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Studies of the Mechanism of the Δ^5-3-Ketosteroid Isomerase Reaction by Substrate, Solvent, and Combined Kinetic Deuterium Isotope Effects on Wild-Type and Mutant Enzymes†

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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 using 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]. On deuteration of the 4β-position (97.0%) of the substrate, $k_{cat}(H)/k_{cat}(4\beta\text{-D})$ is 6.1 in H_2O and 6.3 in D_2O. The solvent isotope effect, $k_{cat}(H_2O)/k_{cat}(D_2O)$, is 1.6 for both the 4$\beta$-H and 4$\beta$-D substrates. Mutation of Tyr-55 to Phe lowers k_{cat} 4.3-fold; $k_{cat}(H)/k_{cat}(4\beta\text{-D})$ is 5.3 in H_2O and 5.9 in D_2O, and $k_{cat}(H_2O)/k_{cat}(D_2O)$ with the 4β-H and 4β-D substrates is 1.5 and 1.7, respectively, indicating concerted general acid–base catalysis in either the enolization or the ketonization step of both the wild-type and the Tyr-55 → Phe (Y55F) mutant enzymes. An additional slow step occurs with the Y55F mutant. Smaller isotope effects on K_m are used to estimate individual rate constants in the kinetic schemes of both enzymes. On deuteration of the 4α-position (88.6%) of the substrate, the secondary isotope effect on k_{cat}/K_m corrected for composition is 1.11 ± 0.02 with the wild-type enzyme and 1.12 ± 0.02 with the Y55F mutant. These effects decrease to 1.06 ± 0.01 and 1.07 ± 0.01, respectively, when the 4β-position is also deuterated, thereby establishing these to be kinetic (rather than equilibrium) secondary isotope effects and to involve a proton-tunneling contribution. Deuteration of the 6-position of the substrate (92.0%) produces no kinetic isotope effects on k_{cat}/K_m with either the wild-type (1.00 ± 0.01) or the Y55F mutant (1.01 ± 0.01) enzyme. Since a change in hybridization from sp^3 to sp^2 occurs at C-4 only during enolization of the substrate and a change in hybridization at C-6 from sp^2 to sp^3 occurs only during reketonization of the dienol intermediate, enolization of the substrate constitutes the concerted rate-limiting step. Concerted enolization is consistent with the right angle or antarafacial orientations of Tyr-14 and Asp-38 with respect to the enzyme-bound substrate and with the additive effects on k_{cat} of mutation of these catalytic residues [Kuliopulos, A., Talalay, P., & Mildvan, A. S. (1990) *Biophys. J. 57*, 39a].

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, accelerating the rate of this process by a factor of $10^{9.5}$ (Kuliopulos et al., 1987) (Figure 1). The enzyme-catalyzed reaction is a stereospecific, intramolecular, cis, diaxial conservative transfer of the 4β-proton to the 6β-position at a rate that approaches the diffusion limit. As previously pointed out (Kuliopulos et al., 1989), the overall reaction is unlikely to be a concerted process on theoretical grounds since concerted, suprafacial, 1,3-sigmatropic shifts are forbidden by orbital symmetry rules (Woodward & Hoffmann, 1970; Alder et al., 1971). Moreover, spectroscopic, isotopic, and kinetic studies have provided evidence for the existence of an enolic intermediate (Wang et al., 1963; Malhotra & Ringold, 1965; Bantia & Pollack, 1986; Eames et al., 1990). The enolization of the substrate would be facilitated by a proton donor that protonates the carbonyl group of the steroid and a proton acceptor that removes the 4β-proton and transfers it to the 6β-position.

NMR docking of the spin-labeled substrate analogue spiro[doxyl-2,3′-5α-androstane]-17′β-ol into the 2.5-Å resolution X-ray structure of isomerase revealed that either Tyr-55 or Tyr-14 might function as the proton donor and that Asp-38 was appropriately positioned to function as the proton acceptor (Kuliopulos et al., 1987b). Cloning and expression of the gene

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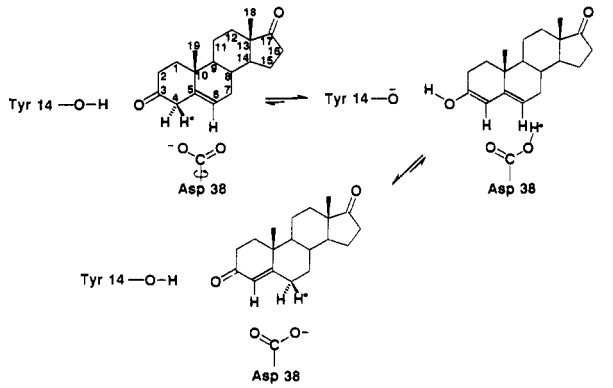


FIGURE 1: Reaction catalyzed by Δ^5-3-ketosteroid isomerase. The enzyme converts androst-5-ene-3,17-dione to androst-4-ene-3,17-dione by the intramolecular and stereospecific transfer of the 4β-proton to the 6β-position, via an enolic intermediate.

for isomerase in *Escherichia coli* (Kuliopulos et al., 1987a) and site-directed mutagenesis of these residues (Kuliopulos et al., 1989) provided direct support for the role of Tyr-14 as the proton donor and of Asp-38 as the proton acceptor in the isomerase reaction. Thus, while mutation of Tyr-55 to Phe (Y55F) resulted in only a 4.3-fold decrease in k_{cat}, mutation of Tyr-14 to Phe (Y14F) resulted in a $10^{4.7}$-fold decrease, and mutation of Asp-38 to Asn (D38N) resulted in a $10^{5.7}$-fold decrease of this parameter (Kuliopulos et al., 1989). Moreover, these effects on k_{cat} were additive since the Y14F and D38N double mutant showed no catalytic activity ($\geq 10^{9.8}$-fold decrease in k_{cat}) yet retained the ability to bind the substrate tightly (Kuliopulos et al., 1990a).

Computer modeling studies of the substrate docked into the 2.5-Å X-ray structure of the enzyme disclosed that Asp-38 and Tyr-14 could not be positioned to interact suprafacially with the bound substrate (Kuliopulos et al., 1989). A detailed reexamination of the structure of the enzyme–substrate complex by computer graphics shows that Asp-38 and Tyr-14 could approach the substrate most favorably, with minimal distortion of the enzyme, at right angles to each other (Figure 2A), less favorably in an antarafacial orientation (Figure 2B), and least favorably in a suprafacial orientation (Figure 2C), because of steric interactions with neighboring residues. The stereoelectronically favorable right angle (Figure 2A) and antarafacial (Figure 2B) orientations of the catalytic groups are consistent with, but do not establish, the concerted enolization of ketosteroid substrates in the first half of the isomerase reaction (Hand & Jencks, 1975; Rebek, 1988). A concerted enolization would also help to explain how a relatively weak base such as Asp-38 could deprotonate a carbon atom and a relatively weak acid such as Tyr-14 could protonate a carbonyl group, by functioning jointly in the same chemical step. Cleland and co-workers (Hermes et al., 1982; O'Leary, 1989) and, independently, Belasco et al. (1983, 1986) have developed the combined kinetic isotope effect method for determining whether two chemical events occur in the same or in separate steps. The present paper examines whether the enolization catalyzed by ketosteroid isomerase is concerted and identifies the rate-limiting step by the use of substrate, solvent, and combined substrate and solvent deuterium isotope effects on the isomerase reaction rates.

Since the transferred proton is conserved in the overall isomerase reaction (Talalay & Wang, 1955; Wang et al., 1963; Malhotra & Ringold, 1965), a primary kinetic isotope effect could occur with the 4β-deuterated substrate in the enolization step, in the reketonization step, or in both steps of the overall reaction. To resolve this ambiguity, secondary kinetic isotope

