomerase.

Purification of the Protein. The procedure for purifying the double mutant was carried out as previously described (Kuliopulos et al., 1989) with slight modification. To prevent proteolysis of this mutant, 1 mM EDTA and 0.5 mM phenylmethanesulfonyl fluoride were added to the sonication buffer. The isomerase was precipitated from the poly(ethylenimine) (Polymin P, BRL) supernatant solution by 65% saturation with ammonium sulfate. The precipitate was desalted in 1 mM bis-Tris–propane–HCl (BTP-Cl), pH 7.0, by passage through a Sephadex G-25M column (Pharmacia, PD-10). The double mutant was then purified by three successive anion-exchange chromatographies. The first separation was on a Fast Flow Q-Sepharose (Pharmacia) column (15 cm × 2.8 cm) with a linear gradient from 1 to 200 mM BTP-Cl, pH 7.0. Column fractions containing isomerase were exchanged into 1 mM BTP-Cl, pH 8.25, with the use of Centriprep microconcentrators (Amicon, Danvers, MA) and then chromatographed on a Mono-Q column (Pharmacia) with a linear gradient from 1 mM BTP-Cl, pH 8.25, to 100 mM BTP-Cl, pH 7.5. Fractions containing the isomerase were rechromatographed through the Mono-Q column under identical conditions. The purest fractions were pooled, concentrated with Centriprep microconcentrators to at least 5 mg/mL, and crystallized at 4 °C by the addition of saturated ammonium sulfate (which had been neutralized to pH 7.0 with 3 M NH_4OH) to a concentration of 35% saturation. After 2 days at 4 °C, the crystalline suspension was centrifuged, the crystals were redissolved in a minimal volume of 50 mM Tris-HCl, pH 7.5, and the enzyme was recrystallized twice at progressively lower levels of ammonium sulfate, 30% and 25% saturation.

In separate experiments, the addition of phenylmethanesulfonyl fluoride or EDTA or the use of Fast Flow Q-Sepharose did not alter the activity of the wild-type enzyme. The purity of the Y14F + D38N double-mutant enzyme was assessed by polyacrylamide gel electrophoresis in the presence of SDS and staining with Coomassie Blue R 250.

Protein Concentrations. The protein concentration of the double-mutant enzyme was determined by absorbance measurements at 280 nm, assuming that a solution containing 1.00 mg/mL enzyme had an A_{280} value of 0.226 (Kuliopulos et al., 1989).

Kinetic Studies. The upper limit estimate of the specific activity of the isomerase double mutant was made by monitoring the change in absorbance at 248 nm resulting from formation of the product, Δ^4-androstene-3,17-dione. Kinetic

measurements were performed at 25 °C in 600-μL total reaction volumes containing 50 mM Tris-HCl, pH 7.5, and 58.2 μM Δ^5-androstene-3,17-dione in methanol (1.7% v/v). Final concentrations of enzyme used in the assays were from 0.5 to 3.16 mg/mL. The kinetic data were analyzed as described (Kuliopulos et al., 1989).

EPR Spectroscopy. EPR spectra were obtained on a Varian E4 EPR spectrometer as described (Kuliopulos et al., 1987b) with the following changes: the time constant was 3 s, modulation amplitude was 3.2 G, microwave frequency was 9.097 GHz, and temperature was 22.6 °C.

$1/T_{1p}$ Measurements and Data Analysis. The paramagnetic effects on the longitudinal relaxation rates ($1/T_{1p}$) of water protons were obtained at 24.3 MHz as described (Mildvan & Engle, 1972; Kuliopulos et al., 1987b). Solutions with fixed amounts of doxyl-DHT (D) were titrated with enzyme (E), the molar relaxivity, $1/(T_{1p}[\text{doxyl-DHT}])$, being measured (Mildvan & Weiner, 1969; Kuliopulos et al., 1987b). Plots of $1/(T_{1p}[\text{doxyl-DHT}])$ against the reciprocal enzyme subunit concentration were fit with theoretical curves by use of

$$1/(T_{1p}D_t) = (D_b/D_t)MR_b + (D_f/D_t)MR_f \quad (1)$$

$$K_D = D_fE_f/D_b \quad (2)$$

By substituting the relationships

$$D_t = D_f + D_b \quad (3)$$

$$E_t = E_f + E_b \quad (4)$$

$$D_b = \{(K_D + E_t + D_t) - [(K_D + E_t + D_t)^2 - 4E_tD_t]^{1/2}\}/2 \quad (5)$$

into eqs 1 and 2, we obtain

$$1/(T_{1p}D_t) = \frac{(K_D + E_t + D_t) - [(K_D + E_t + D_t)^2 - 4E_tD_t]^{1/2}}{2D_t}MR_b + \frac{(D_t - K_D - E_t) + [(K_D + E_t + D_t)^2 - 4E_tD_t]^{1/2}}{2D_t}MR_f \quad (6)$$

where K_D is the dissociation constant, E is the concentration of enzyme subunits, D is the concentration of doxyl-DHT, MR is the molar relaxivity, and the subscripts b, f, and t refer to the bound, free, and total species, respectively. Curve fitting of the data yielded the K_D and the MR_b values for the double mutant–doxyl-DHT complex. The MR_f was measured independently from the slope of the line derived from MR_{obs} plotted against D in the absence of enzyme. The ratio MR_b/MR_f was used to determine the enhancement factor, ϵ_b, of the bound nitroxide on the longitudinal relaxation rate of water protons (Kuliopulos et al., 1987b). In alternative titrations, in which constant concentrations of the enzyme were titrated with variable concentrations of spin-label, it was more convenient to plot $1/T_{1p}$ against D_t. These data were fit by theoretical curves described by eq 6 (multiplied by D_t). In both cases, the fit of the data to the theoretical curves was optimized by nonlinear regression analysis.

The substrate dissociation constant, K_s, was obtained by monitoring the change in the $1/T_{1p}$ values of water protons upon displacement of doxyl-DHT from the steroid-binding site by Δ^5-androstene-3,17-dione. The data were fit by assuming competition between doxyl-DHT and substrate at the single steroid-binding site found, per subunit of the enzyme, according to [see Mullen et al. (1989)]

$$D_b^3(K_D - K_s) + D_b^2(K_sE_t + 2K_sD_t + K_DS_t - K_DK_s - K_DE_t - K_DD_t - K_D^2) + D_b(K_DE_tD_t - K_DD_tS_t - K_DK_sD_t - 2K_sE_tD_t - K_sD_t^2) + K_sE_tD_t^2 = 0 \quad (7)$$

where S_t is the concentration of substrate and K_s is the dissociation constant of substrate from the enzyme. The equation was solved for D_b, which was used with eq 1 (multiplied by D_t) to calculate the observed $1/T_{1p}$ in the displacement titration.

