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Studies of the Catalytic Mechanism of an Active-Site Mutant (Y14F) of Δ^5-3-Ketosteroid Isomerase by Kinetic Deuterium Isotope Effects†

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ABSTRACT: Δ^5-3-Ketosteroid isomerase (EC 5.3.3.1) from *Pseudomonas testosteroni* catalyzes the conversion of androst-5-ene-3,17-dione to androst-4-ene-3,17-dione by a stereoselective transfer of the 4β-proton to the 6β-position. The rate-limiting step has been shown to be the concerted enolization of the enzyme-bound substrate comprising protonation of the 3-carbonyl oxygen by Tyr-14 and abstraction of the 4β-proton by Asp-38 [Xue, L., Talalay, P., & Mildvan, A. S. (1990) *Biochemistry 29*, 7491–7500]. Primary, secondary, solvent, and combined kinetic deuterium isotope effects have been used to investigate the mechanism of the Y14F mutant, which lacks the proton donor and is $10^{4.7}$-fold less active catalytically than the wild-type enzyme. With [4β-D]androst-5-ene-3,17-dione as a substrate in H_2O, a lag in product formation is observed which approaches, by a first-order process, the rate observed with protonated substrate. With the protonated substrate in D_2O, a burst in product formation is detected by derivative analysis of the kinetic data which approaches the rate observed with the 4β-deuterated substrate in D_2O. The absence of such lags or bursts with the protonated substrate in H_2O or with the 4β-deuterated substrate in D_2O, as well as the detection of buffer catalysis by phosphate at pH 6.8, indicates that one or more intermediates dissociate from the enzyme and partition to substrate 31.6 times faster than to product. When corrected for these exchange effects, the k_{cat} and k_{cat}/K_m values both show a primary kinetic isotope effect of 2.4 ± 0.2 for the 4β-D substrate. The detection of a secondary kinetic isotope effect on k_{cat}/K_m of 1.06 ± 0.02 with the 4α-D substrate and the absence of an inverse secondary kinetic isotope effect with the 6-D substrate (1.02 ± 0.02) indicate that enolization is rate limiting for the Y14F mutant. The primary kinetic isotope effects on k_{cat}/K_m with the 4β-D substrate of 2.33 ± 0.06 found in H_2O decreases to 1.16 ± 0.08 in D_2O, and the solvent isotope effect of 7.69 ± 0.18 observed with protonated substrate decreases to 3.85 ± 0.21 with the 4β-D substrate, establishing a stepwise enolization mechanism. A minimal mechanism of the reaction catalyzed by the Y14F mutation thus involves the initial stereoselective removal of the 4β-proton by Asp-38 to form the dienolate carbanion intermediate, which dissociates from the enzyme and is protonated in solution either at C-4 to regenerate the substrate or more slowly on the C-3 oxyanion to form the dienol, which reketonizes rapidly to form the product. Comparison of the k_{off} of the intermediate from the Y14F mutant with that found with the D38N mutant indicates that the phenolic hydroxyl group of Tyr-14 contributes at least 7.6 Kcal/mol to the free energy of binding of the intermediate. Tyr-14 thus appears to play a major role not only in the formation of the dienolic intermediate but also in binding it tightly to the enzyme. A reaction coordinate free energy contour diagram is used to compare the concerted enolization mechanism catalyzed by the wild-type enzyme with the stepwise carbanion mechanism catalyzed by the Y14F mutant and the stepwise oxycarbonium ion mechanism catalyzed by the D38N mutant.

The Δ^5-3-ketosteroid isomerase (EC 5.3.3.1) of *Pseudomonas testosteroni* promotes the highly efficient isomerization of a variety of Δ^5-3-ketosteroids to the conjugated α,β-unsaturated Δ^4-3-ketosteroids by a predominantly conservative and stereoselective transfer of the 4β-proton of the substrate to the 6β-position [see reviews by Talalay and Benson (1972), Pollack et al. (1989), and Schwab and Henderson (1990)]. There is convincing evidence (Kuliopulos et al, 1987b, 1989, 1990; Xue et al., 1990; Eames et al., 1990) that the enzymatic reaction proceeds via an enzyme-bound enolic intermediate that is formed through the concerted participation of two critical catalytic residues: Tyr-14 which serves as the general acid, and Asp-38, which acts as the general base (Figure 1) (Kuliopulos et al., 1989). The overall enzymatic reaction involves two sequential steps: (a) a rate-limiting and concerted enolization reaction in which Tyr-14 protonates the 3-carbonyl oxygen and Asp-38 removes the 4β-proton of the steroid to generate the enolic intermediate and (b) the rapid reketonization of this intermediate in which the functions of these residues are reversed, i.e., tyrosinate-14 functions as a base to deprotonate the oxygen of the dienol, and Asp-38, in its protonated form, transfers the proton it has acquired from the 4β-position and inserts it stereospecifically into the 6β-position. NMR studies have established that these catalytic residues are arranged orthogonally and trans with respect to the A/B

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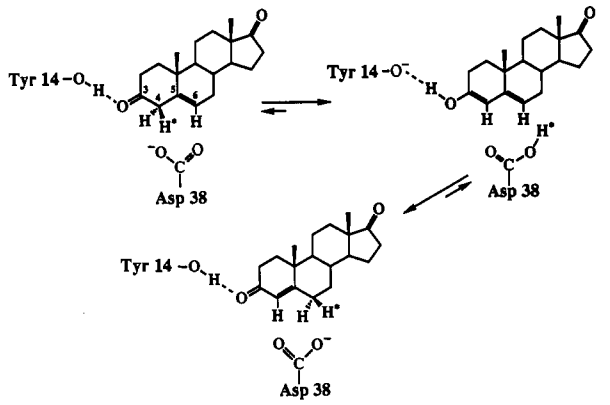


FIGURE 1: Mechanism of the conversion of androst-5-ene-3,17-dione to androst-4-ene-3,17-dione by the wild-type Δ^5-3-ketosteroid isomerase of *P. testosteroni*. In the enolization reaction, Asp-38 removes the axial 4β-proton of the steroid, concerted with the orthogonal and trans protonation of the 3-carbonyl group oxygen by Tyr-14, to give the enzyme-bound $\Delta^{3,5}$-dienol intermediate. Reketonization then occurs by protonation at the 6β-position by Asp-38 and deprotonation of the oxygen at C-3 by Tyr-14 to form the conjugated Δ^4-3-ketosteroid.

rings of the bound steroid and are thereby favorably positioned to effect rapid concerted enolization (Kuliopulos et al., 1991).