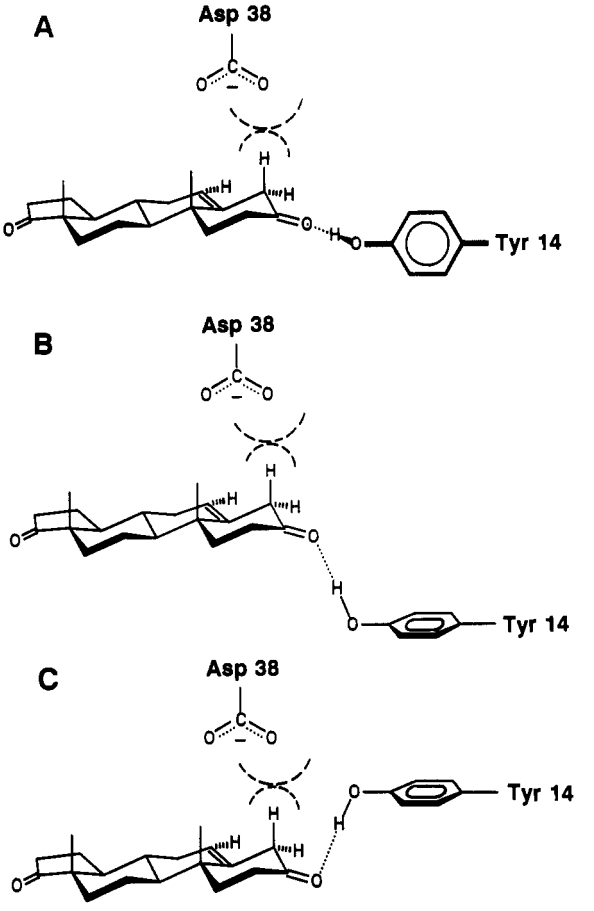


FIGURE 2: Three possible geometric arrangements of the catalytic residues, Asp-38 and Tyr-14, of isomerase while in contact with the substrate, based on solution NMR and X-ray crystallographic data (Kuliopulos et al., 1989). (A) Right angle orientation with Asp-38 positioned directly above the C-4 and C-6 positions in van der Waals contact with the 4β-proton of the substrate and Tyr-14 on the side of the 3-carbonyl oxygen at about a right angle toward readers and at a hydrogen-bonding distance. This orientation requires minimal distortion of the enzyme. (B) Antarafacial orientation with Asp-38 above the C-4 and C-6 positions and Tyr-14 below the 3-carbonyl oxygen atom at a hydrogen-bonding distance. A change of the backbone is required to obtain this conformation. (C) Suprafacial orientation with Asp-38 above the C-4 and C-6 positions and Tyr-14 above the 3-carbonyl oxygen atom. To obtain this conformation, Tyr-14 must overlap with other residues.

effects were measured with the 4α-deuterated and 6-deuterated forms of the substrate androst-5-ene-3,17-dione. A change in hybridization from sp^2 to sp^3 occurs at C-4 only in the enolization step, while the opposite change in hybridization occurs at C-6 only during the reketonization step. Hence, a normal secondary kinetic isotope effect would be expected to occur when the 4α-position is deuterated if enolization were the rate-limiting step, an inverse secondary kinetic isotope effect would be expected when the 6-position is deuterated if reketonization were rate limiting, or both would be observed if both enolization and reketonization were partially rate limiting. Such uses of secondary kinetic isotope effects have been reviewed by Hogg (1978) and by Cook et al. (1981). Preliminary reports of this work have been published (Xue et al., 1989, 1990).

Experimental Procedures

Materials and Equipment

Materials. Solutions of Tris base were adjusted to the desired pH at 25 °C with HCl. For experiments in D_2O, Tris buffers were lyophilized, and the residues were dissolved in

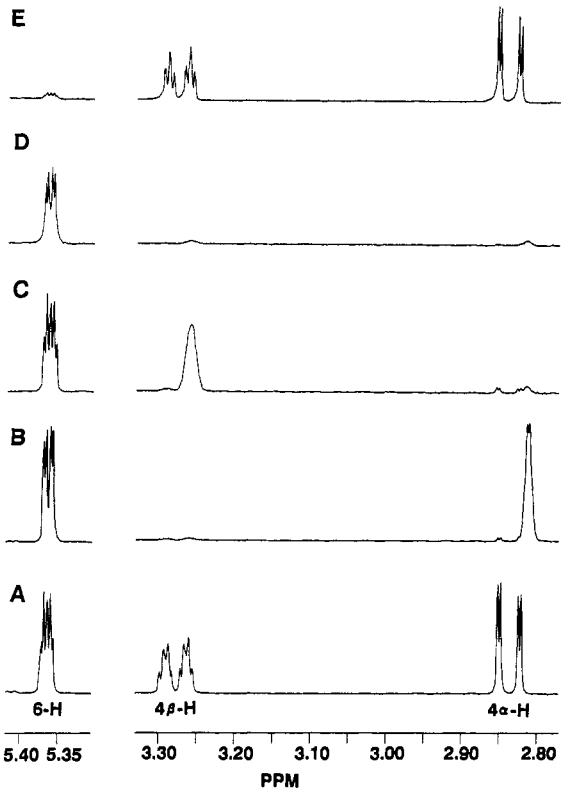


FIGURE 3: Portions of the ^1H NMR spectra of unlabeled and deuterated androst-5-ene-3,17-dione. Spectra were obtained at 600 MHz on a Bruker AM600 NMR spectrometer at 25 °C with a 90° pulse width, quadrature detection, 16-bit A/D conversion, 64 transients with 32K data points, a spectral width of 6024 Hz, an acquisition time of 2.72 s, and a relaxation delay of 10.0 s. Increasing the relaxation delay to 30 s did not alter the spectra. Substrate concentrations were 2–3 mM in 0.5 mL of $CDCl_3$. No line broadening was applied. Chemical shifts refer to internal $CHCl_3$ (δ = 7.24 ppm). (A) Unlabeled substrate (androst-5-ene-3,17-dione). (B) 4β-D substrate. Note that deuteration at the 4β-position removes major coupling to the 4α-H and minor coupling to the 6-H and induces an 0.02 ppm upfield shift of the 4α-H. (C) 4α-D substrate. Deuteration at the 4α-position removes major coupling to the 4β-H and induces an 0.02 ppm upfield shift of the 4β-H. (D) 4,4-D_2 substrate. (E) 6-D substrate. Deuteration at C-6 removes minor coupling to the 4β-H.

D_2O and then lyophilized a second time before being dissolved in D_2O. D_2O (99.9 atom % D), CH_3OD (99.5 atom % D), and $LiAlD_4$ (98 atom % D) were obtained from Aldrich.

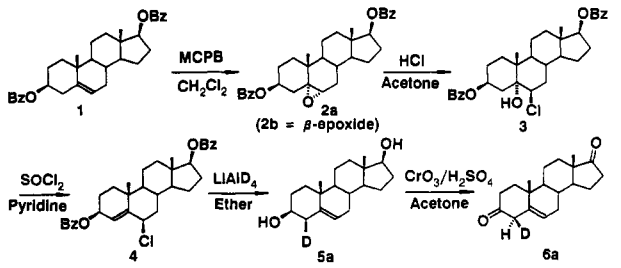
NMR Spectra. Proton NMR spectra of the steroids were obtained in $CDCl_3$ with reference to traces of $CHCl_3$ (δ = 7.24 ppm), at 250 MHz on a Bruker AM250 NMR spectrometer and at 600 MHz on a Bruker AM600 NMR spectrometer. All deuterium labeling (atom percent D) analyses were obtained at 600 MHz (Figure 3).

Enzymes. Recombinant wild-type isomerase and the Y55F mutant were prepared as described (Kuliopulos et al., 1987a, 1989) and stored as crystalline suspensions 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 proteins were determined by measuring the absorbance at 280 nm, assuming values of 0.336 (Benson et al., 1975) and 0.225 (Kuliopulos et al., 1989) for the absorbances of solutions containing 1.00 mg/mL wild-type and Y55F mutant enzymes, respectively.

Synthetic Procedures

Preparation of [4β-D]Androst-5-ene-3,17-dione (**6a**). This substrate was prepared according to modifications of the procedure of Malhotra and Ringold (1965) as shown in Scheme I, and described below.

Scheme I



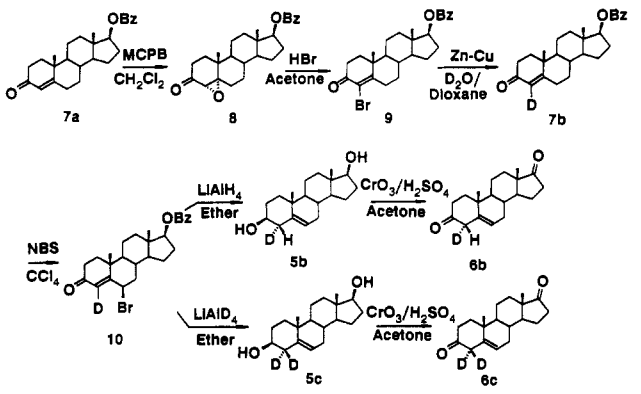
(*A*) *5α,6α-Oxidoandrostane-3β,17β-diol Dibenzoate* (**2**). The crude epoxide was a mixture of 68% 5α,6α- and 32% 5β,6β-epoxides (**2a** and **2b**, respectively), as determined by 250-MHz NMR spectra and HPLC. Successful synthesis of the stereochemically desired **6a** depends critically on obtaining the pure 5α,6α-epoxide (**2a**). Three careful fractional crystallizations with the use of slow cooling and seeding provided the desired pure 5α,6α-epoxide (**2a**) in adequate yield and purity (less than 1% 5β,6β-epoxide as determined by integration of ^1H NMR signals). ^1H NMR ($CDCl_3$): δ (α-epoxide) 7.4–8.1 (m, 10 H, aromatic protons), 4.82 (t, 1 H, J = 8.4, 17α-H), 5.22 (tt, 1 H, $J_{3\alpha,2(4)\beta}$ = 11.3, $J_{3\alpha,2(4)\alpha}$ = 5.4, 3α-H), 2.97 (d, 1 H, J = 4.4, 6β-H), 0.91 (s, 3 H, 18-Me), 1.16 (s, 3 H, 19-Me); δ (β-epoxide) 7.4–8.1 (m, 10 H, aromatic protons), 4.84 (t, 1 H, J = 8.4, 17α-H), 5.05 (tt, 1 H, $J_{3\alpha,2(4)\beta}$ = 11.3, $J_{3\alpha,2(4)\alpha}$ = 5.4, 3α-H), 3.17 (d, 1 H, J = 1.0, 6α-H), 0.96 (s, 3 H, 18-Me), 1.11 (s, 3 H, 19-Me).