Results

Mutagenesis, Homogeneity, and Molecular Properties of the Y14F + D38N Double Mutant. The Y14F + D38N double mutant was successfully constructed from template DNA of the D38N single mutant and the oligonucleotide containing the Tyr-14 → Phe mutation at extremely high yield (approaching nearly 100% of the desired mutation) by the method outlined under Experimental Procedures. The entire gene was sequenced, and the absence of adventitious base changes was verified. When the double mutant was expressed in *E. coli*, the amount of enzyme in soluble form was low, resulting in an overall yield of only 10 mg of homogeneous enzyme from 8 L of late-log-phase cell cultures by the purification procedure described above. When this was overloaded on a sodium dodecyl sulfate–polyacrylamide gel (≈30 μg/lane) and stained with Coomassie Blue R 250, one major band was visible that migrated identically with wild type and the Y14F and D38N single mutants. Scanning of the gel indicated that this major band constituted at least 95% of the detectable protein. Apparently, much of the expressed isomerase was insoluble and sedimented with the cell debris by low-speed centrifugation, suggesting that this particular mutant enzyme might have been processed into inclusion bodies. Attempts to solubilize the precipitated isomerase by lengthy sonication, detergents, and organic solvents such as 80% ethanol (Jarabak et al., 1969) or by renaturation from 6 M urea were unsuccessful. This behavior is much more severe than that of the wild type or either of the single mutants, possibly due to the greater hydrophobicity of the double mutant (Kuliopulos et al., 1989).

The ultraviolet spectrum of the double mutant was also obtained in order to determine protein concentration, to ascertain purity, and to confirm the aromatic amino acid composition (Kuliopulos et al., 1989). The ultraviolet spectrum of the double mutant (Figure 2) is identical with that of the Y14F single mutant with a principal maximum at 277 nm, shoulder at 282 nm, and four smaller absorbance peaks at 253, 259, 265, and 269 nm, consistent with the absence of tryptophan. As with all homogeneous isomerase preparations, the UV minimum was at 250 nm. The phenylalanine to tyrosine ratio, based on the 277 nm/259 nm absorbance ratio (Kuliopulos et al., 1989), for the Y14F + D38N and the Y14F mutants was identical, giving further evidence that the aromatic amino acid composition of the two enzymes was the same.

The double-mutant enzyme was recrystallized twice, and the crystals were examined microscopically. The crystals were found to be elongated needles and thin platelets, characteristic of the monoclinic form of the enzyme and indistinguishable from crystals of the wild-type enzyme (Kawahara & Talalay, 1960; Westbrook, 1976).

Kinetic Parameters of Single and Double Mutants. The specific activity was measured for three different homogeneous preparations of the Y14F + D38N double mutant and was found to be $\leq 1.0 \times 10^{-5}$ IU/mg or at least $10^{9.7}$-fold less than the recombinant wild-type value. This would correspond to a value of $k_{cat} \leq 2.3 \times 10^{-6}$ s^{-1} (Table I). The specific activity measurement of the double mutant was an upper limit estimate because the observed activity was *half* of the spontaneous isomerization rate at pH 7.5 or 0.0005 ΔA/min under standard assay conditions, implying that the double-mutant enzyme

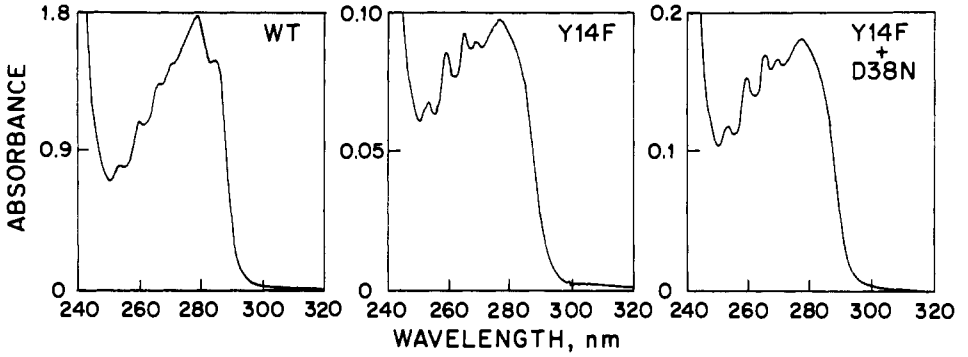

FIGURE 2: Ultraviolet spectra of wild type (WT), Y14F, and the Y14F + D38N double mutant. Spectra were obtained at 25 °C, in 50 mM Tris-HCl, pH 7.5, and blanked against buffer alone. Concentrations for WT, Y14F, and Y14F + D38N were 4.76, 0.407, and 0.739 mg/mL, respectively.

Table I: Kinetic Parameters of Wild Type and Single and Double Mutants of Ketosteroid Isomerase[a]

enzyme	sp act. (IU mg^{-1})[b]	k_{cat} (s^{-1})	K_M (μM)	K_{cat}/K_m (M^{-1} s^{-1})	rel k_{cat}	rel k_{cat}/K_M
wild type[c]	45 300	53 600	340 ± 20	1.58×10^8	1.00	1.00
Y14F[c]	2.28	1.18	123 ± 13	9.59×10^3	$10^{-4.7}$	$10^{-4.2}$
D38N[c]	0.255	0.13	102 ± 22	1.27×10^3	$10^{-5.6}$	$10^{-5.1}$
Y14F + D38N[d]	$\leq 1.0 \times 10^{-5}$	$\leq 2.3 \times 10^{-6}$	17 ± 10[e]	≤0.14	$\leq 10^{-10.4}$	$\leq 10^{-9.1}$

[a] All assays were done at 25 °C. Because of changes in K_M, there is no direct proportionality between specific activity and k_{cat}. [b] The specific activity for wild-type enzyme was determined under standard conditions in 3.0-mL systems containing 50 mM Tris-HCl, pH 7.5, and 58.2 μM Δ^5-androstene-3,17-dione in methanol (1.7%, v/v); the specific activity for the double mutant was determined as with wild type, except that 0.6-mL systems were used. [c] Data from Kuliopulos et al. (1989). [d] All rates are considered upper limit estimates because the measured apparent rate was equal to half of the base-line rate in the absence of enzyme. [e] K_s was determined by direct binding measurements based on the effects of the complex of doxyl-DHT and Y14F + D38N on the $1/T_{1p}$ values of water protons with Δ^5-androstene-3,17-dione in competition with doxyl-DHT (see text).

