Catalytic Mechanism of an Active-Site Mutant (D38N) of Δ^5-3-Ketosteroid Isomerase. Direct Spectroscopic Evidence for Dienol Intermediates†

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ABSTRACT: The Δ^5-3-ketosteroid isomerase (EC 5.3.3.1) of *Pseudomonas testosteroni* catalyzes the conversion of androst-5-ene-3,17-dione to androst-4-ene-3,17-dione by a stereospecific transfer of the 4β-proton to the 6β-position. The reaction involves two steps: (a) a rate-limiting concerted enolization, comprising protonation of the 3-carbonyl oxygen by the phenolic hydroxyl group of Tyr-14 and abstraction of the 4β-proton by the carboxylate group of Asp-38, and (b) rapid reketonization of the dienol, which may or may not be concerted. The active-site mutant D38N, which lacks the base responsible for proton transfer, is about $10^{6.0}$-fold less active catalytically than the wild-type enzyme. With the D38N mutant it was demonstrated spectroscopically that the enzymatic reaction involves the conversion of the substrate to both the dienol and its anion as tightly enzyme-bound intermediates, which are then converted much more slowly to the α,β-unsaturated product. In contrast to the mechanism of the wild-type enzyme, the enolization reaction promoted by the D38N mutant is not stereospecific with respect to removal of the 4β-proton and shows primary kinetic isotope effects on enolization when either 4α or 4β or both of these protons are replaced by deuterium. Kinetic isotope effects obtained with deuterated substrates, solvent, or combinations of the two indicate that, unlike in the wild-type enzyme, protonation of the carbonyl oxygen and removal of the C-4 proton are not concerted in the D38N mutant. Furthermore, the spectroscopic detection of both dienolate and dienol intermediates and the very slow rate of product formation indicate that reketonization of the intermediate(s) is likewise a stepwise process. Loss of the active-site base (Asp-38) thus profoundly alters the mechanism of the ketosteroid isomerase reaction.

The enzyme Δ^5-3-ketosteroid isomerase (EC 5.3.3.1) of *Pseudomonas testosteroni* promotes the isomerization of Δ^5-3-ketosteroids to the conjugated Δ^4-3-ketosteroids by a predominantly conservative and stereospecific intramolecular transfer of the 4β-proton to the 6β-position [see reviews by Talalay and Benson (1972), Batzold et al. (1974), Pollack (1989), and Schwab and Henderson (1990)]. The catalytic rate with the standard substrate, androst-5-ene-3,17-dione, expressed as k_{cat}/K_m, approaches the limit controlled by diffusion, and k_{cat} is $10^{9.5}$-fold greater than the spontaneous rate in the absence of enzyme. Considerable progress has recently been made in understanding the mechanistic details of this reaction. Cloning and sequencing of the isomerase gene and overexpression of the protein (Kuliopulos et al., 1987a; Choi & Benisek, 1987) have made it possible to construct active-site-directed mutant enzymes (Kuliopulos et al., 1989). From these experiments, in conjunction with NMR and X-ray diffraction studies (Kuliopulos et al., 1987b), it was concluded that Asp-38 is the general base responsible for the 4β-proton abstraction and that Tyr-14 is the general acid that protonates the 3-carbonyl oxygen of the steroid to form the dienolic intermediates in the same rate-limiting step that constitutes the first half of the isomerase reaction (Kuliopulos et al., 1989; Xue et al., 1990). In the second half, or reketonization reaction, the roles of these two residues are reversed, with Tyrosinate-14 functioning as a base to deprotonate the dienolic intermediate and Asp-38 in its protonated form functioning as an acid to protonate C-6. The participation of these two residues, which are stereoelectronically favorably positioned orthogonally and trans with respect to the bound substrate in the hydrophobic steroid-binding cavity of the enzyme, can account for the total increase in catalytic rate produced by the enzyme (Kuliopulos et al., 1989, 1990, 1991).

The kinetic scheme for the wild-type isomerase reaction may therefore be formulated as

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E{\cdot}D \rightarrow E + P \qquad (1)$$

where E, S, D, and P refer to enzyme, substrate, dienol intermediate, and product, respectively. Because the rate-limiting step that determines k_{cat} is the concerted enolization (Xue et al., 1990), this scheme can be simplified to

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}S \xrightarrow{k_{cat}} E + P \qquad (2)$$

thus providing only indirect evidence for a dienolic intermediate. Nevertheless, many lines of evidence support the participation of a dienolic intermediate in the isomerase reaction. Stereoelectronic considerations mandate the participation of an intermediate in the reaction (Kuliopulos et al., 1989). Spectroscopic studies and hydrogen isotope exchange between the protons of the steroid product and medium provided the first experimental evidence for dienolic intermediates (Wang et al., 1963). At neutral pH, A-ring phenolic steroids such as 17β-estradiol and 17β-dihydroequilenin upon binding to the isomerase displayed ultraviolet spectral changes consistent with generation of a phenolate anion of the steroid. Similarly, α,β-unsaturated 3-ketosteroids showed bathochromic absorption shifts consistent with protonation of the 3-carbonyl group and similar to those observed upon the addition of strong acid