(*B*) *[4β-D]Androst-5-ene-3,17-dione* (**6a**). Pure **2a** was carried through the reaction sequence to **5a** according to Malhotra and Ringold (1965). The final Jones oxidation of **5a** was modified and strictly controlled. Compound **5a** (100 mg) was dissolved in 25 mL of acetone, and the solution was cooled in an ice–water bath. To this stirred solution was added dropwise 0.50 mL of a freshly prepared saturated solution of Jones reagent (obtained by dissolving 2.0 g of CrO_3 in 7.5 mL of 6 M H_2SO_4). The oxidation was allowed to proceed for exactly 180 s from the beginning of the addition of the Jones reagent. The reaction mixture was then rapidly added to a mixture of 20% NaCl in ice–water with stirring. The precipitate was collected by filtration, and the product was recrystallized from acetone–water, the temperature being maintained below 50 °C.

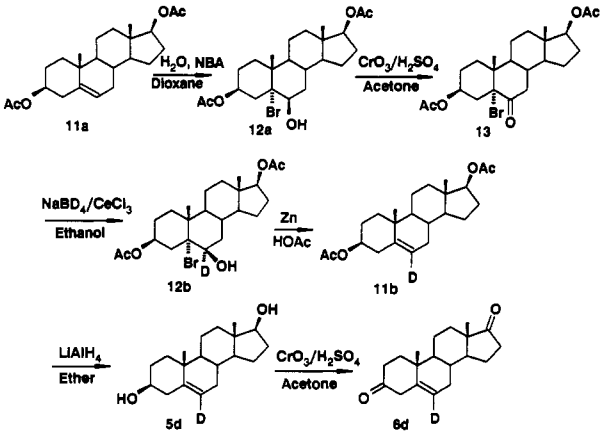
(*C*) *Criteria for Purity of [4β-D]Androst-5-ene-3,17-dione* (**6a**). UV spectra of solutions of **6a** and the product obtained by complete enzymatic isomerization (under enzyme assay conditions) indicated the initial presence of less than 1% of androst-4-ene-3,17-dione. ^1H NMR analysis of **6a** confirmed this finding and indicated that the integrated 4β-proton signal (3.28 ppm, $J_{4\alpha,4\beta}$ = 16.6) of **6a** was 3.0 atom %. This trace of 4β-proton signal was due to the presence of unlabeled androst-5-ene-3,17-dione (characteristic geminal coupling pattern at 3.28 ppm) (Figure 3B). ^1H NMR ($CDCl_3$): δ (unlabeled androst-5-ene-3,17-dione) 3.28 (dq, 1 H, $J_{4\alpha,4\beta}$ = 16.6, $J_{4\beta,x}$ = 3.3, 4β-H), 2.84 (dd, 1 H, $J_{4\alpha,4\beta}$ = 16.6, $J_{4\alpha,2\alpha}$ = 2.2, 4α-H), 5.36 (dt, 1 H, $J_{6,7\beta}$ = 5.3, $J_{6,7\alpha}$ = 2.3, 6-H), 1.20 (s, 3 H, 19-Me), 0.90 (s, 3 H, 18-Me); δ ([4β-D]androst-5-ene-3,17-dione) 3.28 (dq, 0.03 H, $J_{4\alpha,4\beta}$ = 16.6, $J_{4\beta,x}$ = 3.3, 4β-H), 2.84 (dd, 0.03 H, $J_{4\alpha,4\beta}$ = 16.6, $J_{4\alpha,2\alpha}$ = 2.2, 4α-H), 2.81 (broad band, 0.97 H, $\Delta\nu_{1/2}$ = 6.4, 4α-H), 5.36 (dd, 1 H, $J_{6,7\beta}$ = 5.3, $J_{6,7\alpha}$ = 2.3, 6-H), 1.20 (s, 3 H, 19-Me), 0.90 (s, 3 H, 18-Me).

Preparation of [4α-D]Androst-5-ene-3,17-dione (**6b**). This compound was prepared according to Viger et al. (1987) (see

Scheme II



Scheme III



Scheme II). Treatment of 4-bromotestosterone benzoate (**9**) with a Zn–Cu catalyst in the presence of D_2O gave the desired [4-D]testosterone benzoate (**7b**). ^1H NMR analysis indicated that 93.3% of the C-4 position was deuterium labeled, and this isotopic composition remained the same in the final product (**6b**). [4α-D]Androst-5-ene-3β,17β-diol (**5b**) was obtained by reduction of [4-D]-6β-bromotestosterone benzoate (**10**) with $LiAlH_4$. We found that the syn relationship of entering and leaving groups was highly selective but not absolute. High-resolution ^1H NMR (600 MHz) indicated a mixture of 95% of syn (**5b**) and 5% of anti (**5a**; Scheme I) elimination product. The product was then oxidized with the Jones reagent as described for **5a**. The final product, androst-5-ene-3,17-dione, contained three components: **6b** (88.6%), **6a** (4.7%), and unlabeled steroid (6.7%). ^1H NMR ($CDCl_3$) δ 3.26 (broad band, 0.886 H, $\Delta\nu_{1/2}$ = 10.3, 4β-H), 2.81 (broad band, 0.047 H, $\Delta\nu_{1/2}$ = 6.4, 4α-H), 3.28 (dq, 0.067 H, $J_{4\alpha,4\beta}$ = 16.6, $J_{4\beta,x}$ = 3.3, 4β-H), 2.84 (dd, 0.067 H, $J_{4\alpha,4\beta}$ = 16.6, $J_{4\alpha,2\alpha}$ = 2.2, 4α-H), 5.36 (dt, 1 H, $J_{6,7\beta}$ = 5.3, $J_{6,7\alpha}$ = 2.3, 6-H), 1.20 (s, 3 H, 19-Me), 0.90 (s, 3 H, 18-Me) (Figure 3C).

Preparation of [4,4-D_2]Androst-5-ene-3,17-dione (**6c**). Treatment of **10** with $LiAlD_4$ provided [4,4-D_2]androst-5-ene-3β,17β-diol (**5c**) (Scheme II). Because of the presence of the 3α-deuterium, the Jones oxidation rate of the 3β-hydroxyl group of **5c** was about 5-fold slower, and the oxidation was carried out at 25 °C for 20 min. The desired product (**6c**) was obtained by HPLC on a reverse-phase column (Partisil M9 10/50, ODS-2, Whatman), by use of an isocratic mixture of methanol and water (70/30). After HPLC purification, high-resolution ^1H NMR analysis indicated that the final product contained four components in the following ratios: **6c** (87.8%), **6a** (5.7%), **6b** (4.9%), and unlabeled steroid (1.6%). ^1H NMR ($CDCl_3$) δ 3.26 (broad band, 0.049 H, $\Delta\nu_{1/2}$ = 10.3, 4β-H), 2.81 (broad band, 0.057 H, $\Delta\nu_{1/2}$ = 6.4, 4α-H), 3.28 (dq, 0.016 H, $J_{4\alpha,4\beta}$ = 16.6, $J_{4\beta,x}$ = 3.3, 4β-H), 2.84 (dd, 0.016 H, $J_{4\alpha,4\beta}$ = 16.6, $J_{4\alpha,2\alpha}$ = 2.2, 4α-H), 5.36 (dd, 1 H, $J_{6,7\beta}$ = 5.3, $J_{6,7\alpha}$ = 2.3, 6-H), 1.20 (s, 3 H, 19-Me), 0.90 (s, 3 H, 18-Me) (Figure 3D).

Preparation of [6-D]Androst-5-ene-3,17-dione (**6d**). This synthesis is shown in Scheme III.