Table II: Free Energy Barriers to Catalysis of Isomerization of Δ^5- to Δ^4-Androstene-3,17-dione Relative to That with the Wild-Type Enzyme

catalyst	k_{cat}/K_M[a,b] or k'_2[c] (M^{-1} s^{-1})	$\Delta\Delta G_T^{\ddagger}$ (kcal/mol)	k_{cat} or k'_1[d] (s^{-1})	$\Delta\Delta G_T^{\ddagger}$ (kcal/mol)
enzymes				
wild type	1.58×10^8	0	5.36×10^4	0
Y14F	9.59×10^3	5.7	1.18	6.3
D38N	1.27×10^3	6.9	0.13	7.6
Y14F + D38N	≤0.14	≥12.3	$\leq 2.3 \times 10^{-6}$	≥14.1
nonenzymatic				
OH^-	1.82	10.8	5.76×10^{-7}	14.9
H_3O^+	1.5×10^{-2}	13.6	4.7×10^{-10}	19.1
H_2O	8.9×10^{-8}	20.7	5.0×10^{-6}	13.6
Tris	1.3×10^{-3}	15.1	1.3×10^{-5}	13.1
total nonenzymatic			1.86×10^{-5}	12.9

[a] k_{cat}/K_M values are from Kuliopulos et al. (1989) with the following conditions: 25 °C, 50 mM Tris-HCl, pH 7.50, μ = 0.05. [b] The k_{cat}/K_M values for the Y14F and D38N mutants reflect the V_{max} values of the *initial* velocity in these isomerizations, namely, *enolization*. [c] The second-order rate constants (k'_2) for base, k_{OH^-}, and acid, $k_{H_3O^+}$, were determined by Perera et al. (1980) at 30 °C and had to be corrected to the corresponding values at 25 °C. These temperature corrections were made with the activation parameters calculated by Jones and Wigfield (1969), from Arrhenius plots of the isomerization of substrate by acid and base. In addition, the second-order rate constant for water, k_{H_2O}, could be obtained by first measuring the pseudo-first-order rate constant, k_{obs}, at differing Tris-HCl concentrations and extrapolating to zero buffer concentration. The residual rate was equal to 45% (1.78×10^{-5} s^{-1}) of the 50 mM Tris-HCl catalyzed rate, in excellent agreement with the value determined by Perera et al. (1980). The second-order rate constant for Tris-HCl, k_{Tris}, was not sensitive to ionic strength in the range μ = 0.05–0.5. Since $k_{obs} = k_{OH^-}[a_{OH^-}] + k_{H_3O^+}[a_{H^+}] + k_{H_2O}[H_2O] + k_{Tris}f_B[Tris]$ (where f_B is the fraction of free amine in the buffer), the k_{H_2O} value could then be determined by extrapolating the rates of hydroxide and hydronium ion catalysis to their respective values at pH 7.5. [d] Pseudo-first-order rate constants (k'_1) were calculated for 50 mM Tris-HCl, pH 7.50, at 25 °C.

actually inhibited the spontaneous reaction. Presumably, this inhibition resulted from the binding of the steroid within the hydrophobic active site, thereby making the steroid less accessible to acid and base catalysts in the medium. Because of the propensity of isomerase to aggregate at high concentrations of enzyme (Benson et al., 1975), the highest enzyme concentration used in the rate measurements was 3.16 mg/mL. The K_M value for the double mutant was not measurable since the apparent rate was unchanged over the accessible range of substrate concentrations (<60 μM). Hence, in the following calculations, only upper limits to k_{cat} and to k_{cat}/K_M can be estimated, by use of the measured K_s of the Y14F + D38N double mutant instead of K_M (vide infra).

To facilitate comparisons of rate constants for enzyme-catalyzed steroid isomerization with those of acid-, base-, or water-catalyzed reactions, the increases in the free energy barriers ($\Delta\Delta G_T^{\ddagger}$) above that of the most active wild-type enzyme were calculated (Table II). These calculations were made for both the second-order rate constants, such as k_{cat}/K_M and k_{OH}, and for the first-order and pseudo-first-order rate constants, such as k_{cat} and $k_{OH}[OH^-]$, under identical experimental conditions. As seen from the ($\Delta\Delta G_T^{\ddagger}$) values for the second-order rate constants, the wild-type enzyme has greatly lowered the free energy barrier for steroid isomerization below those of the reactions catalyzed by OH^-, H_3O^+, and water, by 11–21 kcal/mol. The $\Delta\Delta G_T^{\ddagger}$ values of the single mutants Y14F and D38N indicate that the loss of the phenolic hydroxyl group of Tyr-14 or of the carboxylate oxygen of Asp-38 has destabilized the transition state on the enzyme by 5.7 and 6.9 kcal/mol, respectively. These inhibitory effects

Table III: Binding of Doxyl-DHT to Y14F + D38N As Measured by the Effects on $1/T_{1p}$ of Water Protons[a]

concn					
doxyl-DHT	Y14F + D38N	n	K_D (μM)	MR_b (M^{-1} s^{-1})	ϵ_b[b]
constant[c]	varied	1.00 ± 0.05	200 ± 60	1400 ± 300	9 ± 4
varied[d]	constant	1.0 ± 0.1	100 ± 40	2200 ± 400	14 ± 5
	average	1.0 ± 0.1	150 ± 75	1800 ± 500	12 ± 5

[a] Conditions were 45–50 mM Tris-HCl, pH 7.5, 22.6 °C, and 2–7% (v/v) methanol. [b] MR_f = 160 M^{-1} s^{-1} as determined from three separate experiments. [c] Doxyl-DHT concentration was 49.0 μM during the entire titration. The concentration of Y14F + D38N mutant enzyme was varied from 29.1 to 245 μM (subunits). [d] Doxyl-DHT concentration varied from 24.8 to 185 μM. The Y14F + D38N concentration was 90.7–98.0 μM.

appear to be additive in the double mutant Y14F + D38N on which the free energy barrier is greater than on the wild-type enzyme by at least 12.3 kcal/mol. However, since the double mutant is completely inactive, synergistic inhibitory effects of the two mutations cannot be excluded.

The same apparent additivity (or synergy) may be seen by comparing the $\Delta\Delta G_T^{\ddagger}$ values of the first-order and pseudo-first-order rate constants. At pH 7.5 and 25 °C the total catalytic effects of OH^-, H_3O^+, water, and Tris base result in a free energy barrier for the isomerization of steroid free in solution which is 12.9 kcal/mol greater than in the Michaelis complex of the wild-type enzyme. The Y14F mutation adds 6.3 kcal/mol, and the D38N mutation adds 7.6 kcal/mol to the kinetic barrier on the enzyme, and in the double mutant a kinetic barrier of ≥14.1 kcal/mol is seen, comparable to that observed in the absence of enzyme. Thus, Tyr-14 and Asp-38 account for essentially all of the catalytic power of steroid isomerase, assuming (as shown below) that the Y14F + D38N double-mutant enzyme is otherwise intact, i.e., that it can bind substrates and hold them in a proper orientation.

Binding of Doxyl-DHT and Substrate to the Double Mutant As Determined by EPR Spectroscopy. To determine whether the Y14F + D38N double mutant has retained enough structure to bind steroids, the interaction of the spin-labeled steroid doxyl-DHT was studied. The EPR spectrum of the rapidly tumbling, free nitroxide is shown in Figure 3. The spectrum consists of three narrow peaks of equal amplitude, as expected for coupling of the unpaired electron with a ^{14}N nucleus with three allowable spin orientations (−1, 0, 1). When stoichiometric amounts of the double mutant were added to the spin-label, the intensity of the EPR spectrum of the free spin-label was greatly decreased and broad peaks appeared, downfield and upfield from the signal of the free spin-label (Figure 3). These broadened peaks represent the highly immobilized enzyme-bound spin-label, which was previously seen with the wild-type enzyme (Kuliopulos et al., 1987b). The central peak contains contributions from both free and bound spin-label; hence, it is also significantly broadened. The bound spin-label could be completely displaced with saturating amounts of the substrate Δ^5-androstene-3,17-dione as shown by the loss of the broadened upfield and downfield peaks and the return of the free peaks to their original amplitude and line shape (Figure 3). Previous experience had shown that quantitation of these effects was difficult due to incipient aggregation of the unbound spin-label (Kuliopulos et al., 1987b). Therefore, an independent method was used to measure the binding of doxyl-DHT to the double mutant, making use of a property of the bound spin-label.