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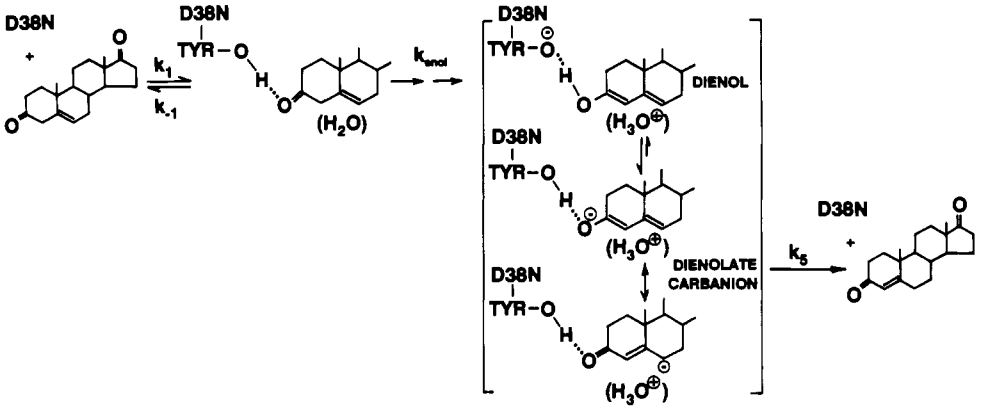


FIGURE 1: Kinetic scheme and mechanism of D38N mutant of Δ^5-3-ketosteroid isomerase showing the participation of phenolic hydroxyl group of Tyr-14 as proton donor and the dienol and dienolate anion as intermediates in the conversion of androst-5-ene-3,17-dione to androst-4-ene-3,17-dione. Water is shown as the base that is postulated to replace Asp-38 of the wild-type enzyme.

(Wang et al., 1963; Kuliopulos et al., 1989). Such spectral shifts were not observed with the Y14F mutant enzyme, and it was concluded that Tyr-14 (in a step concerted with 4β-proton removal by Asp-38) is responsible for the enolization of the substrate (Kuliopulos et al., 1989). From kinetic studies with stereospecifically deuterated substrates, Malhotra and Ringold (1965) suggested that the 4β-proton was removed in a rate-limiting step. Subsequently primary, secondary, and combined kinetic isotope studies with deuterium-labeled substrates and solvent provided evidence for a rate-limiting concerted enolization in the first step of the isomerase reaction (Xue et al., 1990). Moreover, because of the conformational rigidity of 19-nortestosterone, an enzyme-bound product analogue, it was possible to ascertain from interproton distances determined by the nuclear Overhauser effect that the stereochemistry of the concerted enolization reaction was orthogonal and trans (Kuliopulos et al., 1991). Recently, Pollack et al. (1987, 1989) have obtained spectral evidence for the formation of a dienolate intermediate in the base-catalyzed isomerization of androst-5-ene-3,17-dione and have also determined the partitioning of a chemically generated dienol between substrate and product when the enzymatic reaction was carried out under mildly acidic conditions (Eames et al., 1990).

Despite substantial indirect evidence for the involvement of an enolic intermediate in the Δ^5-3-ketosteroid isomerase reaction, no direct evidence has been obtained for the existence of such an intermediate in the enzymatic conversion of the natural substrate. Studies with the D38N mutant, which lacks the base responsible for the proton transfer reaction and has a catalytic rate that is about one-millionth that the wild-type enzyme, provide direct spectral evidence for the presence of enzyme-bound dienolic intermediates in the isomerase reaction. Our experiments establish that both the neutral dienol and the dienolate anion coexist on the mutant enzyme, as shown schematically in Figure 1. We have also determined the dissociation constant ($K_s = k_1/k_{-1}$) of the enzyme substrate complex[1] and the apparent first-order rate constants for enolization (k_{enol}) and for product formation (k_{ket}), and we have estimated the position of the dienol-dienolate equilibrium on the enzyme.

Experimental Procedures

Materials. Solutions of Tris base were adjusted to the desired pH at 25 °C with HCl. For experiments in D_2O, the pH-adjusted Tris buffers were lyophilized and the residues were dissolved in D_2O and then lyophilized a second time before being dissolved in D_2O giving pD 7.2 as measured. D_2O (99.9 atom % D) and CH_3OD (99.5 atom % D) were obtained from Aldrich.

Enzymes. Recombinant wild-type isomerase and the D38N mutant were prepared as described (Kuliopulos et al., 1987a, 1989), recrystallized 2 or 3 times, and stored as crystalline suspensions at 4 °C in 30% saturated solutions of ammonium sulfate neutralized with NH_4OH. For kinetic studies, suitable 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 proteins were determined by measuring the absorbance at 280 nm, assuming absorbance values of 0.336 and 0.343 in 10-mm light path cuvettes for the solutions containing 1.00 mg/mL of the wild-type and D38N mutant enzymes, respectively (Benson et al., 1975; Kuliopulos et al., 1989).

Substrates. [4β-D]Androst-5-ene-3,17-dione substrate was prepared according to modifications of the procedure of Malhotra and Ringold [1965; see also Xue at al. (1990)], and [4α-D]androst-5-ene-3,17-dione was prepared according to Viger et al. (1978). 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).

Kinetic Measurements of k_{enol} and K_s. Standard isomerase activity measurements for the D38N mutant were obtained in a Beckman DU-7 spectrophotometer in quartz cuvettes of 10-mm light path at 25 ± 0.1 °C and at 248 nm, which is the absorption maximum of the product androst-4-ene-3,17-dione (a_m = 16300 M^{-1} cm^{-1}). Reactions were carried out in systems of 1.5-mL volume containing 50 mM Tris-HCl, pH 7.5, 58.3 μM androst-5-ene-3,17-dione in 50 μL of methanol, and appropriate quantities of enzyme. The k_{enol} and K_s values were obtained from pseudo-first-order reaction rates by varying substrate concentrations. A Perkin-Elmer Lambda-9 spectrophotometer equipped with a rapid kinetics accessory (Model

[1] The minimal kinetic scheme needed to explain the present results obtained with the D38N mutant is given in Scheme I. On the assumption that E·S and E^-·SH^+ are in a steady state during the observed accumulation of dienol and dienolate intermediates, the following kinetic formulations are obtained (King & Altman, 1956): $k_{enol} = k_2k_3/(k_2 + k_3 + k_{-2})$ and $K_m(app) = (k_{-1}k_{-2} + k_{-1}k_3 + k_2k_3)/(k_1k_2 + k_1k_3 + k_1k_{-2})$, where $K_m(app)$ is the substrate concentration that gives half-maximum rate of accumulation of the intermediates. If it is further assumed that $k_{-2} \gg k_2$ and k_3, on the basis of the present data, these formulations simplify to $k_{enol} = k_2k_3/k_{-2}$ and $K_m(app) = k_{-1}/k_1 = K_s$.