The wild-type isomerase accelerates the reaction (k_{cat}) by a factor of $10^{9.5}$ when compared to the pseudo-first-order nonenzymatic rate of product formation under the same conditions. The D38N mutant of isomerase is 10^6-fold less active than the wild-type enzyme, and the Y14F mutant has a k_{cat} that is $10^{4.7}$-fold lower than that of the wild-type enzyme. Simultaneous mutation of both of these critical residues (D38N + Y14F) abolishes the entire catalytic activity of the enzyme but preserves the tight binding of substrate, suggesting that the entire catalytic power of the enzyme results from the action of these two residues (Kuliopulos et al., 1990).

Recent studies (Xue et al., 1991) have shown that the catalytic mechanism of the D38N mutant differs significantly from that of the wild-type enzyme in three respects: (a) In the enolization reaction, protonation of the steroid carbonyl oxygen and removal of the 4-proton are not concerted, as shown by kinetic isotope effects obtained with deuterated substrates, deuterated solvent, and combinations of the two. (b) Accordingly, two enzyme-bound intermediates, the steroid dienol and its anion, are detected spectroscopically. They form rapidly but accumulate as tightly bound and long-lived intermediates on the enzyme before undergoing slow conversion to the product. (c) The removal of the proton at C-4 is not stereoselective and shows large primary kinetic isotope effects with either 4β- or 4α-deuterated substrates.

The present study examines the mechaism of the Y14F mutant enzyme, which lacks the phenolic hydroxyl group of the tyrosine responsible for the protonation of the steroid 3-carbonyl oxygen. Experiments with deuterated substrates and solvent lead to the conclusions that deprotonation at C-4 remains stereoselective, that the enolization is not concerted but stepwise, that both steps of the enolization are partially rate limiting, and that the intermediates are largely released into the medium where the reaction is completed spontaneously. Tyr-14 thus appears to play a major role not only in the formation of the dienolic intermediate but also in binding it tightly to the enzyme.

Experimental Procedures

Materials. Solutions of Tris base were adjusted to the desired pH at 25 °C with HCl. For kinetic experiments in D_2O, Tris-HCl buffers were lyophilized and the residues were dissolved in D_2O and then lyophilized a second time before being dissolved in D_2O. D_2O (99.9 atom % D) and CH_3OD (99.5 atom % D) were obtained from Aldrich.

Enzymes. Recombinant Y14F mutant isomerase was prepared as described (Kuliopulos et al., 1987a, 1989), and stored as a crystalline suspension in neutral 30% saturated solutions of ammonium sulfate at 4 °C. For kinetic studies, aliquots of the enzyme suspensions were dissolved in 50 mM Tris-HCl, pH 7.5, and filtered through 0.45-μm Millipore HV filters. Concentrations of the Y14F mutant were determined by measuring the absorbance at 280 nm in 10-mm light path cells, assuming the values of 0.226 for the absorbance of solutions containing 1.00 mg/mL (Kuliopulos et al., 1989). All enzyme concentrations are expressed in terms of subunits.

Substrates. [4β-D]Androst-5-ene-3,17-dione was prepared according to modifications of the procedure of Malhotra and Ringold (1965) [see also Xue et al. (1990)], and [4α-D]-androst-5-ene-3,17-dione was prepared according to Viger et al. (1987). Methods of preparation of [4,4-D_2]androst-5-ene-3,17-dione, and [6-D]androst-5-ene-3,17-dione have been described (Xue et al., 1990). All substrates were purified by silica gel flash chromatography or HPLC on a reverse-phase column when necessary, and the purity and deuterium composition were determined by high-resolution proton NMR at 600 MHz (Xue et al., 1990).

Measurements of k_{cat} and K_m Values. Determinations of the kinetic constants, k_{cat} and K_m, were made with a Beckman DU-7 spectrophotometer in quartz cuvettes of 10-mm light path at 25 °C and at 248 nm, which is the maximum wavelength of absorption of androst-4-ene-3,17-dione (A_m = 16 300 M^{-1} cm^{-1}) in H_2O or D_2O. These spectral constants were not affected by the presence of deuterium in the steroids or by D_2O as solvent. Reactions were carried out in final volumes of 1.50 mL containing 50 mM Tris-HCl (or 33 mM potassium phosphate) at the desired pH, 50 μL of methanol (3.3% by vol), and 100 μg of bovine serum albumin. To minimize random errors, all reaction components were always added in the same order. Each cuvette received 75 μL of 1.0 M buffer; then 1360 μL of H_2O or D_2O were added, followed by 50 μL of CH_3OH or CH_3OD containing 18–90 mM substrate. After the spontaneous rate of isomerization had been measured (usually an absorbance change of 0.001/min), the enzymatic reaction was initiated by addition of 15 μL of isomerase appropriately diluted in 1% neutralized albumin. The reaction was then followed for 3–5 min, and the initial linear reaction velocities were determined when less than 5% conversion of the substrate had occurred. Kinetic constants were obtained from measurements at seven or eight substrate concentrations. The values of k_{cat} and K_m were obtained by a hyperbolic weighted least-squares program (Wilkinson, 1961). Standard errors in k_{cat} and K_m were calculated as described by Wilkinson (1961), and those of k_{cat}/K_m were calculated from the variations in the slopes of double-reciprocal plots as described by Zar (1984).

Substrate Isotope Effects. Both labeled and unlabeled substrates were weighed, dissolved in twice-distilled methanol, and their concentrations were determined by enzymatic conversion to androst-4-ene-3,17-dione (A_m = 16 300 M^{-1} cm^{-1} at 248 nm). An appropriate enzyme concentration was used for each substrate in order to obtain similar ranges of absorbance changes and reaction times.