Measurement of Binding of Doxyl-DHT and Substrate to the Double Mutant Based on Changes in the $1/T_{1p}$ Values of Water Protons. As discussed previously (Kuliopulos et al., 1987b; Mildvan & Weiner, 1969), the paramagnetic effects of nitroxides, when complexed with macromolecules, on the longitudinal relaxation rate ($1/T_1$) of water protons can be used to obtain thermodynamic and structural information on these complexes. If the rotational correlation time of the bound

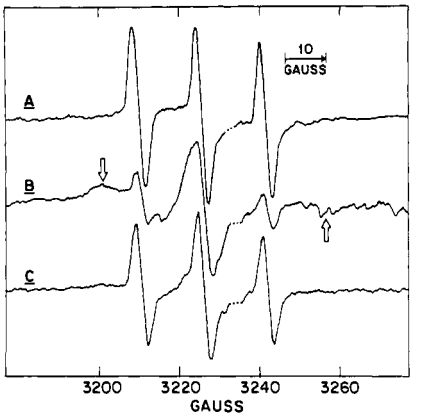

FIGURE 3: Electron paramagnetic resonance spectra of free doxyl-DHT and of the doxyl-DHT–Y14F + D38N complex and displacement of the spin-label by substrate, Δ^5-androstene-3,17-dione. The upper curve (A) was obtained with 98.0 μM doxyl-DHT in 2.0% (v/v) methanol and 49.0 mM Tris-HCl buffer, pH 7.5, in a final volume of 51 μL. Curve B was obtained with 98.0 μM doxyl-DHT, in 2.0% (v/v) methanol, 93.5 μM Y14F + D38N subunits, and 49.0 mM Tris-HCl buffer, pH 7.5. Sample C contained 92.4 μM doxyl-DHT, 88.2 μM Y14F + D38N subunits, and 198 μM Δ^5-androstene-3,17-dione, in 2.0% (v/v) methanol. The instrument settings were scan range 100 G, time constant 3 s, modulation amplitude 3.2 G, microwave power 5 mW, receiver gain 1×10^4, field setting 3227.4 G, scan time 4 min, T = 22.6 °C, and microwave frequency 9.097 GHz. The arrows indicate the broadened upfield- and downfield-shifted signals of the bound (immobilized) spin-label. A small cavity resonance at 3234 G is not shown and is indicated by the dotted line.

nitroxide approaches that of the macromolecule due to immobilization and the nitroxide remains accessible to the solvent, then the paramagnetic effects of the nitroxide on the longitudinal relaxation rate ($1/T_{1p}$) of nearby water protons are greatly enhanced due to the increased dipolar interactions between the unpaired electron and the water protons. The measured molar relaxivity of the free nitroxide (MR_f) was 160 M^{-1} s^{-1}, in reasonable agreement with the previously determined value of 140 M^{-1} s^{-1} (Kuliopulos et al., 1987b). Two types of direct binding titrations were performed. In the first, the molar relaxivity of a solution containing a fixed amount of spin-label was monitored while the enzyme concentration was varied (Figure 4A). These data were fit to a theoretical curve (eq 6) allowing the stoichiometry (n), molar relaxivity (MR_b), and the dissociation constant (K_D) of the bound spin-label to vary. The optimal n, MR_b, and K_D values thus obtained are listed in Table III. In the second titration experiment, the $1/T_{1p}$ values of a solution containing a fixed amount of the enzyme and varying concentrations of doxyl-DHT were measured (Figure 4B). These data were similarly fit and the parameters thus obtained are listed in Table III. The binding stoichiometry of doxyl-DHT was one per subunit of this dimeric enzyme in agreement with that found for the wild-type enzyme, but the dissociation constant was (5 ± 3)-fold weaker. Similar 4–5-fold weakening of steroid binding was previously found with the single mutant Y14F (Kuliopulos et al., 1989), suggesting that the hydroxyl group of Tyr-14 contributes ~1 kcal/mol to steroid binding. The molar re-

laxivity of the spin-label when bound to the Y14F + D38N double mutant (1800 ± 500 M^{-1} s^{-1}) overlaps with that of the wild-type enzyme (1960 ± 300 M^{-1} s^{-1}; Kuliopulos et al., 1987b), yielding comparable enhancement factors of 12 ± 5 and 14 ± 8, respectively. Hence, the double-mutant enzyme Y14F + D38N has retained enough structure to bind tightly and to immobilize partially doxyl-DHT and to orient the paramagnetic nitroxide toward the solvent in a manner indistinguishable from that found with the wild-type enzyme.

In competition experiments, the substrate Δ^5-androstene-3,17-dione displaced doxyl-DHT from the steroid-binding site of the double-mutant enzyme with a K_s value of 17 ± 10 μM (Figure 4B), when corrected for the measured dissociation constant of the spin-label with eq 7. At high total steroid concentrations ([doxyl-DHT] + [Δ^5-androstene-3,17-dione] > 150 μM) the substrate began to aggregate and was unable to displace completely the bound doxyl-DHT, causing the theoretical curve to fall below the measured $1/T_{1p}$ values at substrate concentrations greater than 50 μM.

Discussion

A comparison of k_{cat} of ketosteroid isomerase (5.4 × 10^4 s^{-1}) with the sum of pseudo-first-order rate constants for spontaneous steroid isomerization at the same temperature and pH (10^{-5} s^{-1}) indicates that the enzyme accelerates this process by a factor of $10^{9.5}$ (Tables I and II). If this $10^{9.5}$-fold rate acceleration of steroid isomerization were due entirely to general-acid catalysis by Tyr-14 contributing $10^{4.7}$-fold and to general-base catalysis by Asp-38 contributing $10^{5.6}$-fold, as suggested by the inhibitory effects on k_{cat} of the single mutants Y14F and D38N, respectively (Table I; Kuliopulos et al., 1989), then essentially all activity should be lost in the double mutant.[2] Apparent additivity of the effects of the single mutations was, in fact, observed in the double mutant Y14F + D38N. However, since the double mutant has lost all activity, synergistic effects of the two mutations cannot be excluded. Furthermore, it was important to establish that the enzyme and the substrate binding site were otherwise intact.

The crystals formed by the double mutant were indistinguishable in form from those of the wild-type enzyme suggesting that the double mutation had not altered the overall architecture of the individual enzyme molecules or their packing in the crystal lattice.