SFA-11, Hi-Tech Scientific, Salisbury, England) was used.

Substrate, Solvent, and Combined Deuterium Kinetic Isotope Effects on the Enolization Rate Constants. From apparent second-order rate constants, k_{enol}/K_s values were obtained for unlabeled and deuterated substrates under rapid mixing conditions. Solutions containing equal concentrations (7.5 μM) of substrate and enzyme were loaded into the two reservoirs of the rapid kinetics apparatus. The solutions were then mixed rapidly, and digitized absorbance values at 248 nm were collected as a function of time with a Perkin-Elmer Lambda-9 spectrophotometer. While substrates (unlabeled and deuterium-labeled at different positions) were loaded separately, aliquots of the same enzyme solution were used to determine the k_{enol}/K_s values for the corresponding substrate. Under these conditions, highly reproducible data were obtained. The solvent and double isotope effects on k_{enol}/K_s were measured with substrates dissolved in CH_3OD and enzyme and buffer prepared in D_2O.

Spectra of Enzyme-Bound Intermediates. The time-dependent formation of intermediates and product of the enzymatic reaction was monitored by scanning the UV range from 220 to 320 nm on a Perkin-Elmer Lambda-9 spectrophotometer. The scan time was 5 s for each spectrum. Immediately after the substrate and enzyme were mixed, the first scan was taken as a base line. These scan times are sufficiently rapid in relation to the observed reaction rates that the spectra are very similar to those obtained on a Hewlett-Packard Model HP-8452A diode array spectrophotometer on which spectra were collected in 0.5 s.

Rate of Product Formation. The half-life for the conversion of the enzyme-bound intermediate to product is on the order of several hours. Special conditions were designed for measurement of the rate of product formation. Solutions of substrate (23 μM) and enzyme (30 μM) were loaded into the two separated chambers of paired tandem cuvettes (each compartment had an optical light path of 4.38 mm; Hellma Cells, Jamaica, NY). They were then placed into the cell holders. After the base-line absorbance was determined, the contents of the sample cell compartments were mixed. The spectra from 220 to 320 nm were scanned (5-s scans) at time intervals of 10 min for a 24-h period, and digitized absorbance values were stored. In this way highly reproducible results were obtained, and the absorbance at any wavelength could be plotted as a function of time. After the complete conversion of the substrate to product (about 30 h), a second aliquot of the substrate could be introduced into the cuvettes and the enzyme in both cuvettes was found to retain full activity even after such prolonged storage at 25 °C.

Results and Discussion

Formation of Enzyme-Bound Intermediates. The catalytic activity of wild-type isomerase is extraordinarily high, and under standard assay conditions only about 5 pM enzyme subunit concentrations are needed to obtain conveniently measurable rates in the spectrophotometric assay. The D38N mutant, while binding the androsten-5-ene-3,17-dione substrate efficiently, is $10^{5.7}$-fold less active, and enzyme concentrations need to be raised by a factor of approximately 10^6 in order to obtain easily measurable rates (Kuliopulos et al., 1989). Consequently concentrations of D38N under assay conditions are in the micromolar range and are therefore comparable to those of the substrate, androst-5-ene-3,17-dione (58.3 μM). Under these experimental conditions certain spectroscopic anomalies were noted when the absorption at 248 nm (λ_{max} in water of the product, androst-4-ene-3,17-dione) was monitored as a function of time. A rapid initial rise ("burst") in absorption occurred in the first few minutes and was followed by a slow and prolonged increase in absorption over many hours, until the substrate was completely isomerized (Figure 2). The initial rate of absorption change at 248 nm increased with the added substrate concentration and showed saturation on extrapolation to infinite substrate concentration. However, the magnitude of the initial absorption burst was constant for a given concentration of enzyme and proportional to the enzyme concentration when the substrate was present in excess. When the substrate was limiting and enzyme was in excess, the magnitude of the absorption burst was determined by the substrate concentration (results not shown).

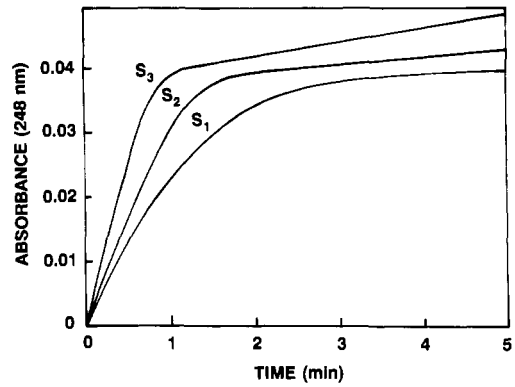


FIGURE 2: Absorption at 248 nm as a function of time when the D38N mutant of isomerase was mixed rapidly with androst-5-ene-3,17-dione and measured against a blank containing the enzyme only. Reactions were carried out at 25.0 ± 0.1 °C in 10-mm light path cuvettes in a final volume of 1.5 mL containing 50 mM Tris-HCl, pH 7.5, and 50 μL of methanol. The enzyme subunit concentration was 2.98 μM, and the substrate concentrations were as follows: S_1 = 18.0 μM, S_2 = 34.0 μM, and S_3 = 68 μM.