Solvent Isotope Effects. For measurements of the solvent isotope effects, both labeled and unlabeled substrates were dissolved in CH_3OD and their concentrations were calibrated spectrophotometrically as described above. All other com-

Table I: Kinetic Isotope Effects on k_{cat} and K_m with the Y14F Mutant of Ketosteroid Isomerase[a]

expt	substrate	solvent	k_{cat} (s^{-1})	K_m (μM)	k_{cat} corrected (s^{-1})	K_m corrected (μM)	substrate isotope effects $^{H}k_{cat}/^{D}k_{cat}$	solvent isotope effects k_{cat} (H_2O)/k_{cat} (D_2O)
1	4β-H	H_2O	1.26 ± 0.10	145 ± 7	1.26 ± 0.10	145 ± 7		
2	4β-D	H_2O	<0.57 ± 0.02	>98 ± 10	0.522 ± 0.02[b]	140 ± 20[c]	2.41 ± 0.21[d]	
3	4β-H	D_2O	>0.15 ± 0.01	<166 ± 18	0.158 ± 0.01[b]	140 ± 20[c]		7.97 ± 0.81[f]
4	4β-D	D_2O	0.130 ± 0.007	134 ± 18	0.130 ± 0.007	134 ± 18	1.22 ± 0.10[e]	4.02 ± 0.27[g]

[a] Obtained from double-reciprocal plots of initial velocity versus substrate concentration. [b] Calculated from k_{cat}/K_m (Table II) and corrected K_m. [c] Corrected by averaging the K_m values obtained in experiments 1 and 4. [d] 4β-D substrate isotope effect in H_2O. [e] 4β-D substrate isotope effect in D_2O. [f] Solvent isotope effect with unlabeled substrate. [g] Solvent isotope effect with the 4β-D substrate.

ponents of the reaction mixture were dissolved in D_2O. Tris-HCl buffers (1.0 M) were prepared by lyophilizing buffers as described above. For the buffers used under standard assay conditions, the measured pH values were 7.37 in H_2O and 7.54 in D_2O as determined against standard buffers without correction.[1] The final deuterium content in the assay systems was about 99.6 atom %. Initial dilution of the stored enzyme into D_2O or H_2O had no effect on the kinetic constants obtained.

Analysis of Kinetic Progress Curves. When [4β-D]-androst-5-ene-3,17-dione was used as the substrate and the medium was protonated, an initial lag in product formation was noted. To analyze such effects on progress curves, product formation was monitored in a Perkin-Elmer Lambda-9 spectrophotometer. Digitized data were recorded under conditions described above for k_{cat} and K_m measurements. From the raw data sets, logarithms of absorbance were obtained and first-order plots were made. At substrate concentrations well below the K_m, a straight line should be obtained for a perfect pseudo-first-order reaction (as found with unlabeled substrate with Y14F in H_2O, as well as with deuterated substrate in D_2O) and any departure from first-order kinetics would be detected by deviations from linearity. To facilitate the detection of departures from pseudo-first-order kinetics, derivatives of the first-order plots were computed by using the Lotus software package. The derivative or tangent at any point of a first-order plot measures the instantaneous reaction rate at the given time. While the derivative should be constant with time for a perfect pseudo-first-order reaction, it will show time dependence for reactions with lags or bursts, permitting their detection with high sensitivity.

Effect of Phosphate Buffer Concentration on the Rate of Reaction Catalyzed by the Y14F Mutant. The enzyme was assayed in potassium phosphate buffer, pH 6.8, ranging in concentration from 33 to 660 mM phosphate, with the ionic strength adjusted to 1.00 M with KCl. Initial velocities were measured at a substrate concentration of 58.2 μM and at 25 °C.

Results

Measurements of Substrate, Solvent, and Combined Isotope Effects on k_{cat} and K_m Values. A double-reciprocal plot of initial velocity with the Y14F mutant at various substrate concentrations yielded a k_{cat} of 1.26 ± 0.07 s^{-1} and a K_m of 145 ± 15 μM, in agreement with previous values (Kuliopulos et al., 1989). Under normal assay conditions, with unlabeled substrate in protonated solvent, in which the substrate concentration (58.2 μM) is much lower than the K_m value, less than 0.1 μM Y14F mutant is needed to observe product formation at a convenient rate. Under these conditions, pseudo-first-order appearance of product, reflecting the pseudo-first-order disappearance of substrate, is observed. However, when [4β-D]androst-5-ene-3,17-dione was used with the Y14F mutant in H_2O, a lag in product formation was observed, resulting in a departure from first-order behavior. A slow initial rate, followed by a progressive acceleration with time to a final constant rate after ~100 s, was observed (Figure 2). This progressive increase in initial velocity will be shown in a later section to result from an exchange of the deuterium label with solvent. In principle, the less the substrate is consumed, the more closely the initial velocity reflects the initial composition of the system. However, to measure the increase in absorbance accurately, a minimum amount of product formation is required. Therefore, measurements of k_{cat} and K_m were made at as low and uniform consumption of substrate as possible. To achieve this goal and to minimize random errors, the time intervals for mixing were closely controlled and measurements of the initial rates were obtained over the same time period. Table I gives the k_{cat} and K_m values measured under these conditions. As shown later, the rate of exchange of deuterium at C-4β of the substrate with solvent is 31.6 times faster than that of formation of product. Therefore, despite the above precautions, the kinetic parameters obtained under initial velocity conditions with the 4β-D substrate and protonated solvent, or with unlabeled substrate and deuterated solvent, are subject to systematic errors. Because of the more distant extrapolation to obtain K_m, these systematic errors affect K_m much more than k_{cat}. Hence, under these conditions, the K_m and k_{cat} values are given as lower or upper limits only, and the corresponding primary isotope effects on k_{cat} are only approximate.

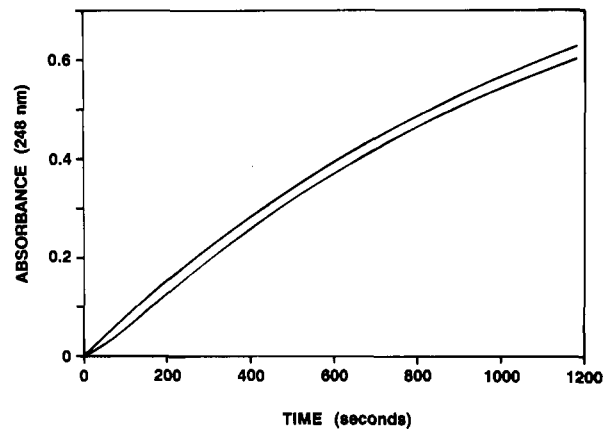


FIGURE 2: Progress of isomerization of androst-5-ene-3,17-dione by the Y14F mutant monitored at 248 nm. The upper curve represents isomerization of unlabeled substrate and the lower curve that of [4β-D]androst-5-ene-3,17-dione. In both cases the initial substrate concentrations were 58.2 μM and the concentration of the Y14F mutant was 0.157 μM in 50 mM Tris-HCl buffer, pH 7.37, in H_2O at 25 °C.