The EPR data revealed that the doxyl-DHT spin-label was bound and highly immobilized by the double-mutant enzyme in a site which overlapped with the substrate site, as had previously been found with the wild-type enzyme (Kuliopulos et al., 1987b). Independent binding studies of doxyl-DHT to the double mutant, obtained by measuring the effects of the nitroxide on the $1/T_{1p}$ values of water protons, confirmed that the spin-label was highly immobilized and also indicated that the nitroxide-containing ring was exposed to the solvent when bound to the double-mutant enzyme, precisely as found with the wild-type enzyme (Kuliopulos et al., 1987b). The 5-fold lower affinity for doxyl-DHT than for the wild-type enzyme may reflect the loss of a hydrogen bond from the Tyr-14 hydroxyl to the 17β-hydroxyl of the steroidal D-ring in the requisite D-ring binding mode. This effect was seen previously with the Y14F single mutant, to which the competitive inhibitors 19-nortestosterone and 17β-estradiol also bound 4–5-fold less tightly than to the wild-type enzyme (Kuliopulos et al., 1989). Interestingly, the substrate Δ^5-androstene-3,17-dione was found to bind more tightly to the double mutant (K_s = 17 ± 10 μM) than to the wild-type enzyme (K_s = 111 μM; Malhotra & Ringold, 1965; Xue et al., 1990) possibly due to a stabilizing effect involving the 19-methyl group of the substrate. The K_s with the double mutant was determined by competition with doxyl-DHT, while the K_s with the wild-type enzyme was obtained by analysis of primary ^2H kinetic isotope effects on both K_M and k_{cat} (Xue et al., 1990; Malhotra & Ringold, 1965).

The tight binding of steroids and the proper orientation and immobilization of the spin-labeled steroid by the double-mutant enzyme argue against significant structural changes at the active site beyond the residues altered. Hence, the apparent additive effects of the individual single mutations on k_{cat}, observed in the Y14F + D38N double mutant, are likely to result from the independent contributions of Tyr-14 and Asp-38 to the rate-limiting step of the wild-type enzyme. As determined by multiple ^2H kinetic isotope effects (Xue et al., 1990), the rate-limiting step with the wild-type enzyme is the concerted enolization of the enzyme-bound substrate, in which Tyr-14 donates a proton to the 3-carbonyl oxygen while Asp-38 removes the 4β-proton of the substrate. With the single mutants, preliminary single turnover, isotope exchange, and kinetic isotope effects suggest that the maximal initial velocities, expressed as k_{cat}, also reflect the rate of substrate enolization with D38N. With Y14F, substrate enolization is partially rate limiting.[3] The three conditions, (a) independent functioning, (b) in the same step, which is (c) rate-limiting, are shown in the Appendix to be sufficient for the effects of mutations of two residues on k_{cat} to be additive in a double mutant (see Table IV). If the step affected by the two mutations were not rate limiting, then synergistic effects of the two mutations on k_{cat} and on k_{cat}/K_M would be observed in a double mutant since the affected step would become relatively more rate limiting in the double mutant than in either of the two single mutants. We, therefore, conclude that it is the independent and concerted functioning of Tyr-14 and Asp-38 in the same rate-limiting step which permits the apparent additivity of the effects of mutating these residues on k_{cat}. The independence of the contributions of Tyr-14 ($10^{4.7}$-fold) and Asp-38 ($10^{5.6}$-fold) to the rate of the concerted enolization step of ketosteroid isomerase is likely to result from the remote distance of these residues from each other in the active complex since they are positioned either perpendicularly or antarafacially, but not suprafacially, with respect to the bound substrate (Figure 1; Kuliopulos et al., 1989; Xue et al., 1990).

As also shown in the Appendix, and as summarized in Table IV, if the two mutated residues functioned independently in consecutive steps, additive effects on k_{cat} would occur if either or both steps were rate-limiting. If the two affected steps were not rate limiting, then partial additivity of the effects of the two mutations would be observed in the double mutant, and in the limiting case, an effect on k_{cat} comparable to that of the more damaging of the two single mutations would be seen, i.e., D38N in the present case. Such partial additivity was not observed in this system.

Conversely, with an enzyme of unknown mechanism, observation of additive effects of mutating two residues on k_{cat} or on k_{cat}/K_M suggests the independent functioning of the two residues in the same step or in consecutive steps, one or both of which are rate-limiting. Synergistic effects on k_{cat} of mutating two residues suggest their independent and concerted

[2] Although the choice of mutated residues (Phe and Asn) was appropriately based on structural similarity to residues of the wild-type enzyme (Tyr and Asp, respectively), the increase in hydrophobicity could have exaggerated the contributions of Tyr-14 and Asp-38 to catalysis.

[3] L. Xue and A. Kuliopulos, unpublished observations.

Table IV: Interactions of Effects of Two Mutations on the Kinetic Parameters of an Enzyme[a]

step affected by		k_{cat}		k_{cat}/K_M	
mutation 1	mutation 2	requirement for additivity	general case[b]	requirement for additivity	general case[b]
k_2	k_2	k_2 rate limiting	synergistic effects	k_2 or k_3 rate limiting	synergistic effects
k_2, k_{-2}	k_2, k_{-2}	both k_2 and $k_{-2} \ll k_3$ or k_3 rate limiting	synergistic effects	both k_2 and $k_{-2} \ll k_3$ or k_{-1} or k_3 rate limiting	synergistic effects
k_2	k_3	k_2 rate limiting or k_3 rate limiting	< additive (effect of more damaging single mutation)	k_2 rate limiting or k_3 rate limiting	synergistic effects
k_2, k_{-2}	k_3	both k_2 and $k_{-2} \ll k_3$ or k_3 rate limiting	< additive (effect of more damaging single mutation)	$k_{-1} \ll k_2$ or $k_{-2} \ll k_2$ or k_3 rate limiting	< additive (effect of more damaging single mutation)

[a] The kinetic scheme of the enzyme is shown in eq 1A (see Appendix). [b] $k_2 \sim k_{-2} \sim k_3$.