(A) 5α-Bromo-6β-hydroxyandrostane-3β,17β-diol Diacetate (**12a**). To a stirred solution containing 15 g of androst-5-ene-3β,17β-diol diacetate (**11a**; Aldrich) in 200 mL of 1,4-dioxane was added 20 mL of 8% $HClO_4$ solution. The cloudy solution then received 5.8 g of recrystallized *N*-bromoacetamide (NBA) over a 30-min period. After an additional 30 min, only traces of starting material could be detected by TLC. The reaction was terminated by addition of 20 mL of 1% $Na_2S_2O_3$ and 10 mL of 10% $NaHCO_3$. The mixture then was dried on a rotary evaporator. The oily residue was dissolved in ethyl acetate, washed with water, and dried with anhydrous Na_2SO_4. After filtration and removal of solvent by rotary evaporation, the bromohydrin (**12a**) was recrystallized from a mixture of ethyl acetate and hexane (yield 8.9 g; 47%). ^1H NMR ($CDCl_3$) δ 4.16 (br s, 1 H, 6α-H), 4.60 (t, 1 H, J = 8.4, 17α-H), 5.45 (tt, 1 H, $J_{3\alpha,2(4)\beta}$ = 11.3, $J_{3\alpha,2(4)\alpha}$ = 5.4, 3α-H), 0.75 (s, 3 H, 18-Me), 0.78 (s, 3 H, 19-Me), 2.00 (s, 6 H, acetate Me's).

(B) 5α-Bromo-6-oxoandrostane-3β,17β-diol Diacetate (**13**). To a solution of 4.5 g of 5α-bromo-6β-hydroxyandrostane-3β,17β-diol diacetate (**12a**) in 100 mL of acetone at 25 °C was added dropwise 8.0 mL of Jones reagent over a 10-min period. After an additional 5 min, the reaction was terminated by pouring the mixture into ice–water, and a white precipitate formed immediately. The suspension was evaporated on a rotary evaporator to reduce the acetone content, and the organic product was extracted with CH_2Cl_2. The extract was dried with anhydrous Na_2SO_4, filtered, and evaporated to dryness on a rotary evaporator (4.0 g of a white solid crude product). The product was recrystallized from a mixture of ethanol–water–ethyl acetate (100/100/15). Short needles (2.9 g) were obtained, and a second recrystallization gave 2.47 g of pure 5α-bromo-6-oxoandrostane-3β,17β-diol diacetate (**13**). ^1H NMR ($CDCl_3$) δ 4.63 (t, 1 H, J = 8.4, 17α-H), 5.30 (tt, 1 H, $J_{3\alpha,2(4)\beta}$ = 11.3, $J_{3\alpha,2(4)\alpha}$ = 5.4, 3α-H), 0.75 (s, 3 H, 18-Me), 0.96 (s, 3 H, 19-Me), 2.00, 2.02 (2 s, 6 H, acetate Me's), 3.15 (dd, 1 H, $J_{7\alpha,7\beta}$ = 14.8, $J_{7\alpha,8\beta}$ = 12.3, 7α-H), 2.37 (dd, 1 H, $J_{4\alpha,4\beta}$ = 14.6, $J_{4\alpha,3\alpha}$ = 5.4, 4α-H), 2.27 (dd, 1 H, $J_{7\alpha,7\beta}$ = 14.8, $J_{7\beta,8\beta}$ = 5.5, 7β-H), 2.05 (dd, $J_{4\alpha,4\beta}$ = 14.6, $J_{4\beta,3\alpha}$ = 11.3, 4β-H).

(C) [6α-D]-5α-Bromo-6β-hydroxyandrostane-3β,17β-diol Diacetate (**12b**). 5α-Bromo-6-oxoandrostane-3β,17β-diol diacetate (**13**; 745 mg) was dissolved in 100 mL of anhydrous ethanol. Then 2.5 g of $CeCl_3 \cdot 7H_2O$ was added, since in its absence the desired bromohydrin was not obtained. Gentle heating was used to dissolve the cerium chloride, and the solution then was cooled to 25 °C in a water bath. A total of 265 mg of $NaBD_4$, (98 atom % D, from Aldrich) was added over an 18-min period. After an additional 12 min, the reaction was terminated by addition of 50 mL of H_2O. The volume of the suspension was reduced to about 50 mL on a rotary evaporator to reduce the ethanol content. The organic material was then extracted with three 50-mL portions of ether. After drying with anhydrous Na_2SO_4 and filtration, the ether was removed on a rotary evaporator to give a white solid crude product. A pilot experiment showed that the crude product contained mainly the *trans*-bromohydrin and only a trace amount of the *cis*-bromohydrin and that both underwent elimination to give **11b** although the elimination rates were quite different. Therefore, no further purification was carried out.

(*D*) [*6-D*]*Androst-5-ene-3β,17β-diol Diacetate* (**11b**). The crude product **12b** obtained from reduction was dissolved in 80 mL of glacial acetic acid. Then 250 mg of Zn dust was added with stirring. After 5 min, another aliquot of 250 mg of Zn dust was added. An additional 5 min later, the reaction mixture was filtered through a sintered glass funnel. The filtrate was then heated to 70 °C, and 150 mL of H_2O was added gradually. After overnight cooling, 344 mg of fine crystals was obtained (58% yield). Trace amounts of several polar impurities were removed by silica gel flash chromatography (50% benzene, 8% ethyl acetate, 43% hexane). ^1H NMR indicated 92.0 atom % D at the vinylogous C-6 position. ^1H NMR ($CDCl_3$) δ 5.35 (d, 1 H, J = 5.1, 6-H), 4.58 (t, 1 H, J = 8.4, 17α-H), 4.58 (tt, 1 H, $J_{3\alpha,2(4)\beta}$ = 11.3, $J_{3\alpha,2(4)\beta}$ = 5.4, 3α-H), 0.78 (s, 3 H, 18-Me), 1.00 (s, 3 H, 19-Me), 2.01–2.02 (2s, 6 H, acetate Me's).

(*E*) [*6-D*]*Androst-5-ene-3β,17β-diol* (**5d**). Compound **11b** (340 mg) was dissolved in 50 mL of anhydrous ether, and the solution was cooled in ice–water. Over a 2-min period, 100 mg of $LiAlH_4$ was added. After an additional 3 min, the ice bath was removed, and 30 min later, the solution was heated to reflux under an argon atmosphere. After 2 h of refluxing, TLC indicated that the reaction was complete. Water was then added to quench the reaction, and ethyl acetate was used to extract the organic materials. The extract was dried with anhydrous Na_2SO_4 and filtered, and solvent was removed by rotary evaporation. The resultant white solid crude product was then recrystallized from an ethanol–water mixture. The yield was 160 mg (68%) of small crystals. ^1H NMR analysis indicated 92.0 atom % D at the vinylogous C-6 position. ^1H NMR ($CDCl_3$) δ 5.33 (d, 0.08 H, J = 5.1, 6-H), 3.62 (t, 1 H, J = 8.4, 17β-H), 3.50 (tt, 1 H, $J_{3\alpha,2(4)\beta}$ = 11.3, $J_{3\alpha,2(4)\alpha}$ = 5.4, 3α-H), 0.74 (s, 3 H, 18-Me), 1.00 (s, 3 H, 19-Me).

(*F*) [*6-D*]*Androst-5-ene-3,17-dione* (**6d**). Compound **5d** was oxidized with Jones reagent under the conditions described for **6a**. High-resolution ^1H NMR analysis indicated that there were two components in the final product, **6d** (92.0%) and unlabeled androst-5-ene-3,17-dione (8.0%). ^1H NMR ($CDCl_3$) δ 3.28 (dt, 1 H, $J_{4\alpha,4\beta}$ = 16.6, $J_{4\beta,X}$ = 3.3, 4β-H), 2.84 (dd, 1 H, $J_{4\alpha,4\beta}$ = 16.6, $J_{4\alpha,2\alpha}$ = 2.2, 4α-H), 5.36 (dt, 0.080 H, $J_{6,7\beta}$ = 5.3, $J_{6,7\alpha}$ = 2.3, 6-H), 1.20 (s, 3 H, 19-Me), 0.90 (s, 3 H, 18-Me) (Figure 3E).

Kinetic Measurements

All determinations of kinetic constants 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 (λ_m) of absorption of androst-4-ene-3,17-dione (a_m = 16 300 M^{-1} cm^{-1}) in H_2O or D_2O. It was established that there were no observable isotope effects on both λ_m and a_m for all the deuterium-labeled substrates used in this work. All substrates were purified by silica gel flash chromatography or HPLC (on a reverse-phase column when necessary) before use. Reactions were carried out in final volumes of 1.50 mL, containing 50 mM Tris-HCl (or 33 mM potassium phosphate) of the desired pH, 50 μL of methanol (3.3% by volume), and 100 μg of bovine serum albumin. Since the highest steroid concentrations appoach the limits of solubility, all reaction components were always added in the same order to minimize solubility problems. Each cuvette received 10 μL of 1% bovine serum albumin, followed by 75 μL of 1.0 M buffer; then, 1350 μL of H_2O or D_2O was added, followed by 50 μL of CH_3OH or CH_3OD containing 18–90 mM substrate. The substrate is more slowly soluble in D_2O, and the cuvette contents were always stirred for 1–3 min. After the spontaneous rate of isomerization had stabilized (usually an absorbance change of 0.001/min), the enzymatic reaction was initiated by addition of 15 μL of isomerase appropriately diluted in 1% 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 determinations at seven or eight substrate concentrations, and the values of k_{cat} and K_m were calculated 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).