These findings suggested that the rapid binding of the substrate, which is not directly observable, was followed by the much slower formation of an enzyme-bound intermediate(s), which is responsible for the initial burst. The extinction coefficient of the intermediate was ~12 500 M^{-1} cm^{-1} at 248 nm, assuming a binding stoichiometry of unity. Whereas the magnitude of this estimated absorption coefficient made it unlikely that it represented binding of the substrate to the D38N isomerase mutant, since the unconjugated androst-5-ene-3,17-dione has no significant absorption at this wavelength, the presence of bound product (androst-4-ene-3,17-dione, a_m = 16 300 M^{-1} cm^{-1} at 248 nm) could not be unequivocally excluded on the basis of these findings alone. Nevertheless, product binding is unlikely to be responsible for the observed absorbance changes for the following reasons. First, when the product analogue, 19-nortestosterone, is bound to D38N, the absorption spectrum shows a peak at 260 nm and no additional bands (Kuliopulos et al., 1989), unlike the more complicated spectrum of the present species (see below). Second, the product analogue, 19-nortestosterone, binds and dissociates rapidly from D38N, well within conventional mixing times (Kuliopulos et al., 1989), unlike the present species (see below). Third, the rate of appearance of the present species is not affected by deuteration at the 6-position of the substrate (Table I), indicating that a rate-limiting reketonization is not part of the process by which this species forms (see below). Consequently, the most plausible explanation for the initial absorption burst is the formation of enzyme-bound intermediates such as dienolic species that are tightly bound to the active site of the enzyme.

Insight into the nature of these intermediates was obtained by scanning the absorption spectra between 230 and 310 nm as a function of time (Figure 3). These spectra show the early

Table I: Kinetic Deuterium Isotope Effects on the Enolization (k_{enol}/K_s) of Androst-5-ene-3,17-dione by the D38N Mutant Isomerase

substrate	solvent	k_{enol}/K_s[a] (M^{-1} s^{-1})	k_{enol}/K_s[b] (M^{-1} s^{-1})	kinetic isotope effects[c]	combined isotope effect
4,4-H_2[d]	H_2O	650	650	1.0	
4α-D	H_2O	280	244	2.66 ± 0.06	
4β-D	H_2O	428	421	1.54 ± 0.03	
4,4-H_2[d]	D_2O	401	–[e]	1.62 ± 0.08[f]	2.54 ± 0.12[f]
4,4-D_2	D_2O	158	–[e]	4.11 ± 0.08	1.12 ± 0.05–1.28 ± 0.06[g]
4,4-D_2	H_2O	202	177	3.67 ± 0.07	
6-D	H_2O	650	650	1.0 ± 0.01	

[a] The k_{enol}/K_s values were obtained at 25 °C under rapid mixing conditions. The solutions of 15.0 μM D38N mutant enzyme and 15.0 μM substrate, both containing 50 mM Tris-HCl, pH 7.5, and 3.3% MeOH, were loaded into the two reservoirs of the rapid kinetic accessory. Absorbance at 248 nm was monitored during the enolization process. The plot of reciprocal concentration with respect to time yielded apparent second-order enolization rates that are equal to k_{enol}/K_s. [b] Values are corrected for isotopic composition. The compositions of androst-5-ene-3,17-dione (in atom %) were as follows (Xue et al., 1990). 4β-D: 4β-D (97.0%), 4,4-H_2 (3.0%). 4α-D: 4α-D (88.6%), 4β-D (4.7%), 4,4-H_2 (6.7%). 4,4-D_2: 4,4-D_2 (87.8%), 4β-D (5.7%), 4α-D (4.9%), 4,4-H_2 (1.65%). 6-D: 6-D (92.0%), 6-H (8.0%). [c] Isotope effects are presented as the ratios of the corrected (when available) k_{enol}/K_s values for unlabeled substrate (and solvent) to those of the deuterium-labeled substrates (or solvent). [d] Unlabeled. [e] Could not be corrected for isotopic composition since the 4-monodeuterated substrates were not studied in D_2O. [f] Substrate (4,4-D_2) isotope effect in D_2O. [g] Solvent isotope effect with 4,4-D_2-labeled substrate, calculated with both corrected and uncorrected values of k_{enol}/K_s.

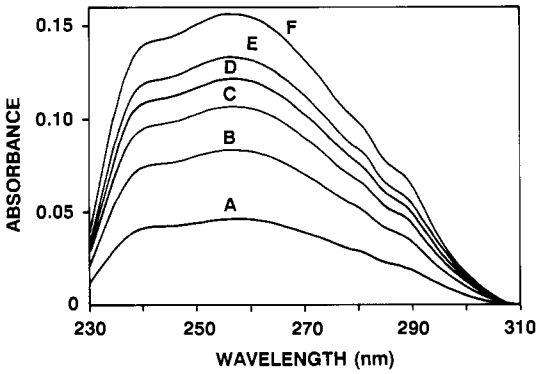


FIGURE 3: Ultraviolet spectrum as a function of time after rapid mixing of the D38N mutant isomerase with substrate. The enzyme concentration was 16.0 μM and the substrate concentration was 12.0 μM. The spectra were collected at time intervals of 20 s in a diode array spectrophotometer, with a dwell time of 0.5 s under the same conditions as described in Figure 2. The spectra shown were obtained at the following times: A = 20 s, B = 40 s, C = 60 s, D = 80 s, E = 100 s, and F = 900 s.

development of two major absorption bands at 241 nm (a_m ~ 11 800 M^{-1} cm^{-1}) and 258 nm (a_m ~ 13 000 M^{-1} cm^{-1}), and two smaller bands near 280 and 288 nm, before significant amounts of free product, with maximal absorption at 248 nm, were formed (Figure 4). The two major absorption bands correspond closely in position to those reported for the unbound dienol and dienolate in solution at 236 nm (a_m ~ 17 500 M^{-1} cm^{-1}) and 256 nm (a_m ~ 15 000 M^{-1} cm^{-1}), respectively (Pollack et al., 1986, 1990; R. M. Pollack, personal communication, 1990). While the extinction coefficients of the free species of dienols appear to be higher than those of the bound ligands, it should be noted that the bound species are a mixture of the two intermediates.