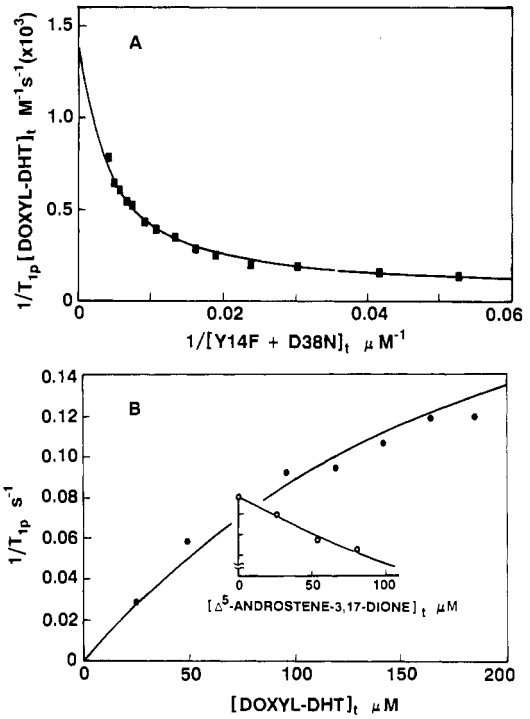

FIGURE 4: Titrations measuring the binding of doxyl-DHT to the Y14F + D38N double mutant by the $1/T_{1p}$ of water protons. (A) The spin-label was titrated with Y14F + D38N, and the molar relaxivity of the water protons was plotted against reciprocal enzyme concentration. The theoretical titration curve was fit to eq 6 with K_D = 200 ± 60 μM, MR_b = 1400 ± 300 M^{-1} s^{-1}, MR_f = 50 M^{-1} s^{-1}, and n = 1.00 ± 0.05. The doxyl-DHT and enzyme concentrations were as described in the legend to Table III. (B) The double mutant was titrated with doxyl-DHT and the $1/T_{1p}$ plotted against spin-label concentration. The theoretical curve (eq 6) multiplied by [doxyl-DHT], was fit with K_D = 100 ± 40 μM, MR_b = 2200 ± 400 M^{-1} s^{-1}, MR_f = 160 M^{-1} s^{-1}, and n = 1.0 ± 0.1. (Insert) Displacement of doxyl-DHT by substrate, Δ^5-androstene-3,17-dione. The theoretical curve was fit to eq 7 multiplied by [doxyl-DHT] with a K_i = 17 ± 10 μM, MR_b = 2610 ± 500 M^{-1} s^{-1}, MR_f = 160 ± 40 M^{-1} s^{-1}, and K_D = 80 ± 30 μM. Conditions were as described in the legend to Table III.

functioning in the same step if the general structure of the enzyme has otherwise been preserved (Table IV). Since catalysis formally reflects the binding of transition states, equivalent statements can be made for additive effects of mutations on the ground-state binding of substrates, activators, and inhibitors (Weber et al., 1990). Partially additive and nonadditive effects on k_{cat} indicate that the residues function in consecutive steps. Analogous interactions have been found in considering combined kinetic isotope effects as a test for concerted versus separate chemical steps (O'Leary, 1989).

Despite these restrictive conditions, additive effects of mutations on catalysis are not uncommon. With carboxypeptidase, small effects of mutations on k_{cat}/K_M have been shown to be additive. Thus, the Y198F and Y248F single mutations decrease k_{cat}/K_M by factors of 1.9- and 14.6-fold, respectively, while the double mutation decreases k_{cat}/K_M 33.4-fold (Gardell et al., 1985, 1987). Similarly with tyrosyl-tRNA synthetase, the C35G and H48G single mutations decrease k_{cat}/K_M by factors of 6.8- and 6.6-fold, respectively, while the double mutation decreases k_{cat}/K_M by 50-fold (Carter et al., 1984). These residues are both proposed to act independently and, in the same step, to stabilize the transition state for the tyrosine attack on ATP (Fersht, 1987). Because of the small effects in these cases, the possibility remains that the magnitudes observed are coincidental and bear no direct relevance to the mechanism of the wild-type enzyme.

In many cases, however, simple additivity of the individual effects of mutations on k_{cat} or on k_{cat}/K_M is not seen. Fersht et al. (1988) in their extensive analysis of the individual contributions of active site residues of tyrosyl-tRNA synthetase on transition-state stabilization as reflected in k_{cat}/K_M have demonstrated that the sum of the contributions of Thr-40, His-45, Lys-82, Lys-230, Lys-233, and Arg-86, on the basis of the effects of individual mutations, equals a rate enhancement of $\geq 10^{17}$, which is greater than that expected. Nonadditivity in this case is ascribed to an overestimation of the binding energies of charged active site residues involved in hydrogen bonds to the substrate (Fersht et al., 1988; Fersht, 1988). An alternative explanation for nonadditivity is the collaborative effect of several residues to yield a single interaction as in the catalytic triad of the serine proteases (Carter & Wells, 1988). If each member of the catalytic triad of Ser-221, His-64, and Asp-32 of subtilisin is individually mutated to alanine, decreases in k_{cat} relative to that of wild-type of $10^{6.3}$, $10^{6.3}$, and $10^{4.5}$, respectively, are seen. The three different double-alanine mutants are only 1–8-fold more active than the triple-alanine mutant which has the same k_{cat} as the S221A and H64A single mutants. If additivity of effects had occurred, the k_{cat} of the triple mutant should have dropped to 10^{-17} of k_{cat} of the wild-type, a meaningless value since the overall rate acceleration by subtilisin is only $10^{9.5}$-fold. As these authors have stated, the histidine and aspartate residues are merely acting to increase the nucleophilicity of the serine residue and are not contributing significant transition-state stabilization independently of the serine, and thus, the full effect of the catalytic triad residues on the overall rate enhancement is limited by the full effect of the serine on the rate.

With ketosteroid isomerase, we conclude that the entire rate enhancement of $10^{9.5}$-fold can be accounted for by the Tyr-14 and Asp-38 residues acting concertedly and independently as general-acid and -base catalysts in the rate-limiting conversion of the substrate to the dienol intermediate. Our results also suggest a general test for the independent and concerted or consecutive functioning of two residues in catalysis.

ACKNOWLEDGMENTS

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Appendix: Analysis of the Effects of Two Mutations on the Kinetic Constants of an Enzyme

A kinetic scheme for ketosteroid isomerase based on initial velocity substrate kinetics (Batzold et al., 1976), kinetic isotope effects (Xue et al., 1990), and transient-state kinetics (Eames et al., 1990) is

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ED \xrightarrow{k_3} E + P \qquad (1A)$$

where ES is the enzyme–substrate complex and ED is the enzyme complex of the dienol intermediate. With the steady-state assumption, the King and Altman (1956) method being used, this scheme leads to the rate equation

$$v = \frac{k_{cat}[E]}{1 + K_M/[S]} \qquad (2A)$$

in which

$$k_{cat} = \frac{k_2k_3}{k_2 + k_3 + k_{-2}} \qquad (3A)$$

$$K_M = \frac{k_{-1}k_{-2} + k_{-1}k_3 + k_2k_3}{k_1k_2 + k_1k_3 + k_1k_{-2}} \qquad (4A)$$

and

$$k_{cat}/K_M = \frac{k_1k_2k_3}{k_{-1}k_{-2} + k_{-1}k_3 + k_2k_3} \qquad (5A)$$