In order to obtain strictly comparable kinetic isotope effects, closely similar reaction conditions including identical volumes of methanol were used for each pair of substrates in all measurements. It was shown in separate experiments that prior incubation of the substrate with bovine serum albumin (15–100 μg/1.5 mL) did not affect the k_{cat} or K_m values for either the wild-type or the Y55F mutant enzyme.

Substrate Isotope Effects. Both labeled and unlabeled substrates were weighed and 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). In order to maintain the same albumin concentration in the assay system and an equivalent absorbance range, the enzyme concentration was raised 5-fold for the 4β-deuterated substrate when the primary isotope effects were studied. For measurements of secondary isotope effects, identical enzyme solutions were used for each pair of substrates.

Solvent Isotope Effects. For measurements of the solvent isotope effects, both labeled and unlabeled substrates were dissolved in CH_3OD and their concentrations calibrated spectrophotometrically as described above. All other components of the reaction mixture were dissolved in D_2O. Tris-HCl buffers (1.0 M) were prepared by lyophilizing buffers adjusted with HCl to the appropriate pH in H_2O, redissolving the residue in D_2O, lyophilizing the solution again, and then reconstituting the buffer salts in D_2O. For the buffers used under standard assay conditions, the final pH values were 7.37 in H_2O and 7.54 in D_2O as determined against standard buffers without correction. The final deuterium content in the assay systems was about 99.6 atom %. Dilution of the enzyme into D_2O or H_2O had no effect on the kinetic constants obtained.

Dependence on pH of k_{cat} and K_m Values for the Wild-Type and the Y55F Mutant Enzymes. A series of potassium phosphate and Tris-HCl buffer solutions with pH values ranging from 6.1 to 8.6 were prepared in H_2O and D_2O. The k_{cat} and K_m values were determined as described above. The final pH values of the diluted buffer solutions, which were determined on a pH meter without corrections, were as follows: 6.12 and 6.90 (33 mM phosphate in D_2O); 6.48 (33 mM phosphate in H_2O); 7.65 and 8.22 (50 mM Tris-HCl in D_2O); 7.43 and 8.55 (Tris-HCl in H_2O).

RESULTS

Substrate Deuterium Isotope Effects on the Reaction Catalyzed by Wild-Type Isomerase. As indicated in Figure 4 and the derived kinetic parameters (Table I), a comparison of k_{cat} using 4β-protonated androst-5-ene-3,17-dione with that obtained with the 4β-deuterated substrate yielded a primary substrate deuterium isotope effect of 6.13 ± 0.30 in H_2O. Hence, either deprotonation (during enolization) at the 4β-position of the substrate or reprotonation (during reketonization) at the 6β-position of the dienol intermediate by

Table I: Substrate and Solvent Isotope Effects on Kinetic Parameters of Wild-Type Isomerase

substrate[a]	solvent	k_{cat} ($\times 10^{-3}$ s^{-1})[b]	K_m (μM)	substrate isotope effects: $k_{cat}(H)/k_{cat}(4\beta\text{-D})$	substrate isotope effects: $(k_{cat}/K_m)(H)/(k_{cat}/K_m)(4\beta\text{-D})$[c]	solvent isotope effects: $k_{cat}(H_2O)/k_{cat}(D_2O)$	solvent isotope effects: $(k_{cat}/K_m)(H_2O)/(k_{cat}/K_m)(D_2O)$
4β-H	H_2O	53.0 ± 2.6	285 ± 16	6.13 ± 0.30[d]	2.99 ± 0.06[d]	1.59 ± 0.10[f]	1.15 ± 0.03[f]
4β-D	H_2O	8.64 ± 0.32	139 ± 7				
4β-H	D_2O	33.3 ± 4.7	206 ± 35	6.25 ± 0.30[e]	3.31 ± 0.07[e]	1.62 ± 0.10[g]	1.27 ± 0.03[g]
4β-D	D_2O	5.32 ± 0.10	109 ± 3				

[a] 4β-D substrate contains 97.0 atom % deuterium. [b] The standard errors in the kinetic parameters were computed as described by Wilkinson (1961). [c] The standard errors in k_{cat}/K_m were calculated from the variations in the slopes of the double-reciprocal plots (Figure 4) as described by Zar (1984). [d] In H_2O. [e] In D_2O. [f] With protonated substrate. [g] With 4β-D substrate.

Asp-38 constitutes a major rate-limiting step of the isomerase reaction. The reason for this ambiguity is that the 4β-proton removed from the substrate during the enolization step is largely conserved and transferred to the dienol intermediate to yield the final product (Talalay & Wang, 1955; Malhotra & Ringold, 1965).

Use of the 4β-deuterated substrate also resulted in a 2-fold decrease in K_m, which rules out the simplest kinetic scheme involving the prior equilibration of E with S followed by the rate-limiting enolization of the bound substrate.

Solvent Deuterium Isotope Effects on the Reaction Catalyzed by Wild-Type Isomerase. Comparison of the kinetic parameters of the isomerase reaction in H_2O with those measured in D_2O with the 4β-protonated substrate (Figure 4) revealed a solvent deuterium isotope effect $k_{cat}(H;H_2O)/k_{cat}(H;D_2O) = 1.59 \pm 0.10$ (Table I),[1] indicating that either protonation of the carbonyl group of the substrate or deprotonation of the hydroxyl of the dienol intermediate by Tyr-14 also constitutes a rate-limiting step in the isomerase reaction.

Combined Solvent and Substrate Deuterium Isotope Effects on the Reaction Catalyzed by Wild-Type Isomerase. To determine whether the solvent deuterium isotope effect reflects the same rate-limiting step as does the substrate 4β-deuterium isotope effect, or a different one, the substrate deuterium isotope effect was determined in D_2O (Figure 4). The finding of essentially the same primary substrate deuterium isotope effect at the 4β-position [$k_{cat}(H;D_2O)/k_{cat}(4\beta\text{-D};D_2O) = 6.25 \pm 0.30$] in D_2O as in H_2O [$k_{cat}(H;H_2O)/k_{cat}(4\beta\text{-D};H_2O) = 6.13 \pm 0.30$] and, conversely, the detection of nearly the same solvent deuterium isotope effect [$k_{cat}(4\beta\text{-D};H_2O)/k_{cat}(4\beta\text{-D};D_2O) = 1.62 \pm 0.10$ and $k_{cat}(H;H_2O)/k_{cat}(H;D_2O) = 1.59 \pm 0.10$] (Table I) indicates that both isotope effects are operating on the same chemical step since slowing down one step would competitively reduce the isotope effect on the other step if the two steps were separate. Similar effects are noted on k_{cat}/K_m with somewhat smaller experimental errors due to the greater precision in the measurement of slopes of double-reciprocal plots (Table I). Hence, the rate-limiting step in the isomerase reaction is a concerted process, in accord with the relative orientation of Asp-38 (the general base) and Tyr-14 (the general acid) (Kuliopulos et al., 1989) (Figure 2). In the presence of D_2O the K_m values were 20–25% lower than those in H_2O, whether the 4β-deuterated or the protonated substrate was used.

Variation of the pH over the range 6.1–8.6 in H_2O and D_2O revealed no significant change in the solvent isotope effect, indicating that it probably did not result from a change in pK_a of groups on the enzyme.

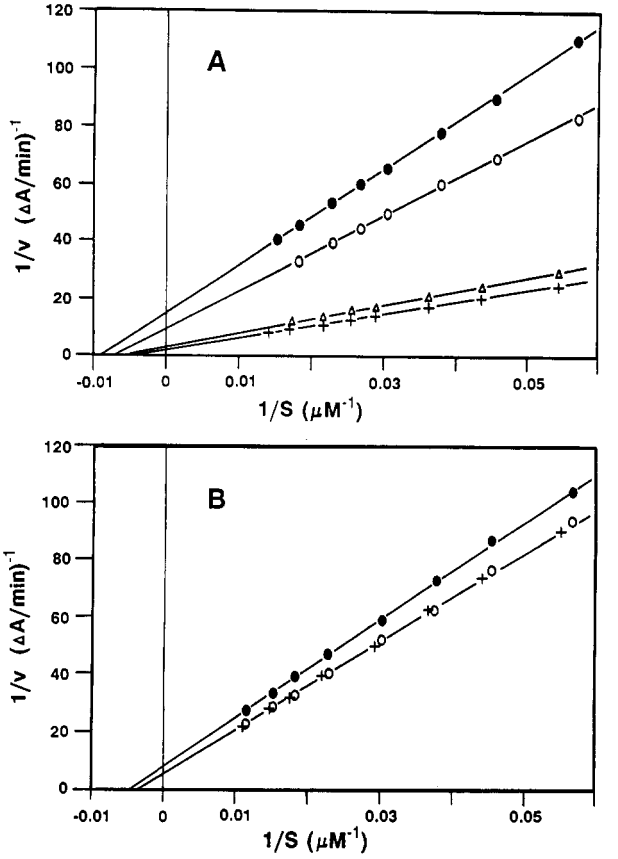


FIGURE 4: Double-reciprocal plots of the initial velocity of the isomerase reaction with respect to substrate concentration catalyzed by the wild-type isomerase, with unlabeled and deuterated substrates. Measurements were carried out in a Beckman DU-7 spectrophotometer at 25 °C and at 248 nm (a_m = 16 300 M^{-1} cm^{-1}), which is the absorption maximum of the product. Initial rates were measured in a final volume of 1.5 mL containing 50 mM Tris-HCl buffer at pH 7.5 and 3.3% MeOH. (A) Primary kinetic isotope effects on k_{cat} and K_m for the wild-type isomerase. All rates were normalized to an enzyme site concentration of 12.6 pM: unlabeled substrate in H_2O (+); unlabeled substrate in D_2O (Δ); 4β-D substrate in H_2O (○); 4β-D substrate in D_2O (●). (B) Secondary kinetic isotope effects on k_{cat}/K_m ratio for the wild-type isomerase. All rates were normalized to an enzyme site concentration of 2.20 pM: unlabeled substrate in H_2O (+); 6-D substrate in H_2O (○); 4α-D substrate in H_2O (●).