[1] The k_{cat} and K_m values of the wild-type and the Y14F and D38N mutants of ketosteroid isomerase show little or no variation with pH over this range (Kuliopulos et al., 1989).

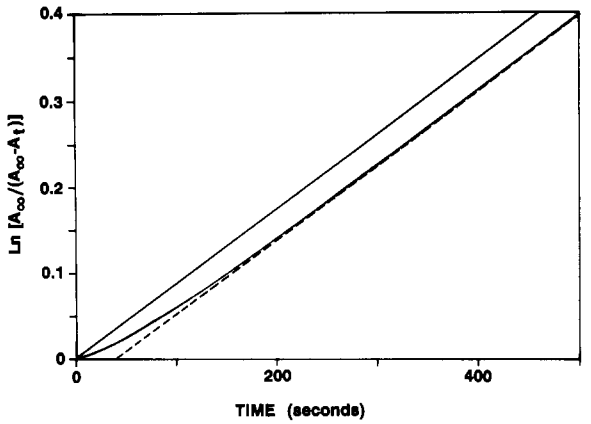


FIGURE 3: Progress of the isomerization of androst-5-ene-3,17-dione by the Y14F mutant as a function of time. The absorbance values were obtained from Figure 2. The upper solid line represents perfect pseudo-first-order behavior when unlabeled substrate was used. The lower solid curve shows the lag in isomerization that occurs when the 4β-D substrate was isomerized. The dashed line indicates the extrapolated reaction rate in the final phase when the rate is equal to that observed with the unlabeled substrate (the slopes are parallel).

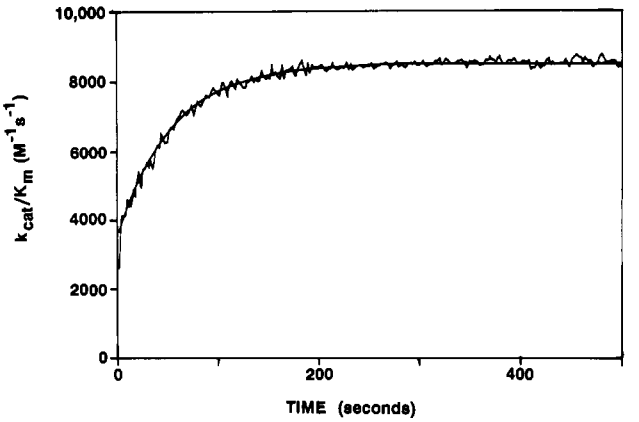


FIGURE 4: Determination of deuterium exchange rate and kinetic isotope effect (on k_{cat}/K_m) when [4β-D]androst-5-ene-3,17-dione was isomerized in H_2O by the Y14F mutant. The experimental curve was obtained by taking first derivatives of the plot for the 4β-D substrate in D_2O shown in Figure 3. These derivatives yield a pseudo-first-order reaction rate constant at each point in time, equal to $(k_{cat}/K_m)[E]$. Here the reaction rate (k_{cat}/K_m) is plotted with respect to time, and a lag is obvious. The smooth curve is a theoretical first-order fit to the measurements indicating that the rate of isomerization (k_{cat}/K_m) of the 4β-D substrate, catalyzed by the Y14F mutant in H_2O, undergoes a first-order acceleration ($t_{1/2}$ = 37.4 ± 0.1 s), resulting from substitution of 4β-deuterium by a proton.

However, the k_{cat} values may be corrected for the exchange as follows. Since deuteration of both the 4β-position of the substrate and of the solvent produced no significant change in K_m from that found with unlabeled substrate in H_2O (Table I), it may reasonably be assumed that separate deuteration of either produces no isotope effect on K_m. The absence of a kinetic isotope effect on K_m results from a profound slowing, by the Y14F mutation, of steps occurring after substrate binding, making the K_m value approach closely the true dissociation constant of the enzyme–substrate complex. Hence the K_m value with either deuterated substrate or solvent may be assumed to be the average value of 140 μM. From this value, and by using the accurate values of k_{cat}/K_m obtained from an analysis of the exchange process (see below), corrected k_{cat} values and isotope effects on k_{cat} have been calculated (Table I). Since these corrected isotope effects on k_{cat} parallel those on k_{cat}/K_m, they will be discussed in a later section.

Studies of Deuterium Exchange with Solvent and Evidence for Release of Intermediates from the Active Site of Y14F Mutant. The initial lag in reaction velocity observed when [4β-D]androst-5-ene-3,17-dione was isomerized by Y14F in H_2O was highly reproducible, and mixing artifacts could therefore be excluded. In principle, this lag could have been due either to activation of the enzyme with time or to the loss of an isotope effect due to the exchange of deuterium out of the substrate with time. Since the isomerizations of unlabeled substrate by Y14F in H_2O or of the 4β-D substrate in D_2O do not show such lags, it is reasonable to conclude that the lag results from the loss of deuterium from the substrate by an enzyme-dependent exchange reaction with solvent.

After the absorbance profile for the isomerization of the 4β-D substrate in H_2O was recorded, the logarithm of the absorbance was used to construct a first-order plot with respect to time (Figure 3). The tangent at each point of the first-order curve was then computed and plotted as a function of time (Figure 4). It was confirmed that the rate of product formation increased with time when the 4β-D substrate was isomerized in H_2O and reached a plateau value (Figure 4). Furthermore, the rate of attainment of the final rate obeyed first-order kinetics quite well, as shown by curve fitting (Figure 4). The final rate (k_{cat}/K_m = 8600 M^{-1} s^{-1}) closely approaches the rate found with unlabeled substrate isomerized by the Y14F mutant under identical conditions (8690 M^{-1} s^{-1}). The results are consistent with the suggestion that, during the time period when only a small portion of the substrate was consumed, most of the 4β-deuterium of the remaining substrate had been removed by an exchange process. The rate acceleration reflects the rising population of the unlabeled substrate molecules and the resulting loss of a primary kinetic deuterium isotope effect at the 4β-position. The pseudo-first-order rate constant used to fit the rate acceleration (0.0185 s^{-1}) when corrected for the enzyme concentration (0.157 μM) yields an apparent second-order rate constant of 1.18 × 10^5 M^{-1} s^{-1} for the replacement of a deuteron by a proton at the 4β-position of the substrate. Since the 4β-deuterium on the substrate is stable in the absence of enzyme, it is clear that the Y14F mutant of ketosteroid isomerase catalyzes the loss of the 4β-deuterium from the substrate. The mechanism probably involves the removal of the 4β-deuterium by the intact base, Asp-38, to form an enolate carbanion. The carbanion intermediate is then protonated by H_2O on the enzyme or by H_2O or buffer in the medium to form unlabeled substrate. If so, the rate of acceleration of product formation measures the rate of exchange of the label. Comparison of the rate constant for this acceleration with the rate constant for isomerization to product with deuterated substrate yielded a ratio of 31.6:1, favoring the exchange. This rate ratio measures the ratio of two processes, both catalyzed by the Y14F mutant: deuterium exchange with solvent and product formation from deuterated substrate.[2]