Two Mutations Affecting the Same Step. Assume that two mutations 1 and 2 affect separate residues which function independently in the k_2 step, altering k_2 by the factors $1/a$ and $1/b$, respectively, in the single mutants, such that

$$(k_{cat})_1 = \frac{(k_2/a)k_3}{k_2/a + k_3 + k_{-2}} \qquad (6A)$$

$$(k_{cat})_2 = \frac{(k_2/b)k_3}{k_2/b + k_3 + k_{-2}} \qquad (7A)$$

If the two residues function independently, then in the double mutant k_2 is altered by the factor $1/ab$, such that

$$(k_{cat})_{1+2} = \frac{(k_2/ab)k_3}{k_2/ab + k_3 + k_{-2}} \qquad (8A)$$

If k_2 were rate-limiting, then terms containing k_2 would disappear from the denominators of eqs 6A–8A and additivity of the effects of the mutations on k_{cat} would occur. More generally, from eqs 3A, 6A, 7A, and 8A we obtain the ratio of the effect of the double mutation on k_{cat} of the wild-type enzyme to the product of the corresponding effects of the two single mutations:

$$\frac{\frac{(k_{cat})_{1+2}}{(k_{cat})}}{\frac{(k_{cat})_1}{(k_{cat})}\frac{(k_{cat})_2}{(k_{cat})}} = \frac{\frac{k_2}{k_3 + k_{-2}} + \frac{ab(k_3 + k_{-2})}{k_2} + [a + b]}{\frac{k_2}{k_3 + k_{-2}} + \frac{ab(k_3 + k_{-2})}{k_2} + [ab + 1]} \qquad (9A)$$

Note that for additivity of effects this ratio must equal 1 and that any deviation from unity results from the square bracketed terms in the numerator and denominator. If k_2 were very small compared to k_3 or k_{-2}, i.e., rate limiting, then both the numerator and denominator of eq 9A are dominated by the middle term, $ab(k_3 + k_{-2})/k_2$, and unity is approached. Thus, for additive effects on k_{cat}, both mutations must affect residues which function independently in the same step, as initially assumed, and this step must be rate-limiting as shown in eq 9A, by letting k_2 approach zero.

Alternatively, if k_2 were not rate limiting and the factors a and b were large, the terms in brackets would predominate, and the double mutant would show synergistic damage, beyond that predicted by the product of the effects of the two single mutations. In the limiting case, the additional factor of synergy would be equal to that of the less damaging single mutation.

An analysis of the effects on k_{cat}/K_M with the same assumptions, namely, independent effects of two mutations on k_2, yields, from eq 5A, the relationship

$$\frac{\frac{(k_{cat}/K_M)_{1+2}}{(k_{cat}/k_M)}}{\frac{(k_{cat}/K_M)_1}{(k_{cat}/K_M)}\frac{(k_{cat}/K_M)_2}{(k_{cat}/K_M)}} = \frac{\frac{k_2k_3}{k_{-1}(k_{-2} + k_3)} + \frac{abk_{-1}(k_{-2} + k_3)}{k_2k_3} + [a + b]}{\frac{k_2k_3}{k_{-1}(k_{-2} + k_3)} + \frac{abk_{-1}(k_{-2} + k_3)}{k_2k_3} + [ab + 1]} \qquad (10A)$$

From eq 10A it is seen that rate limitation by either k_2 or k_3 or both would increase the middle term in the numerator and denominator and lead to additive effects on k_{cat}/K_M of mutations affecting k_2 alone. Thus, under these conditions, the effect of a double mutation on k_{cat}/K_M is less discriminating than on k_{cat} alone. If neither k_2 nor k_3 were rate-limiting and a and b were large factors, then in the limiting case synergistic effects of the two mutations on k_{cat}/K_M would be observed.

If both k_2 and k_{-2} were affected by mutation 1 decreasing these rate constants by $1/a$ and by mutation 2 decreasing these constants by $1/b$, the resulting ratio of effects on k_{cat} is given by

$$\frac{\frac{(k_{cat})_{1+2}}{k_{cat}}}{\frac{(k_{cat})_1}{k_{cat}}\frac{(k_{cat})_2}{k_{cat}}} = \frac{\frac{k_2 + k_{-2}}{k_3} + \frac{abk_3}{k_2 + k_{-2}} + [a + b]}{\frac{k_2 + k_{-2}}{k_3} + \frac{abk_3}{k_2 + k_{-2}} + [ab + 1]} \qquad (11A)$$

For additivity of effects on k_{cat}, both k_2 and k_{-2} must be rate-limiting, i.e., small compared with k_3, to permit the middle term to predominate. If either k_2 or k_{-2} or both were large, additivity would not be observed, unless k_3 were rate-limiting, enlarging the first term in both the numerator and denominator of eq 11A, i.e., allowing the k_2 step to equilibrate. In the general case, without restrictions as the rate-limiting steps, where $k_2 \sim k_{-2} \sim k_3$, additivity would not be observed, and if a and b were large, synergistic effects on k_{cat} would be observed in the double mutant.

Similar conditions for additivity occur with k_{cat}/K_M when both k_2 and k_{-2} are affected by mutations 1 and 2 as may be seen in the relevant ratio of predicted effects:

$$\frac{\frac{(k_{cat}/K_M)_{1+2}}{(k_{cat}/K_M)}}{\frac{(k_{cat}/K_M)_1}{(k_{cat}/K_M)}\frac{(k_{cat}/K_M)_2}{(k_{cat}/K_M)}} = \frac{\frac{k_{-1}k_{-2} + k_2k_3}{k_{-1}k_3} + \frac{abk_{-1}k_3}{k_{-1}k_{-2} + k_2k_3} + [a + b]}{\frac{k_{-1}k_{-2} + k_2k_3}{k_{-1}k_3} + \frac{abk_{-1}k_3}{k_{-1}k_{-2} + k_2k_3} + [ab + 1]} \qquad (12A)$$

Two Mutations Affecting Consecutive Steps. Now we make the different assumption that mutation 1 affects a residue

which functions in the k_2 step and alters k_2 by the factor $1/a$. Mutation 2 independently affects a residue involved in the k_3 step, altering k_3 by the factor $1/b$.

$$(k_{cat})_1 = \frac{k_2k_3/a}{k_2/a + k_3 + k_{-2}} \quad (6A)$$

$$(k_{cat})_2 = \frac{k_2k_3/b}{k_2 + k_3/b + k_{-2}} \quad (13A)$$

$$(k_{cat})_{1+2} = \frac{k_2k_3/ab}{k_2/a + k_3/b + k_{-2}} \quad (14A)$$

By inspection of eqs 6A, 13A, and 14A it may be noted that additivity of the effects of the two mutations on k_{cat} would occur only if either k_2 or k_3 or both were rate-limiting, i.e., smaller than k_{-2}. More generally, from eqs 3A, 6A, 13A, and 14A we obtain the ratio of the effect of the double mutant on k_{cat} relative to that of the wild-type enzyme, compared to the product of the corresponding effects of the two single mutations, as

$$\frac{\frac{(k_{cat})_{1+2}}{k_{cat}}}{\frac{(k_{cat})_1}{k_{cat}}\frac{(k_{cat})_2}{k_{cat}}} = \frac{a\frac{k_3 + k_{-2}}{k_2} + b\frac{k_2 + k_{-2}}{k_3} + abk_{-2}\frac{k_3 + k_2 + k_{-2}}{k_2k_3} + [ab + 1]}{a\frac{k_3 + k_{-2}}{k_2} + b\frac{k_2 + k_{-2}}{k_3} + abk_{-2}\frac{k_3 + k_2 + k_{-2}}{k_2k_3} + [a + b]} \quad (15A)$$

Note in eq 15A that the nonidentical terms, shown in square brackets, are inverted from those of the previous assumption.