Further evidence for the formation of enzyme-bound dienolic intermediates was provided by experiments in which the rates of formation of the intermediates were measured with substrates deuterated at the 4-position (Table I). The rate of the initial absorption burst was markedly slower for [4β-D]-, [4α-D]-, and [4,4-D_2]androst-5-ene-3,17-dione compared to the unlabeled substrate. Such kinetic isotope effects would

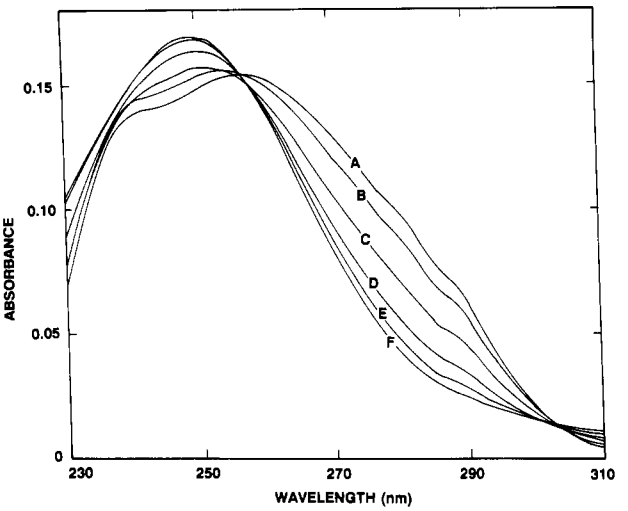


FIGURE 4: Time course of ultraviolet spectral changes associated with the conversion of the isomerase-intermediate complex to product. Conversion of intermediate to product was monitored by scanning the full spectrum from 230 to 310 nm at the indicated time intervals. The spectra displayed were obtained at the following times: A = 0.5 h, B = 1.0 h, C = 3.0 h, D = 6.0 h, E = 9.0 h, and F = 18.0 h. Solutions of substrate (23 μM) and enzyme (30 μM) were loaded into the two separated chambers of paired tandem cuvettes (each cell chamber had an optical light path of 4.38 mm). After the base-line absorbance was determined, the sample cell contents were mixed, and spectra from 230 to 310 nm were obtained at time intervals of 10 min for a 24-h time period. After 30 h the enzyme was found to retain full activity.

not be expected if the substrate were merely bound without undergoing any chemical changes.

Thus, the combined spectroscopic and kinetic isotope experiments argue for the formation of enzyme-bound dienolic intermediates. The rate of formation of these intermediates was measured under pseudo-first-order conditions, with the substrate concentrations ranging from 21 to 86 μM and the enzyme concentration at 2.49 μM. Initial velocities were obtained and the double-reciprocal plot yielded the values k_{enol} = 0.052 ± 0.005 s^{-1} and K_m(app) = K_s = 80 ± 10 μM.[1]

From the relative intensities of the peaks at 241 and 258 nm, we infer that the dienol and dienolate coexist in an equilibrium on the enzyme. If we assume that the extinction coefficients of the dienol and dienolate species are similar (Pollack et al., 1987, 1990), the dienolate appears to be favored thermodynamically by a factor of 2–4, and this may reflect an apparent ΔpK_a between Tyr-14 and the enzyme-bound dienol of 0.3–0.6.

Substrate Kinetic Deuterium Isotope Effects on Rate of Enolization. The kinetic isotope effects on k_{enol}/K_s values are expressed as ratios for the unlabeled to the deuterated substrates. For [4α-D]-, [4β-D]-, and [4,4-D_2]androst-5-ene-3,17-dione, these ratios are 2.66 ± 0.06, 1.54 ± 0.03, and 3.67 ± 0.07, respectively (Table I). The isotope effect of 2.66 for the 4α-deuterated substrate is much larger than the secondary kinetic isotope effect on k_{cat}/K_m observed with the wild-type enzyme (1.12 ± 0.02; Xue et al., 1990) and is too large to be a secondary kinetic or equilibrium isotope effect. Hence the ratio of 2.66 reflects a primary kinetic isotope effect, indicating that, unlike the wild-type enzyme, which stereoselectively removes the 4β-proton (Malhotra & Ringold 1965; Xue et al., 1990), the D38N mutant enzyme is much less selective and carries out deprotonation of the 4α-position as well. The loss of stereoselectivity is also reflected in the kinetic isotope effect for the 4β-deuterated substrate of 1.54, which is significantly lower than that found with the wild-type enzyme (2.99 ± 0.06; Xue et al., 1990) and is also too large to be a secondary kinetic

or equilibrium isotope effect. The larger kinetic isotope effect of 3.67 for the 4,4-dideuterated substrate reflects both primary and mutual secondary isotope effects. These large kinetic isotope effects indicate that the removal of a proton from C-4 is a major rate-limiting step for enolization of the substrate by the D38N mutant and for the overall reaction of the wild-type enzyme (in which enolization is rate-limiting). However, the removal of the 4β-proton (or 4β-deuteron) is no longer favored in the mutant. This lack of stereospecificity is not surprising since the Asp-38 of the wild-type enzyme, which is located directly above the 4β-proton, has been mutated to Asn, which cannot act as a base, and alternative or mobile bases might interact with either the 4α- or 4β-proton. The fact that the 4α-deuterated substrate has a higher kinetic isotope effect than the 4β-deuterated substrate suggests that the 4α-proton is preferentially removed in the mutant, possibly because it is sterically more accessible to an alternative base than the 4β-proton,[2] although the 4β-proton is more acidic (Corey & Sneen, 1956; Pollack et al., 1989). As with the wild-type enzyme, the 6-D-labeled substrate does not show a kinetic isotope effect, thereby establishing that the initial burst in ultraviolet absorption is substrate enolization and also indicating that deuteration at C-6 does not produce a remote secondary isotope effect on the enolization reaction in which a proton at C-4 is removed.