The nonenzymatic isomerization of androst-5-ene-3,17-dione catalyzed by hydroxide was studied by Pollack et al. (1987),

[2] Direct evidence for the occurrence of part of the reaction promoted by Y14F in free solution should in principle be provided by the failure of deuterium to appear in the product when [4-D]androst-5-ene-3,17-dione is isomerized in H_2O, since the dienolate anion is expected to be converted in solution to both unlabeled substrate and product. Conversely, if the isomerization of unlabeled substrate by Y14F were conducted in D_2O, the product would be expected to acquire deuterium at C-6, unlike the wild-type enzyme. These experiments are not easy to carry out with Y14F, which is catalytically greatly handicapped, requiring the addition of stoichiometric amounts of enzyme in relation to the amount of steroid substrate isomerized. Under these conditions of high enzyme concentration, the product can undergo rebinding to the enzyme and exchanges with protons of the medium, thereby complicating the interpretation of the results.

Table II: Kinetic Isotope Effects on k_{cat}/K_m with the Y14F Mutant of Ketosteroid Isomerase

expt	substrate	solvent	k_{cat}/K_m (M^{-1} s^{-1})	substrate isotope effects $^{H}(k_{cat}/K_m)/^{D}(k_{cat}/K_m)$	solvent isotope effects k_{cat}/K_m (H_2O)/k_{cat}/K_m (D_2O)
1	4β-H	H_2O	8690 ± 120[a]		
2	4β-D	H_2O	3730 ± 75[b]	2.33 ± 0.06[d]	
3	4α-D	H_2O	8200 ± 116[a]	1.06 ± 0.02[e]	
4	6-D	H_2O	8520 ± 120[a]	1.02 ± 0.02[f]	
5	4β-H	D_2O	1130 ± 60[c]		7.69 ± 0.18[h]
6	4β-D	D_2O	970 ± 50[a]	1.16 ± 0.08[g]	3.85 ± 0.21[i]

[a] Obtained from slopes of double-reciprocal plots of initial velocity versus substrate concentration. [b] Obtained by extrapolation of the initial phase of derivative plot (Figure 4) to zero time. [c] Obtained as in footnote b from data exemplified in Figure 5. [d] Primary 4β-D substrate isotope effect in H_2O obtained by extrapolation of initial and final phases of derivative plot (Figure 4) to zero time and to infinite time, respectively. [e] Secondary 4α-D substrate isotope effect in H_2O. [f] Secondary 6-D substrate isotope effect in H_2O. [g] Primary 4β-D substrate isotope effect in D_2O obtained as in footnote d from Figure 5. [h] Solvent isotope effect with unlabeled substrate. [i] Solvent isotope effect with the 4β-D substrate.

who concluded that the 4β-proton is initially removed by the base to form a carbanion intermediate, which is subsequently protonated both at C-4 to form substrate as well as at C-6 to form product, at a ratio of rates of 25:1 in favor of substrate formation. The similarity of rate ratios observed in the absence of enzyme and with the Y14F mutant suggests that, under our experimental conditions, the Y14F mutant is most likely to carry out only the first part of the isomerization, namely, the removal of 4β-D (or 4β-H when an unlabeled substrate is used), and that subsequent reactions, either proceeding forward to form product or backward to form substrate, occur in solution and are facilitated by solvent and by buffer molecules.

If this were the mechanism, an unlabeled substrate in the presence of the Y14F mutant in D_2O should show the opposite effect, namely, a burst of product formation followed by a decrease in the rate of isomerization as more and more substrate becomes deuterated in solution. The progress curves of absorbance with time under these conditions did not reveal an obvious departure from simple pseudo-first-order behavior. However, when a first-order plot was made and derivatives were taken, a small burst was detected, followed by a first-order decrease in rate to a constant value (Figure 5) which agreed within 6% with that obtained by double-reciprocal plots with the 4β-D substrate in D_2O (Table II). When the pseudo-first-order rate constant of 0.0175 s^{-1} is corrected for enzyme concentration, the apparent second-order rate constant for the replacement of a proton by a deuteron at the 4β-position of the substrate is 1.11×10^4 M^{-1} s^{-1}, reflecting a large solvent deuterium isotope effect of ~10.6 on the exchange process, consistent with its occurrence in solution. The small overall decrease in rate observed with protonated substrate in D_2O results from a small 4β-D substrate isotope effect in D_2O (see below).

Buffer Effects on the Rate of the Isomerase Reaction Catalyzed by Y14F. To obtain further evidence for the dissociation of intermediates from the enzyme, the effect of phosphate buffer on the rate of product formation was studied between 33 and 660 mM potassium phosphate at pH 6.8, while the ionic strength was kept constant at 1.0 M by adding KCl. The rate of product formation increased linearly with phosphate concentration over this range by 0.241 s^{-1}, indicating that the reaction was first order in phosphate buffer. The overall second-order rate constant for the activation by phosphate was 0.385 M^{-1} s^{-1} at 25 °C. In a model reaction, phosphate catalysis of the isomerization of 3-cyclohexenone in aqueous solution has been reported (Whalen et al., 1976). If the reasonable assumption is made that phosphate buffer does not gain access deep into the active site of ketosteroid isomerase where the 3-keto group of the substrate is positioned, the sizeable acceleration of product formation by phosphate buffer suggests that phosphate protonates the dienolate carbanion in solution. Hence this intermediate can dissociate from the enzyme. Although there is no direct evidence for it, the dienol may also form on the enzyme and dissociate rapidly into solution.