Substrate, Solvent, and Combined Deuterium Isotope Effects on the Reaction Catalyzed by the Y55F Mutant Isomerase. As previously found (Kuliopulos et al., 1989), mutation of Tyr-55 to Phe resulted in a 4.3-fold reduction in k_{cat} and a 1.9-fold decrease in K_m (Table II), indicating that although the enzyme has been slightly damaged catalytically by this mutation, Tyr-55 is not an essential residue for catalysis. With this mutant enzyme, the substrate deuterium isotope effect on k_{cat} at the 4β-position was reduced to 5.31 ± 0.27, and the

[1] The kinetic solvent and substrate deuterium isotope effects are specified in the text by designating the isotopic composition of both the substrate and the solvent in parentheses following the kinetic constant that is being measured. For example, $k_{cat}(4\beta\text{-D};H_2O)$ is the k_{cat} value obtained for [4β-D]androst-5-ene-3,17-dione in H_2O, and $k_{cat}(H;D_2O)$ is the k_{cat} value obtained with protonated androst-5-ene-3,17-dione in D_2O.

Table II: Substrate and Solvent Isotope Effects on Kinetic Parameters of the Y55F Mutant Isomerase

substrate[a]	solvent	k_{cat} ($\times 10^{-3}$ s^{-1})[b]	K_m (μM)	substrate isotope effects: $k_{cat}(H)/k_{cat}(4\beta\text{-D})$	substrate isotope effects: $(k_{cat}/K_m)(H)/(k_{cat}/K_m)(4\beta\text{-D})$[c]	solvent isotope effects: $k_{cat}(H_2O)/k_{cat}(D_2O)$	solvent isotope effects: $(k_{cat}/K_m)(H_2O)/(k_{cat}/K_m)(D_2O)$
4β-H	H_2O	12.2 ± 0.3	152 ± 4	5.31 ± 0.27[d]	4.03 ± 0.08[d]	1.50 ± 0.09[f]	1.16 ± 0.02[f]
4β-D	H_2O	2.29 ± 0.17	115 ± 12				
4β-H	D_2O	8.11 ± 0.68	117 ± 14	5.93 ± 0.30[e]	4.65 ± 0.09[e]	1.68 ± 0.10[g]	1.34 ± 0.03[g]
4β-D	D_2O	1.37 ± 0.05	92 ± 5				

[a] 4β-D substrate contains 97.0 atom % deuterium. [b] The standard errors in the kinetic parameters were computed as described by Wilkinson (1961). [c] The standard errors in k_{cat}/K_m were calculated from the variations in the slopes of the double-reciprocal plots (Figure 5) as described by Zar (1984). [d] In H_2O. [e] In D_2O. [f] With protonated substrate. [g] With 4β-D substrate.

solvent deuterium isotope effect was reduced to 1.50 ± 0.09. These findings raise the possibility that an additional partially rate-limiting step has been introduced by the Y55F mutation (Figure 5, Table II). Consistent with this view, the substrate isotope effect $k_{cat}(H;H_2O)/k_{cat}(4\beta\text{-D};H_2O)$ increased to 5.93 ± 0.30 in D_2O, and the solvent isotope effect $k_{cat}(H;H_2O)/k_{cat}(H;D_2O)$ increased to 1.68 ± 0.10 when the 4β-deuterated substrate was used (Table II). These small increases indicate that while the rate-limiting step has remained concerted, the combined substrate and solvent isotopic slowing of this concerted step has made it relatively more rate limiting than another partially rate-limiting step. The same trend is noted with k_{cat}/K_m (Table II). As with the wild-type enzyme, a small but significant decrease in K_m of the Y55F mutant occurred upon deuteration of either the 4β-position of the substrate or the solvent.

Secondary Isotope Effects on the Reaction Catalyzed by the Wild-Type and Y55F Mutant Isomerase. To determine whether enolization of the substrate constitutes the concerted, rate-limiting step, secondary deuterium kinetic isotope effects were measured with the 4α-deuterated substrate. The rationale behind this approach is that a hybridization change from sp^3 to sp^2 occurs at C-4 of the substrate during the enolization and no hybridization change occurs at C-4 during the subsequent reketonization. Hence, rate-limiting enolization would result in a decreased reaction rate with the 4α-deuterated substrate. Such an effect is indeed observed on both k_{cat} and k_{cat}/K_m with the latter effect being more precisely determined from the slope of a double-reciprocal plot (Figure 4; Table III). When corrected for the composition of the substrate (Table III), the magnitude of the secondary isotope effect on k_{cat}/K_m with both the wild-type (1.11 ± 0.02) and the Y55F mutant (1.12 ± 0.02) overlaps with the expected equilibrium deuterium isotope effect (1.127 ± 0.006) (Cook et al., 1980) (Figures 4 and 5; Table III). This similarity raises the possibility that the observed secondary isotope effect is on the keto–enol equilibrium, followed by a rate-limiting reketonization of the dienol intermediate.

Such an equilibrium effect was ruled out, and a predominantly kinetic 4α secondary isotope effect was established by measuring the secondary isotope effect with a deuterium atom rather than a hydrogen atom in the 4β-position undergoing transfer. The significantly lower 4α secondary isotope effect of 1.06 ± 0.01 with the wild-type and 1.07 ± 0.01 with the Y55F mutant enzymes (Table III) observed when a deuteron rather than a proton is the migrating species establishes contributions to the secondary isotope effect resulting from tunneling of the 4β-proton and the coupled motion of the 4β- and 4α-protons in the transition state, requiring this effect to be largely kinetic (Kurz & Frieden, 1980; Cook et al., 1981; Huskey & Schowen, 1983; Klinman, 1990). Hence, concerted enolization of the enzyme-bound substrate constitutes a major rate-limiting step in the isomerase reaction.

To test whether reketonization of the dienol intermediate is also partially rate limiting, inverse secondary kinetic isotope effects on k_{cat}/K_m were sought with the 6-deuterated substrate since the hybridization at C-6 does not change during enolization but changes from sp^2 to sp^3 during reketonization. The absence of any kinetic isotope effects on k_{cat}/K_m at C-6 with both the wild-type (1.00 ± 0.01) (Figure 4; Table III) and the Y55F mutant enzymes (1.01 ± 0.01) (Figure 5; Table III) argues against reketonization of the dienol intermediate as a rate-limiting step and indicates that this process is very rapid. The remote possibility that an inverse secondary kinetic isotope effect at C-6 has been precisely compensated by coupled