Solvent and Combined Kinetic Isotope Effects on Rate of Enolization. A general acid and a general base are necessary to obtain optimal acceleration of the enolization process. However, they can act either stepwise or in concert. Measurements of the solvent isotope effect and the combined substrate and solvent deuterium isotope effects on the enolization rate provide a powerful method to test the concertedness of the general acid and the general base in the enolization process (O'Leary, 1989; Xue et al., 1990). The solvent deuterium isotope effect (ratio of k_{enol}/K_s in H_2O to that in D_2O) was found to be 1.62 ± 0.08 when unlabeled substrate was used (Table I), but it fell to 1.12–1.28 for the 4,4-dideuterated substrate. Similarly, the substrate isotope effect for the 4,4-dideuterated substrate was reduced from 3.67 ± 0.07 (in H_2O) to 2.54 ± 0.12 (in D_2O) (Table I). We conclude that the decreases of the combined isotope effects in both cases indicate that the substrate deuterium isotope effect on the removal of the 4-proton (or deuteron) and the solvent isotope effect (on the protonation of the 3-carbonyl group) occurred in separate partially rate-limiting steps during the enolization process on the D38N mutant. This finding is in marked contrast to the concerted enolization process catalyzed by the wild-type enzyme (Xue et al., 1990).

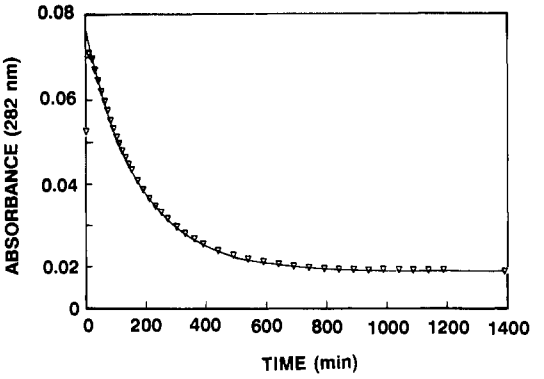
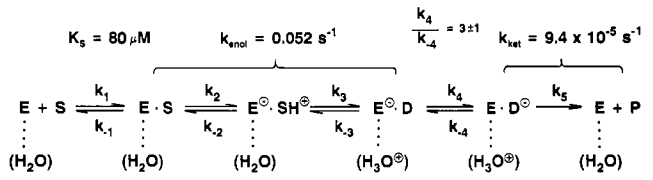


FIGURE 5: Time-dependent decrease in absorbance of the isomerase-intermediate complex at 282 nm. The measurements were retrieved from the reaction described in Figure 4. A theoretical first-order fit was drawn as the solid line; a k value of 9.4×10^{-5} s^{-1} gave the best fit for the data except for the first two points. The fit was constructed with the equation $A_t = A_0e^{-kt} + C$, where $A_0 = 0.0580$, $C = 0.0189$, $k = 5.65 \times 10^{-3}$ min^{-1}, and $t_{1/2} = 123$ min.

The loss of concerted general acid–base catalysis in the D38N mutant is likely to result from the absence of the base, Asp-38. Hence, the enolization catalyzed by the D38N mutant probably occurs by prior protonation of the 3-keto group of the substrate by Tyr-14, followed by deprotonation of the oxycarbonium species by an alternative base. Evidence that Tyr-14 can function in the absence of Asp-38 was provided by the observation that, like the wild-type enzyme, the D38N mutant induces a red shift in the UV spectrum of the bound steroid 19-nortestosterone, while the Y14F mutation does not (Kuliopulos et al., 1989), and by the additivity of the effects on k_{cat} of the Y14F and D38N mutations (Kuliopulos et al., 1990). The alternative base, possibly a trapped water molecule, with a pK_A value of −1.7, is a $10^{6.4}$-fold weaker base than Asp-38, with a pK_A of 4.7 (Pollack et al., 1986), resulting in a $10^{5.7}$-fold decrease in the rate of enolization. On the wild-type enzyme, in the subsequent reketonization of the dienolic intermediate, the protonated form of Asp-38 is the proton donor and the anion of Tyr-14 is the proton acceptor in a highly efficient process, which may be concerted (Kuliopulos et al., 1989; Xue et al., 1990). Because Asp-38 is not available in the D38N mutant, either the intermediate(s) is(are) released from the enzyme or an alternative proton donor (possibly trapped H_3O^+ or H_2O) participates, and proton donation is highly inefficient (see below). Under the circumstances, it is not surprising that an alternative, inefficient proton donor cannot operate in concert with deprotonation of the dienolic intermediate by Tyrosinate-14, resulting in the spectroscopic detection of both dienolate and dienol intermediates. Thus the D38N mutation has prevented both the concerted formation and the concerted reketonization of the dienol intermediate, permitting the accumulation (or equilibration) on the enzyme of the dienolate as well.