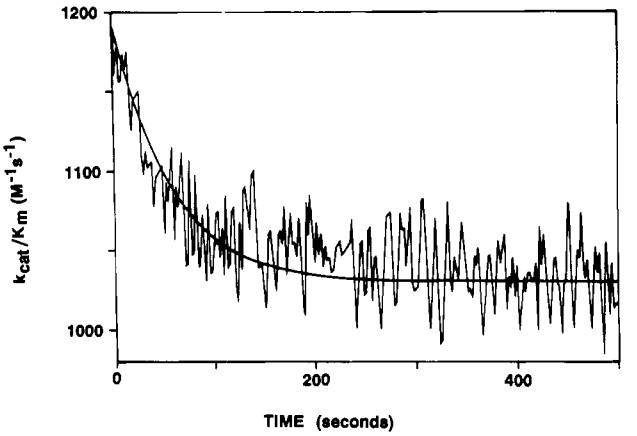


FIGURE 5: Determination of substrate proton exchange rate and substrate deuterium kinetic isotope effect in D_2O on k_{cat}/K_m of the isomerization catalyzed by the Y14F mutant. The experiment was done under the same conditions as in Figure 2, except that unlabeled substrate and D_2O were used. The enzyme concentration was 1.57 μM. The pH was 7.54 as obtained directly from the pH meter (uncorrected). The data analyses were done by the same methods used for Figures 3 and 4. The smooth curve is the theoretical first-order fit, indicating that the rate of isomerization of unlabeled substrate catalyzed by Y14F mutant in D_2O undergoes a pseudo-first-order decrease ($t_{1/2}$ = 39.7 ± 5.5 s), resulting from substitution of the 4β-proton by deuterium.

Primary and Secondary Substrate Isotope Effects on k_{cat}/K_m. As discussed above, measurements of the primary isotope effects on the overall isomerization catalyzed by Y14F were confounded by the isotope exchange process. Hence, isotope effects on k_{cat} obtained from initial reaction rates, before much exchange had occurred, are only estimates (Table I). However, when the initial and final phases of the reaction (Figures 4 and 5) were extrapolated to zero and infinite time, respectively, primary kinetic isotope effects on k_{cat}/K_m could be obtained with considerable accuracy. Table II lists the substrate and solvent isotope effects on k_{cat}/K_m obtained in this way. Experiments 1 and 2 show an isotope effect of 2.33 for the 4β-D substrate, slightly smaller than the value of 2.99 ± 0.06 found with the wild-type enzyme (Xue et al., 1990).

Experiment 3 gives an isotope effect of 1.06 ± 0.02 for the 4α-D substrate, obtained from slopes of double-reciprocal plots. Derivative analyses of first-order plots showed no evidence of a lag in this case. The value of 1.06 is reasonable for a secondary kinetic isotope effect but smaller than that found with wild-type enzyme [1.11 ± 0.02, Xue et al. (1990)]. This result establishes that the Y14F mutant retains the stereoselectivity of the wild-type enzyme for the 4β-proton and further supports the conclusion that the stereoselectivity stems from the ori-

entation of Asp-38, the general base, toward the β-face of the substrate (Kuliopulos et al., 1989; Xue et al., 1991).

The presence of a secondary kinetic isotope effect with the 4α-D substrate and the absence of a secondary kinetic isotope effect with the 6-D substrate (1.02 ± 0.02, Table II, experiment 4) indicate that enolization of the enzyme-bound substrate, rather than reketonization of dienolic intermediates, constitutes the rate-limiting step(s) of the reaction catalyzed by the Y14F mutant, as found with the wild-type enzyme (Xue et al., 1990). If reketonization were rate limiting, a detectable inverse secondary isotope effect approaching 0.88 would be expected with the 6-D substrate (Cook et al., 1980) since a hybridization change from sp^2 to sp^3 occurs at C-6 only during reketonization.

Solvent and Combined Isotope Effects on k_{cat}/K_m. While both primary and secondary kinetic isotope effects at the C-4 position of substrate are smaller with Y14F than with the wild-type enzyme, the solvent isotope effect is much larger. Experiment 5 gives a net solvent isotope effect on k_{cat}/K_m of the isomerization reaction of 7.69, which is 4.8-fold larger than the value of 1.62 found with the wild-type enzyme (Xue et al., 1990). Hence, in the rate-limiting formation of the dienolic intermediate catalyzed completely or in part by the Y14F mutant, both deprotonation at C-4 and protonation of the carbonyl oxygen are partially rate limiting. To determine whether these proton transfers occur in the same or in separate steps, i.e., whether dienol formation is concerted or stepwise, the combined isotope effects were studied.

Table II shows that the substrate primary isotope effect at the 4β-position decreased from 2.33 in H_2O to 1.16 when measured in D_2O. The solvent isotope effect decreased from 7.69 when unlabeled substrate was used to 3.85 when the 4β-D substrate was used. These decreases provide convincing evidence that the substrate and solvent isotope effects occur at different partially rate-limiting steps since slowing of one step by introducing deuterium makes the other step relatively less rate limiting. A stepwise enolization is reasonable because substrate deprotonation occurs on the Y14F mutant, while protonation of the dienolate to form the dienol appears to occur predominantly in solution. Since little or no isotope effects on K_m occurred, these isotope effects are actually on k_{cat} (Table I), although they are more accurately measured on k_{cat}/K_m (Table II).

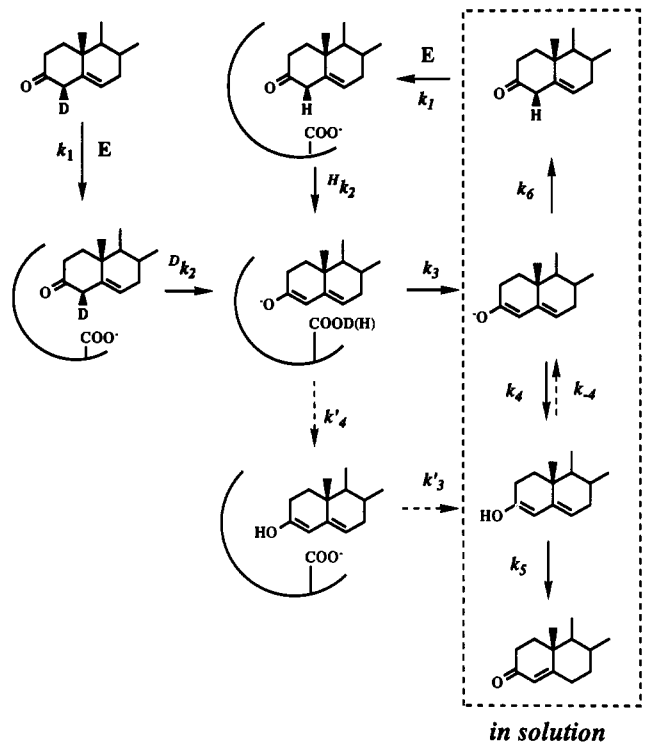
Discussion

The mechanism of the Y14F mutant differs from that of the wild-type enzyme, which shows concerted dienol formation as the rate-limiting step, and resembles that of the D38N mutant for which dienol formation is also a stepwise process (Xue et al., 1990, 1991).