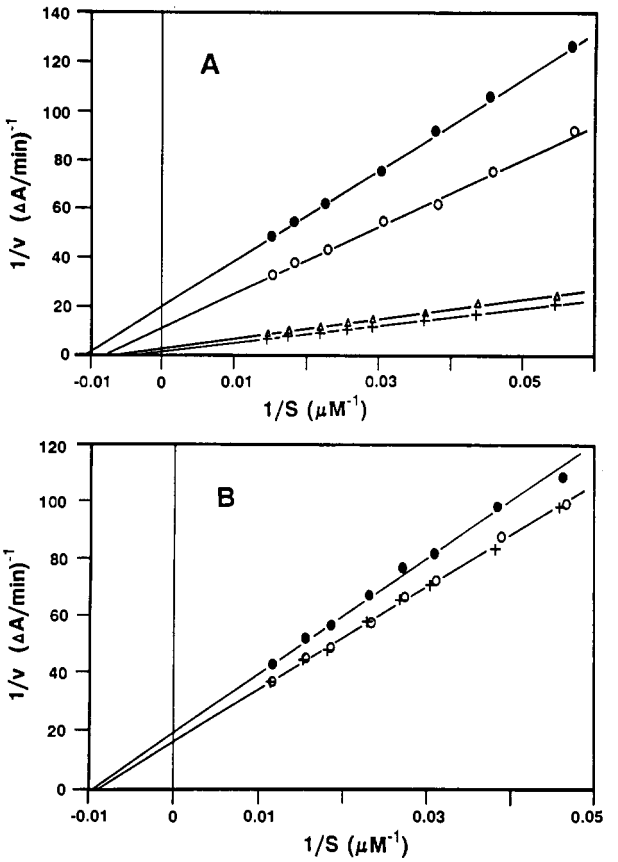


FIGURE 5: Double-reciprocal plots of the initial velocity of the isomerase reaction with respect to substrate concentration catalyzed by the Y55F mutant isomerase, with unlabeled and deuterated substrates. Measurements were carried out in a Beckman DU-7 spectrophotometer at 25 °C and at 248 nm (a_m = 16 300 M^{-1} c^{-1}), which is the absorption maximum of the product. Initial rates were measured in a final volume of 1.5 mL containing 50 mM Tris-HCl buffer at pH 7.5 and 3.3% MeOH. (A) Primary kinetic isotope effects on k_{cat} and K_m for the Y55F mutant isomerase. All rates were normalized to an enzyme site concentration of 36.9 pM: unlabeled substrate in H_2O (+); unlabeled substrate in D_2O (Δ); 4β-D substrate in H_2O (○); 4β-D substrate in D_2O (●). (B) Secondary kinetic isotope effects on k_{cat}/K_m ratio for the Y55F mutant isomerase. All rates were normalized to an enzyme site concentration of 5.85 pM: unlabeled substrate in H_2O (+); 6-D substrate in H_2O (○); 4α-D substrate in H_2O (●).

Table III: Secondary Kinetic Deuterium Isotope Effects on k_{cat}/K_m for Wild-Type and Y55F Mutant Enzymes[a]

	A		B		
enzyme	substrate	k_{cat}/K_m ($\times 10^{-8}$ M^{-1} s^{-1})	substrate	k_{cat}/K_m ($\times 10^{-8}$ M^{-1} s^{-1})	ratios of k_{cat}/K_m (A/B)
wild type	unlabeled	1.98 ± 0.03	4α-D[b]	1.75 ± 0.03	1.11 ± 0.02[c]
wild type	4β-D[b]	0.602 ± 0.06	4,4-D_2[b]	0.568 ± 0.010	1.06 ± 0.01[c]
wild type	unlabeled	1.98 ± 0.03	6-D	1.98 ± 0.03	1.00 ± 0.01[d]
Y55F	unlabeled	0.803 ± 0.008	4α-D[b]	0.717 ± 0.007	1.12 ± 0.02[c]
Y55F	4β-D[b]	0.199 ± 0.002	4,4-D_2[b]	0.186 ± 0.002	1.07 ± 0.01[c]
Y55F	unlabeled	0.803 ± 0.008	6-D	0.795 ± 0.008	1.01 ± 0.01[d]

[a] The standard errors in k_{cat}/K_m were calculated from the variations in the slopes of the double-reciprocal plots (Figures 4 and 5) as described by Zar (1984). The ratios of the k_{cat}/K_m values are given as the means of two experimental determinations ± half of the difference between these values. [b] These data were corrected for the isotopic composition of the substrates determined by 600-MHz ^{1}H NMR spectra. The k_{cat}/K_m(observed) is the sum of the products of the k_{cat}/K_m values for each component multiplied by the fractional abundance of that component. [c] Equilibrium isotope effect predicted to be 1.127 ± 0.006 (Cook et al., 1980). [d] Equilibrium isotope effect predicted to be 0.887 ± 0.005 (Cook et al., 1980).

motion to a value of 1.00, while not totally excluded, is unlikely since it would require exactly the same effect to have occurred on both the wild-type and the Y55F mutant (which shows a 4.3-fold lower k_{cat}) enzymes. Moreover, such a precise compensation of effects does not provide the most parsimonious explanation of a negative result.

Discussion

The present work systematically studied primary and secondary deuterium kinetic isotope effects on the ketosteroid isomerase reaction, confirming and greatly extending the early observations of Malhotra and Ringold (1965), who detected a sizable primary kinetic isotope effect on k_{cat} of the isomerase reaction when the 4β-hydrogen of the substrate was replaced with deuterium (Table I). This effect and the primary solvent and combined deuterium kinetic isotope effects which we have determined (Tables I and II) establish a concerted rate-limiting step in the reactions catalyzed by both the wild-type and the Y55F mutant ketosteroid isomerases. The secondary substrate kinetic isotope effect of deuterium at the 4α-position and the lack of a secondary kinetic isotope effect at the C-6 position argue that enolization of the enzyme-bound substrate constitutes the concerted rate-limiting step on both enzymes and that reketonization of the dienol intermediate is a more rapid step (Figure 6).

On adding a synthetic steroid dienol to ketosteroid isomerase, at pH 5.0, Eames et al. (1990) have observed the formation of both the unconjugated steroid substrate and the conjugated steroid product with relative rate constants of 2:1, resulting in a partitioning ratio of 68 ± 3% substrate formation and 32 ± 3% product formation. These similar rate constants imply similar barrier heights for the reaction of the enolic intermediate in both directions. While these data provide direct evidence for the participation of an enolic intermediate, they appear inconsistent with our observations of the rate-limiting formation and rapid reketonization of this enolic intermediate. Under the experimental conditions of Eames et al. (1990), Asp-38 of ketosteroid isomerase was 67% deprotonated (pK_a = 4.7; Pollack et al., 1986), and Tyr-14 was fully protonated ($pK_a \geq 9.7$; Kuliopulos et al., 1990b). This form of the enzyme is inappropriate to catalyze reactions of the enolic intermediate, which would require the opposite states of protonation of the catalytic residues. Under these conditions, the only protons available in 67% of the enzyme–enol complex are on the 3-hydroxyl group of the added enolic species itself ($pK_a \sim 10$; Pollack et al., 1989) and on Tyr-14. In the low-energy s-cis conformation of the dienol (Capon et al., 1988), the 3-hydroxyl proton is located only 2.5 Å from C-4, approaching van der Waals contact, but it is 4.9 Å distant from C-6. The tyrosine hydroxyl proton, if hydrogen bonded

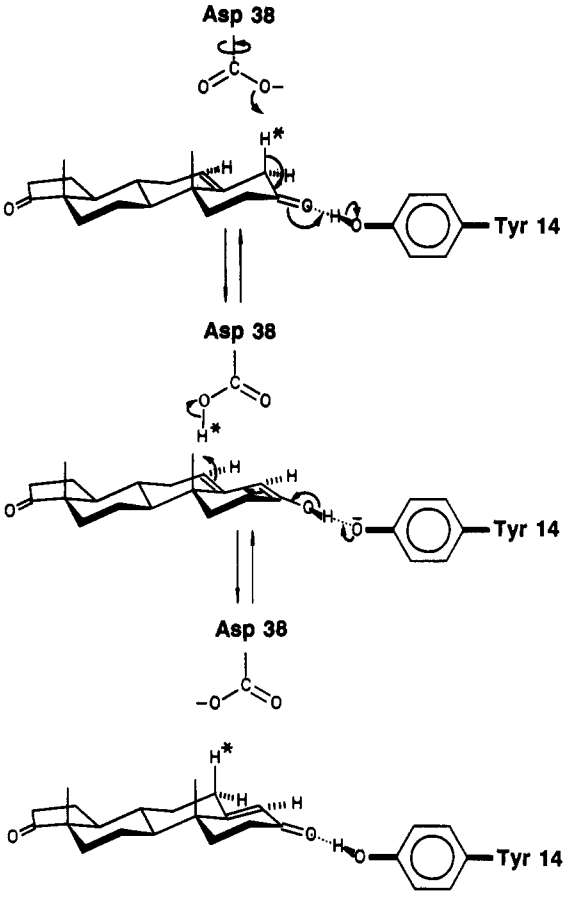


FIGURE 6: Mechanism of isomerization of androst-5-ene-3,17-dione catalyzed by Δ^5-3-ketosteroid isomerase consistent with the present data. Asp-38 is above the 4β-H, and Tyr-14 is on the side of the 3-carbonyl oxygen atom forming a hydrogen bond. Enolization is concerted. Reketonization is also depicted as concerted, although this point has not been established. Rotation of Asp-38 about its β–γ bond enables the original 4β-proton to be transferred to the 6β-position.

to the C-3 oxygen, would be 4.2 Å from C-4 and 6.5 Å from C-6. Since both of these protons are much closer to C-4 than to C-6, an unusually facile proton transfer to C-4 may be taking place on the enzyme-bound dienol, from the 3-hydroxyl to C-4 rather than to C-6, preferentially accelerating substrate formation under the conditions of the experiment of Eames et al. (1990), thus changing the mechanism of the reaction. Evidence suggesting such a change in mechanism resulting in the base-mediated transfer of the hydroxyl proton to the β-carbon of an enol has been obtained with the H95Q mutant of triosephosphate isomerase (Nickbarg et al., 1988).