[2] An apparent inconsistency among the values of the substrate kinetic isotope effects measured in H_2O (Table I), pointed out by a reviewer, can be explained by the existence of inflated secondary kinetic isotope effects resulting from coupled proton motion and proton tunneling (Kurz & Frieden, 1980; Cook et al., 1980; Huskey & Schowen, 1983; Klinman, 1991; Xue et al., 1990) as follows: To permit analysis of the limited data, we make two simplifying assumptions, namely, that the primary kinetic deuterium isotope effects are equal at the 4α- and 4β-positions and that the secondary kinetic deuterium isotope effects are also equal at the 4α- and 4β-positions. From Table I we may write the following equations: rate (4β-D) = 421 = $f_\beta(650)/p + (1 - f_\beta)(650)/s'$; rate (4$\alpha$-D) = 244 = $(1 - f_\beta)(650)/p + f_\beta(650)/s'$; and rate (4,4-$D_2$) = 177 = $f_\beta(650)/ps + (1 - f_\beta)(650)/ps = 650/ps$, where the value 650 is the rate with (4,4-H_2), f_β is the fraction of β-H removed, $1 - f_\beta$ is the fraction of α-H removed, p represents the primary kinetic isotope effect, s' is the inflated secondary isotope effect, and s is the noninflated secondary isotope effect, i.e., when deuterium is in the primary position. Variation of f_β from 0 to 1.00 leads to unrealistic values of s (exceeding the equilibrium isotope effect of 1.13) and of s' ($\geq$1.41) when $f_\beta < 0.17$. Unrealistic values of s (<1.0) are also obtained when $f_\beta > 0.21$. Hence, with the above assumptions, it may reasonably be concluded that $0.17 \leq f_\beta \leq 0.21$. Assuming f_β to be in this range leads to reasonable and precedented values for primary, inflated secondary, and noninflated secondary kinetic isotope effects [Klinman (1991) and references therein]. For example, with $f_\beta = 0.20$, $p = 3.50$, $s' = 1.35$, and $s = 1.05$. With the wild-type enzyme, for which f_β is 1.0, the corresponding kinetic isotope effects on k_{cat}/K_m are 2.99, 1.11, and 1.06, respectively (Xue et al., 1990). Hence the present analysis, albeit simplified, suggests a significantly greater inflation of the secondary kinetic isotope effect with the D38N mutant (1.35) than with the wild-type enzyme (1.11), presumably due to a relatively greater tunneling contribution. This is not surprising since the kinetic barrier to enolization has been raised by 7.4 kcal/mol in the D38N mutant. Furthermore, this analysis suggests that the kinetic barrier to enolization has not been widened and may have been narrowed in the mutant.

Scheme I



Product Formation. After the initial burst of absorption arising from the formation of enzyme-bound intermediate(s), steady-state product formation occurred (Figure 2). This slow process was followed most conveniently and accurately by monitoring the rate of disappearance of the enzyme-bound intermediate(s) at 282 nm (Figures 4 and 5), rather than by the appearance of the product at 248 nm.[3] The data of Figure 5 show an excellent fit to a first-order decay of the enzyme-bound intermediate(s) with a half-life of 123 min, equivalent to a first-order rate constant of 9.4×10^{-5} s^{-1}. While faster than the spontaneous rate of the overall conversion of substrate to product (1.9×10^{-5} s^{-1}; Kuliopulos et al., 1990), this specific rate is much slower than that of the spontaneous reketonization of the dienolate in the absence of enzyme (0.122 s^{-1}; Pollack et al., 1987). Hence it reflects either the slow reketonization of the enzyme-bound intermediate(s) or the slow dissociation of the intermediate(s) followed by rapid reketonization in solution. In either case, it is clear that, on the D38N mutant enzyme, reketonization of the intermediate is slower by a factor of $\geq(1.3 \times 10^3)$-fold compared to free solution and by a factor of $\geq(5.6 \times 10^8)$-fold compared to the wild-type enzyme. These large factors reflect the relative inaccessibility of the active site to proton donors from the bulk solvent, as previously suggested by the unchanged pH–rate profile of the D38N mutant from that of the wild-type enzyme (Kuliopulos et al., 1989).

The slow rate of product formation by D38N (Figure 5), when corrected for the partitioning of the free dienolate (Pollack et al., 1987), sets an upper limit of $\leq 2.3 \times 10^{-3}$ s^{-1} for the rate constant for dissociation of the enzyme-intermediate complex. This value is at least 10^7-fold smaller than k_{-1}, the rate constant for dissociation of the substrate from the wild-type enzyme (3.4×10^4 s^{-1}; Xue et al., 1990). Because of their comparable K_s values, a similar k_1 would be expected for the binding of substrate to the D38N mutant. Further, assuming similar diffusion-controlled values for the rate constants for the binding of both the substrate and the intermediate (Xue et al., 1990; Eames et al., 1990), the intermediate is bound to D38N at least 10^7-fold more tightly than the substrate with a dissociation constant $\leq 10^{-11}$ M. These considerations indicate that the active site of isomerase, even when damaged severely by the D38N mutation, is well adapted to stabilize selectively the reaction intermediate(s) in preference to the substrate or product of the reaction.

[3] Attempts to measure steady-state product formation directly by absorbance at 248 nm were unsuccessful because the rate of this reaction was comparable to that of the uncatalyzed reaction in solution. Furthermore, preparations of D38N were contaminated by traces (0.2–0.5 parts per million) of the wild-type enzyme. Evidence for a contribution to the steady-state rate of product formation by contaminating wild-type enzyme was provided by the variability of this rate in different preparations and by the detection of a large primary kinetic isotope effect on this rate with the 4β-deuterated substrate and a small secondary isotope effect with the 4α-deuterated substrate, similar to those found with the wild-type enzyme (Xue et al., 1990). The presence of traces of wild-type enzyme in our preparations of D38N is most plausibly attributed to errors in translation or to spontaneous hydrolysis of the amide of Asn-38 to restore the wild-type enzyme. Since the Y14F + D38N double mutant, prepared by the same procedures, showed no residual catalytic activity, laboratory contamination of D38N by wild-type enzyme is a less likely possibility (Kuliopulos et al., 1990). The contamination by wild-type enzyme was estimated by measuring the initial rate of product formation at 248 nm and correcting this rate by subtracting (a) the uncatalyzed reaction rate and (b) the true rate of product formation obtained from the rate of disappearance of intermediates (Figure 5).