From eq 15A, if k_2, k_3, or both were small compared to k_{-2}, the identical third terms in both the numerator and denominator would predominate, and additive effects on k_{cat} would be approached. If neither k_2 nor k_3 were rate-limiting, the square bracketed terms would contribute, and less than additive effects of the two mutations would be observed in the double mutant. In the limiting case, with large values for a and b, the bracketed terms would dominate, and the double mutant would show the effect on k_{cat} of the more damaging single mutation.

The parameter k_{cat}/K_M shows similar but not identical behavior. The relevant equation is

$$\frac{\frac{(k_{cat}/K_M)_{1+2}}{(k_{cat}/K_M)}}{\frac{(k_{cat}/K_M)_1}{(k_{cat}/K_M)}\frac{(k_{cat}/K_M)_2}{(k_{cat}/K_M)}} = \left\{\frac{a(k_1k_{-2} + k_{-1}k_3 + k_2k_3)}{k_{-2}k_2} + \frac{abk_{-1}(k_{-2} + k_3)}{k_2k_3} + \frac{k_3(k_{-1} + k_2)}{k_{-1}k_{-2}} + [a + b]\right\} \Big/ \left\{\frac{a(k_1k_{-2} + k_{-1}k_3 + k_2k_3)}{k_{-2}k_2} + \frac{abk_{-1}(k_{-2} + k_3)}{k_2k_3} + \frac{k_3(k_{-1} + k_2)}{k_{-1}k_{-2}} + [ab + 1]\right\} \quad (16A)$$

From eq 16A it is seen that if either k_2 or k_3 or both were rate-limiting, the second term in the numerator and denominator would dominate, and additivity of the effects would occur. However if neither were rate-limiting and if both a and b were large, the square bracketed terms would dominate, and synergistic effects on k_{cat}/K_M would be observed. (Note that under these conditions partially additive effects on k_{cat} would be found.)

Finally we assume that mutation 1 affects both k_2 and k_{-2} by the factor $1/a$ and mutation 2 independently affects k_3 by the factor $1/b$. Since initial velocities are considered, the effect of mutation 2 on k_{-3} is not relevant. The ratio of effects on k_{cat} is given by

$$\frac{\frac{(k_{cat})_{1+2}}{k_{cat}}}{\frac{(k_{cat})_1}{(k_{cat})}\frac{(k_{cat})_2}{(k_{cat})}} = \frac{\frac{ak_3}{k_2 + k_{-2}} + \frac{b(k_2 + k_{-2})}{k_3} + [ab + 1]}{\frac{ak_3}{k_2 + k_{-2}} + \frac{b(k_2 + k_{-2})}{k_3} + [a + b]} \quad (17A)$$

From eq 17A it is seen that for additivity of effects on k_{cat} to occur both k_2 and k_{-2} must be rate-limiting or k_3 must be rate-limiting. Otherwise, effects less than additive would be observed. In the limiting case, with large values of a and b, the double mutant would show the effect on k_{cat} of the more damaging single mutation.

The ratio of effects on k_{cat}/K_M with these assumptions is given by

$$\frac{\frac{(k_{cat}/K_M)_{1+2}}{(k_{cat}/K_M)}}{\frac{(k_{cat}/K_M)_1}{(k_{cat}/K_M)}\frac{(k_{cat}/K_M)_2}{(k_{cat}/K_M)}} = \left\{ak_3\frac{k_{-1} + k_2}{k_{-1}k_{-2}} + b\left(\frac{k_{-2}}{k_3} + \frac{k_2}{k_{-1}}\right) + \frac{k_2}{k_{-1}}\left(1 + \frac{k_3}{k_{-2}} + \frac{k_2k_3}{k_{-1}k_{-2}}\right) + [ab + 1]\right\} \Big/ \left\{ak_3\frac{k_{-1} + k_2}{k_{-1}k_{-2}} + b\left(\frac{k_{-2}}{k_3} + \frac{k_2}{k_{-1}}\right) + \frac{k_2}{k_{-1}}\left(1 + \frac{k_3}{k_{-2}} + \frac{k_2k_3}{k_{-1}k_{-2}}\right) + [a + b]\right\} \quad (18A)$$

For additive effects on k_{cat}/K_M to occur, either k_{-1}, k_{-2}, or k_3 must be very small, causing appropriate identical terms in the numerator and denominator to dominate. Rate limitation by k_2 would not cause additivity of effects on k_{cat}/K_M in the double mutant. In the general case, with comparable values of the relevant rate constants, effects less than additive would be seen. If a and b are both large, the double mutant would show the effects of the more damaging single mutation.

Registry No. Doxyl-DHT, 25521-33-9; L-Tyr, 60-18-4; L-Asp, 56-84-8; Δ^5-3-ketosteroid isomerase, 9031-36-1; Δ^5-androstene-3,17-dione, 571-36-8.

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Amino Acid Sequence of the Cyclic GMP Stimulated Cyclic Nucleotide Phosphodiesterase from Bovine Heart†

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ABSTRACT: The complete amino acid sequence of the cyclic GMP stimulated cyclic nucleotide phosphodiesterase (cGS-PDE) of bovine heart has been determined by analysis of five digests of the protein; placement of the C-terminal 330 residues has been confirmed by interpretation of the corresponding partial cDNA clone. The holoenzyme is a homodimer of two identical N^{α}-acetylated polypeptide chains of 921 residues, each with a calculated molecular weight of 103 244. The C-terminal region, residues 613–871, of the cGS-PDE comprises a catalytic domain that is conserved in all phosphodiesterase sequences except those of PDE I from *Saccharomyces cerevisiae* and a secreted PDE from *Dictyostelium*. A second conserved region, residues 209–567, is homologous to corresponding regions of the α and α' subunits of the photoreceptor phosphodiesterases. This conserved domain specifically binds cGMP and is involved in the allosteric regulation of the cGS-PDE. This regulatory domain contains two tandem, internal repeats, suggesting that it evolved from an ancestral gene duplication. Common cyclic nucleotide binding properties and a distant structural relationship provide evidence that the catalytic and regulatory domains within the cGS- and photoreceptor PDEs are also related by an ancient internal gene duplication.

Cyclic nucleotides mediate a variety of cellular responses to biological stimuli. The concentrations of the cyclic nucleotides are controlled by the opposing action of adenylate or guanylate cyclases and phosphodiesterases (PDEs),[1] which are in turn regulated by transmembrane signals or second-messenger ligands (e.g., calcium/calmodulin or cGMP). Among this milieu of interacting regulatory systems, a variety of cyclic nucleotide phosphodiesterase isozymes (Beavo, 1988; Beavo & Reifsynder, 1990) have been identified; they share the potential to return cyclic nucleotide stimulated systems to the ground state in response to regulatory signals. Different tissues express PDEs with different responsiveness to natural regulatory metabolites. The present study defines the amino acid sequence of one of the larger PDEs, the cGMP-stimulated

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[1] Abbreviations: cGS-PDE, cGMP-stimulated cyclic nucleotide phosphodiesterase; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid.

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