In our experiments, in addition to the substrate deuterium isotope effects on k_{cat}, much smaller but significant primary

Table IV: Derived Individual Rate Constants, K_s Values, and Commitment Factors for Wild-Type and Y55F Enzymes[a]

enzyme	solvent	k_1 ($\times 10^{-8}$ M^{-1} s^{-1})	k_{-1} ($\times 10^{-3}$ s^{-1})	k_2(H) ($\times 10^{-3}$ s^{-1})	K_s (μM)	K_m(H) (μM)	k_2(H)/k_{-1}	K_m(4β-D) (μM)	k_2(4β-D)/k_{-1} (μM)
wild type	H_2O	3.0	34	53.0	111	285	1.6	139	0.26
wild type	D_2O	2.9	26	33.3	91	206	1.3	109	0.20
Y55F	H_2O	2.7	28	12.2	106	152	0.43	115	0.08
Y55F	D_2O	2.7	23	8.1	87	117	0.35	92	0.06

[a] The rate constants and K_s values were calculated by assuming that $k_{cat} = k_2$ in the kinetic scheme of eq 3 and that the primary 4β-deuterium kinetic isotope effect is solely on k_2.

kinetic isotope effects on K_m were detected, generally <2.0 (Table I). These effects may be used to evaluate individual rate constants in the kinetic scheme of ketosteroid isomerase. A general kinetic scheme for the isomerase reaction at zero product concentration may be written as

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ED \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EP \xrightarrow{k_4} E + P \qquad (1)$$

From the present data with both the wild-type and the Y55F mutant isomerases, the k_2 step which leads to the dienol intermediate, D, is rate limiting in the forward direction, i.e., $k_2 \ll k_3$, k_4, which simplifies the kinetic scheme to

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

Since ED does not accumulate and the reverse isomerase reaction is much slower than the forward reaction (Batzold et al., 1976), the k_{-2} step may also be neglected, leading to the simple kinetic scheme

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad (3)$$

in which $k_{cat} = k_2$ and $K_m = (k_{-1} + k_2)/k_1$. Assuming that deuteration at the 4β-position of the substrate affects only k_2, we have used the observed primary substrate isotope effects on k_{cat} and K_m to calculate the individual rate constants for the wild-type and the Y55F mutant enzymes in both H_2O and D_2O (Table IV). From Table IV it is seen that k_1 approaches the diffusion limit and is larger than k_{cat}/K_m. The lower forward commitment factor k_2/k_{-1} in the Y55F mutant enzyme as compared to that in the wild-type enzyme results in a value of K_m which approaches the dissociation constant K_s of the enzyme–substrate complex. The only significant effect of D_2O is on k_2, the rate constant for enolization. In the case of the Y55F mutant, the k_2 step may be more complicated and include another partially rate-limiting step which precedes enolization, since the 4β-deuterium primary kinetic isotope effect on k_{cat} increased slightly in D_2O [see O'Leary (1989)]. This additional step may be the positioning of the catalytic residues near the enzyme-bound substrate. When this is completed, a concerted enolization takes place on the mutant enzyme as well.

The concerted action of Asp-38 in deprotonating C-4 of the bound substrate with that of Tyr-14 in protonating the 3-carbonyl oxygen in the rate-limiting step is consistent with the additive effects on k_{cat} of mutating these two catalytic residues. Thus, k_{cat} of the D38N mutant is $10^{5.7}$-fold lower than that of the wild-type enzyme, k_{cat} of the Y14F mutant is $10^{4.7}$-fold lower, (Kuliopulos et al., 1989) and k_{cat} of the double-mutant enzyme is at least $10^{9.8}$-fold lower, showing no catalytic activity but intact substrate binding (Kuliopulos et al., 1990a). If Asp-38 and Tyr-14 were acting consecutively in a stepwise mechanism, the double mutant would most simply have shown the kinetic effects of the more damaging of the two single mutants, i.e., a $10^{5.7}$-fold decrease in k_{cat}.

The concerted functioning of Asp-38 and Tyr-14 is also consistent with the orientation of these catalytic residues with respect to the bound substrate, as found by computer modeling of the structure of the enzyme–substrate complex, based on NMR and X-ray data (Kuliopulos et al., 1989) (Figure 2). The most likely orientation of Asp-38 and Tyr-14 which allows their simultaneous contact with the 4β-proton and the 3-carbonyl oxygen of the substrate, respectively, with minimal distortion of the enzyme, is at a right angle with respect to the bound substrate (Figure 2A). An antarafacial orientation (Figure 2B) is less likely, while a suprafacial arrangement (Figure 2C) is least likely because of steric interaction. The right angle orientation is stereoelectronically optimal for both a concerted enolization of the substrate (Hand & Jencks, 1975; Rebek, 1988) and a concerted reketonization of the dienol intermediate (Figure 6). Although rate limiting, the concerted enolization step is very rapid since $k_{cat} = 5.3 \times 10^4$ s^{-1} with the wild-type enzyme and 1.3×10^4 s^{-1} with the Y55F mutant. Since reketonization must be even faster, this process may also be concerted, although the present experiments do not test this point. If enolization alone were concerted, then either the right angle or the antarafacial orientation of the acid and base catalysts would seem appropriate to facilitate this process; hence, both orientations are possible. This point is currently being studied by NMR, with a double mutant of ketosteroid isomerase (Y55F + Y88F) in which Tyr-14 is the sole tyrosine residue in the molecule (Kuliopulos et al., 1990b).

The magnitudes of the kinetic isotope effects provide information on the structure of the transition state of the concerted enolization. Thus, the secondary kinetic isotope effects at C-4 of the substrate, with deuterium as the migrating species to minimize the contributions of coupled motion and tunneling, are 47 ± 8% of the way toward the equilibrium isotope effect with the wild-type enzyme and 55 ± 8% toward the equilibrium effect with the Y55F mutant (Table III). These observations, together with the sizable primary kinetic isotope effects at C-4 of the substrate (Tables I and II), suggest that the proton removed from the substrate is approximately equidistant between C-4 and an oxygen of Asp-38 and that the hybridization at C-4 is roughly halfway between sp^3 and sp^2 in the transition state, i.e., that the transition state is symmetric. However, the primary solvent kinetic isotope effect, which reflects the transition state of the proton being donated by Tyr-14 to the 3-carbonyl oxygen of the substrate in the same step, is small (Tables I and II). Three possible explanations for the low solvent kinetic isotope effect of ~1.6 may be considered. First, strong hydrogen bonding between Tyr-14 and the 3-carbonyl oxygen of the enzyme-bound substrate in the ground state would weaken the tyrosine O–H bond more than the tyrosine O–D bond, decreasing the force constant of the former more than that of the latter, thereby decreasing the kinetic isotope effect. Such hydrogen bonding in the ground state, which is essential for the subsequent concerted proton transfer, is suggested by the 10-nm red shift in the ultraviolet absorption spectrum of the product analogue 19-nortestosterone which occurs on binding to the wild-type isomerase but is not observed on binding to the Y14F mutant (Kuliopulos et al., 1989).

A second explanation for the low solvent kinetic isotope effect is an asymmetric transition state at the 3-carbonyl group in which protonation of the carbonyl oxygen by Tyr-14 either leads or lags the more symmetric deprotonation of C-4 by Asp-38. Such an unbalanced transition state is consistent with the greater difference in pK_a values betwen the proton donor and the proton acceptor at the 3-oxygen than at C-4 in the enzyme–substrate complex. Thus, the pK_a of Tyr-14 is ≥ 9.7 (Kuliopulos et al., 1990b) while that of cyclohexanone is −7 (Gordon & Ford, 1972), leading to a $\Delta pK_a \geq 16.7$ at the 3-carbonyl oxygen. The pK_a of Asp-38 is 4.7 (Pollack et al., 1986) while that of the 4β-hydrogen is 12.7 (Pollack et al., 1987), yielding a ΔpK_a of only 8 at C-4. Hence, a transition state with the substrate proton equidistant between C-4 and Asp-38 and with the enzymic proton closer to Tyr-14 than to the 3-carbonyl oxygen would provide a reasonable explanation of the low solvent kinetic isotope effect.

A third explanation, which cannot be excluded, is long range coupled motion in the transition state between the 4β-proton which is being removed and the Tyr-14 proton which is being added to the 3-carbonyl group, since coupled motion decreases primary kinetic isotope effects (Huskey & Schowen, 1983; Klinman, 1990). These three explanations are not mutually exclusive. For example, coupled motion would require strong hydrogen bonding at the 3-oxygen, and strong hydrogen bonding in the ground state does not preclude an unbalanced transition state, asymmetric at C-3 and symmetric at C-4. Although the information is as yet incomplete, it is already clear that the $10^{9.5}$-fold rate acceleration produced by keto-steroid isomerase results from the concerted action of the general acid and base catalysts, Tyr-14 and Asp-38, in a hydrophobic environment and that nature has used sound stereoelectronic principles to bring this about.

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