As summarized in Scheme I, the stepwise enolization catalyzed by the Y14F mutant is likely to proceed via the initial deprotonation of the substrate at C-4 by the intact base, Asp-38, to yield a dienolate carbanion intermediate (k_2), resulting in the observed primary and secondary kinetic isotope effects at C-4. This dienolate carbanion has two possible reaction pathways depending on whether it is protonated to form the dienol on or off the enzyme. Most simply, the dienolate dissociates rapidly from the enzyme (k_3) and is slowly protonated by water and buffer on oxygen to yield the dienol, the next intermediate in the overall isomerization (k_4), or at C-4 to regenerate the substrate (k_6). The dienol intermediate proceeds spontaneously and rapidly to form product (k_5).

Additionally, in a minor pathway designated by broken arrows in Scheme I, the dienolate carbanion may remain enzyme-bound long enough to be protonated slowly at the oxyanion by a surrogate proton donor for Tyr-14, probably H_2O, to yield the dienol (k'_4). The dienol dissociates rapidly from the enzyme (k'_3) and either spontaneously forms product as described above (k_5) or rapidly loses a proton to form the dienolate carbanion (k_{-4}), which can regenerate substrate (k_6). Either or both of these pathways, which differ in the site of formation of the dienol, provide explanations for the exchange of the 4β-proton of the substate with solvent catalyzed by Y14F and for the solvent isotope effect. From model reactions in aqueous solutions, Malhotra and Ringold (1965) concluded that the dienolate species is preferentially protonated at C-4 to regenerate substrate because of the greater charge density at this position, while the dienol is preferentially protonated at C-6 to form product. These model studies, together with the present observation of a 31.6-fold faster rate of substrate regeneration than product formation with the 4β-D substrate in H_2O, suggest that dienol formation on the Y14F enzyme constitutes, at most, a minor pathway.

Scheme I



The unusually large solvent isotope effect obtained with the Y14F mutant (7.69, Table II) provides further evidence for the dissociation of the dienolate intermediate from the enzyme and for formation of the dienol in solution. Comparably large solvent kinetic isotope effects of 6 and 7.7, respectively, have been found in the tertiary amine catalyzed isomerization of androst-5-ene-3,17-dione (Perera et al., 1980) and in the phosphate-catalyzed isomerization of 3-cyclohexenone (Whalen et al., 1976).

As previously pointed out (Xue et al., 1990), the small solvent isotope effect of 1.62 found with the wild-type enzyme could result from one or more of three mechanisms for reducing primary isotope effects: (1) strong hydrogen bonding between Tyr-14 and the 3-carbonyl oxygen of the enzyme-bound substrate in the ground state; (2) a transition state which is asymmetric near C-3, i.e., the proton transfer to the C-3 oxygen is not far advanced; and, (3) long-range coupled motion in a concerted transition state between the 4β-proton and the Tyr-14 hydroxyl proton. Clearly, mechanism 3 for decreasing the primary solvent isotope effect is inoperative with the Y14F