Conclusions

The above observations on the D38N mutant together with previous conclusions derived from studies with a variety of spectroscopic techniques applied to the wild-type isomerase lead to the proposal of Scheme I.

Because D38N retains Tyr-14, the acid catalyst of the enolization and the base catalyst for the reketonization, we require an asymmetric and more complicated kinetic scheme for D38N than for wild-type enzyme (eqs 1 and 2), with possibly water acting as a surrogate for Asp-38 as shown in Scheme I. In this scheme, D and D$^-$ represent the dienol and dienolate intermediates, respectively, and k_5 is limited by the dissociation of the enzyme-bound intermediates or by product formation on the enzyme, whichever is slower.

In accord with Scheme I, our experiments establish that the neutral dienol and the dienolate carbanion are formed and discharged in stepwise reactions and that these intermediates coexist on the enzyme as shown schematically in Figure 1.

By the use of spectroscopic methods we have determined the dissociation constant (K_s) of the enzyme·substrate complex and the apparent first-order rate constants for the enolization (k_{enol}) and for product formation (k_{ket}). We have also estimated the equilibrium constant for step 4, the dienol–dienolate equilibrium on the enzyme.

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Registry No. Asp, 56-84-8; D_2, 7782-39-0; androst-5-ene-3,17-dione, 571-36-8; Δ^5-3-ketosteroid isomerase, 9031-36-1; 3-hydroxyandrosta-3,5-dien-17-one, 1229-13-6; 3-hydroxyandrosta-3,5-dien-17-one anion, 121375-08-4.

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Tryptase from Rat Skin: Purification and Properties[†,‡]

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ABSTRACT: Tryptase was purified 13 000-fold to apparent homogeneity from rat skin. The two-step procedure involved ammonium sulfate fractionation of the initial extract followed by combined sequential affinity chromatography on agarose–glycyl-glycyl-*p*-aminobenzamidine and concanavalin A–agarose. The purified enzyme had a specific activity toward *N*-benzoylarginine ethyl ester (BzArgOEt) of 170 μmol/min mg^{-1} and was obtained in a yield of 28% as determined by the specific substrate, H-D-Ile-Pro-Arg-*p*-nitroanilide. Rat skin tryptase was thermal labile, losing 50% of its activity when preincubated for 30 min at 30 °C. The presence of NaCl (1 M) improved thermal stability and was necessary for long-term storage. Heparin did not stabilize the enzyme against thermal denaturation, and heparin–agarose failed to bind the enzyme. Rat skin tryptase was inhibited by diisopropylphosphofluoridate, antipain, leupeptin, and aprotinin but not by α_1-antitrypsin, ovomucoid, or soybean or lima bean trypsin inhibitors. Substrate specificity studies using a series of tri- and tetrapeptidyl-*p*-nitroanilide and peptidyl-7-amino-4-methylcoumarin substrates demonstrated the existence of an extended substrate binding site. Rat skin tryptase hydrolyzed [Arg^8]vasopressin, neurotensin, and the oxidized B-chain of insulin at the -Arg^8-Gly^9-NH$_2$, -Arg^8-Arg^9-, and -Arg^{22}-Gly^{23}-bonds, respectively. No general proteinase activity was observed toward casein, hemoglobin, or azocoll. Rat skin tryptase had a M_r of 145 000 by gel filtration. The subunit M_r was either 34 000 or 30 000 depending on the electrophoretic technique used. Treatment of the enzyme with peptide N-glycosidase F (N-glycanase) decreased the subunit M_r by 4000. The enzyme exhibited multiple isoelectric forms (p*I*'s of 4.5–4.9). Rat skin tryptase was found to be related statistically to other tryptases on the basis of amino acid composition. The N-terminal amino acid sequence was Ile^1-Val^2-Gly^3-Gly^4-Gln^5-Glu^6-Ala^7-Ser^8-Gly^9-Asn^{10}-Lys^{11}-Trp^{12}-Pro^{13}-Trp^{14}-Gln^{15}-Val^{16}-Ser^{17}-Leu^{18}-Arg^{19}-Val^{20}---^{21}-Asp^{22}Thr^{23}-Tyr^{24}-Trp^{25}-, with a putative glycosylation site at residue 21. This sequence was 72–80% homologous with the N-terminus of other tryptases but only 40% homologous with that of bovine trypsin.

Tryptase is a protease that is stored within the secretory granules of mast cells and released in active form upon mast cell activation (Schwartz et al., 1981a). Tryptase is presumed to play a role in the immunoglobulin E dependent and drug-induced immediate hypersensitivity reactions mediated by mast cells and has recently been used as a specific marker of these events in humans (Schwartz et al., 1987). Although the precise in vivo function of tryptase is unknown, in vitro studies have shown that certain tryptase preparations can activate complement factor C3 to C3a anaphylatoxin (Schwartz et al., 1983), convert prothrombin to thrombin (Kido et al., 1985a), destroy both fibrinogen (Schwartz et al., 1985) and high molecular weight kininogen (Maier et al., 1983), activate latent rheumatoid synovial collagenase (Gruber et al., 1988), and hydrolyze vasoactive intestinal peptide, peptide histidine–methionine (PHM), calcitonin gene-regulated peptide (Tam & Caughey, 1990), and ACTH (1–39) (Cromlish et al., 1987).

To date, tryptase has been purified from various human tissues [pulmonary mast cells (Schwartz et al., 1981b), lung (Smith et al., 1984; Harvima et al., 1988), pituitary (Cromlish et al., 1987), and skin (Harvima et al., 1988)] as well as from dog mastocytoma cells (Caughey et al., 1987) and rat peritoneal mast cells (Kido et al., 1985b). These different tryptase preparations have many properties in common. However,

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