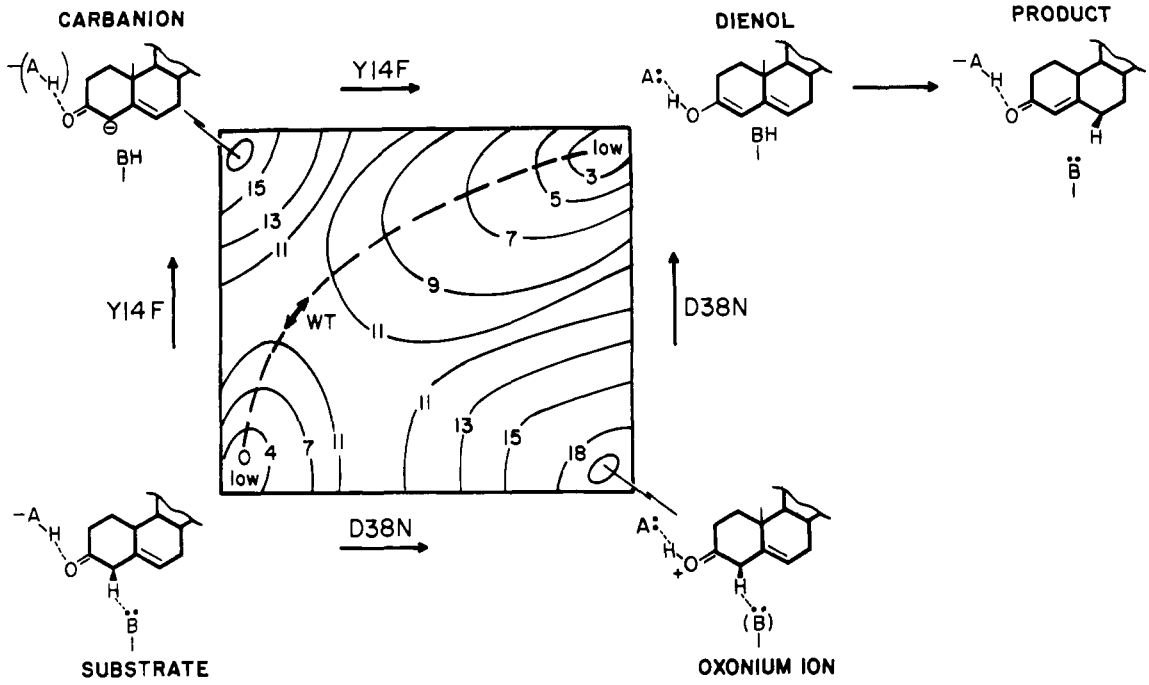


FIGURE 6: Reaction coordinate free energy contour diagram, according to Jencks (1985), comparing the enolization mechanism catalyzed by wild-type ketosteroid isomerase (WT) with those of the Y14F and D38N mutants. The free energy of the enzyme-bound substrate is arbitrarily set at zero. The reaction path for the concerted enolization catalyzed by the wild-type enzyme is shown by the broken curved line, and the unbalanced transition state (Xue et al., 1990) is indicated by the location of the double-headed arrow. The free energy barrier at the transition state (11 kcal/mol) is based on a k_{cat} of 54000 s^{-1} (Kuliopulos et al., 1989). The solid arrows indicate the stepwise enolizations catalyzed by the D38N (Xue et al., 1991) and Y14F mutants (this paper). The energy level of the enzyme-bound oxonium ion intermediate (18 kcal/mol) is based on the 10^6-fold slower enolization found with the D38N mutant (Kuliopulos et al., 1989; Xue et al., 1991). The energy level of the carbanion intermediate (15 kcal/mol) is based on the $10^{3.1}$-fold slower rate of removal of the 4β-proton of the substate on the Y14F mutant. This factor results from the observation that the dienolate carbanion is formed 32.6 times faster than product is formed (see text). With the Y14F mutant, the second step, from the carbanion to the dienol, occurs predominantly or entirely in solution.

mutant since dienol formation is not concerted. Mechanism 1 is also absent on Y14F as shown by ultraviolet spectral studies of the enzyme-bound product analogue 19-nortestosterone, which revealed a 10-nm red shift on the wild-type enzyme, suggesting hydrogen bonding, but no red shift on the Y14F mutant (Kuliopulos et al., 1989). Hydrogen bonding of Tyr-14 to enzyme-bound 19-nortestosterone has also been detected by ultraviolet resonance Raman spectroscopy with the double mutant (Y55F + Y88F) in which Tyr-14 is the only remaining tyrosine residue (Austin et al., 1991). A more symmetric transition state for protonation of the C-3 oxygen of the carbanion with the Y14F mutant is likely since the pK_a difference between the dienol which is forming (pK_a = 10; Zeng & Pollack, 1991) is closer to that of an alternative proton donor such as water (pK_a = 15.7) than was the case with wild-type enzyme. With wild-type enzyme where the transition state is concerted, the 3-carbonyl oxygen (pK_a when protonated $\sim$−7) is protonated by Tyr-14 (pK_a > 10.8; Kuliopulos et al., 1991), resulting in a much larger difference in pK_a values. Since the dienolate dissociates from the Y14F mutant, an additional mechanism for increasing the primary solvent kinetic isotope effect with Y14F would be that the dienolate is protonated via multiple simultaneous pathways, because more degrees of freedom exist in solution, in contrast to the simple protonation of the trans lone pair of the carbonyl oxygen of the substrate by Tyr-14 which occurs on the wild-type enzyme (Kuliopulos et al., 1991).

Since the dienolate and, possibly, the dienolic intermediates dissociate rapidly from the active site of the Y14F mutant but dissociate much more slowly from the active site of the D38N mutant, where they can be directly observed by ultraviolet spectroscopy (Xue et al., 1991), it is reasonably concluded that Tyr-14 plays a major and direct role in the tight binding of the intermediate to the enzyme. On the wild-type enzyme and on the slightly damaged Y55F mutant, such tight binding of the dienolic intermediate by tyrosinate-14 permits an intramolecular proton transfer from the 4β- to the 6β-position to occur. With Y14F, since the intermediate is apparently not converted to product on the enzyme, the rate constant for exchange represents a lower limit for the rate constant for dissociation of intermediates from the active site. As estimated from k_{cat} and the 31.6-fold faster isotopic exchange rate, the rate constant for dissociation of the dienolate (and dienolic) intermediates from Y14F must be at least 39.8 s^{-1}, corresponding to a half-time of 0.0174 s. While this rate constant cannot be measured for the wild-type enzyme because of rapid product formation, these intermediates dissociate from the active site of the D38N mutant, which retains Tyr-14, with a half-time of at least 123 min (Xue et al., 1991). The ratio of these two half-times for dissociation of intermediates reflects the loss of at least 7.6 kcal/mol of binding free energy that results from the absence of the hydroxyl group of Tyr-14. It has been reported that deletion of a charged hydrogen bond weakens binding between tyrosyl-tRNA synthetase and its substrates by 3.5–4.5 kcal/mol (Fersht et al., 1985). The active site of ketosteroid isomerase has a more hydrophobic environment and an optimum geometry and is therefore likely to exhibit stronger hydrogen bonding between tyrosinate-14 and the dienol intermediate on the wild-type enzyme and comparably strong interactions on all mutants that retain Tyr-14. Such tight binding of labile intermediates, together with much weaker hydrogen bonding of substrates in the ground state by Tyr-14 (Austin et al., 1991), contributes to the rate acceleration for enolization. On the Y14F mutant, the dienolate intermediate, which probably forms first, cannot be held tightly and therefore dissociates rapidly. If formed

on the enzyme, the dienolic intermediate also dissociates rapidly. Hence, Tyr-14 contributes significantly, not only to the formation and ketonization of the dienolic intermediate but also to its tight binding by ketosteroid isomerase.

From our studies of kinetic isotope effects with wild-type isomerase (Xue et al., 1990) and with the D38N (Xue et al., 1991) and Y14F mutants, we can now compare the mechanisms of enolization catalyzed by each of these enzymes, making use of a reaction coordinate free energy contour diagram (Jencks, 1985; Julin & Kirsch, 1989) (Figure 6). The wild-type enzyme catalyzes a concerted enolization because the curved pathway across the saddle point is lower in free energy than the two alternative right-angle pathways which are stepwise, involving either an oxonium ion or a carbanion intermediate. The energy levels of these intermediates are obtained from the effects of the D38N and Y14F mutations, respectively, on the rates of formation of the intermediates. The concerted transition state on the wild-type enzyme is unbalanced, symmetric in substrate deprotonation at C-4, but early in protonation of the 3-carbonyl oxygen (Xue et al., 1990), which places the transition state to the left of center in Figure 6, as indicated by the double-headed arrow. The Y14F mutation greatly increases the free energies of the oxonium ion and of the dienol, thereby also increasing the free energy of the concerted transition state, shifting it toward the carbanion intermediate. The stepwise carbanion pathway thus becomes the lowest free energy pathway to the dienol. The D38N mutation has the opposite effect, greatly increasing the free energies of the carbanion and the dienol, thereby shifting the transition state toward the oxonium ion intermediate, making the stepwise oxonium ion pathway the lowest free energy pathway to the dienol. Mutation of either of these two key catalytic residues thus significantly alters the mechanism of enolization catalyzed by ketosteroid isomerase.

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