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Energetics of 3-Oxo-Δ^5-steroid Isomerase: Source of the Catalytic Power of the Enzyme†

David C. Hawkinson, Teresa C. M. Eames, and Ralph M. Pollack*

Laboratory for Chemical Dynamics, Department of Chemistry and Biochemistry, University of Maryland Baltimore County, Baltimore, Maryland 21228-5398, and Center for Advanced Research in Biotechnology, 9600 Gudelsky Drive, Rockville, Maryland 20850

Received June 25, 1991; Revised Manuscript Received August 23, 1991

ABSTRACT: Knowledge of the partitioning of the putative dienol intermediate (**2**) by steroid isomerase (KSI) (Hawkinson et al. 1991), in conjunction with various steady-state kinetic parameters, allows elucidation of the detailed free energy profile for the KSI-catalyzed conversion of 5-androstene-3,17-dione (**1**) to 4-androstene-3,17-dione (**3**). This free energy profile shows four kinetically significant energy barriers (substrate binding, the two chemical steps, and dissociation of product) that must be traversed upon conversion of **1** to **3**. Thus, no single step of the catalytic cycle is cleanly rate-limiting. The source of the catalytic power of KSI is discussed via comparison of the free energy profile for the KSI-catalyzed isomerization with those for the acetate-catalyzed isomerization and the aqueous reaction at pH 7. Similarities between the energetics of the KSI-catalyzed and triosephosphate isomerase catalyzed reactions are also noted.

A detailed understanding of the mechanism of action of an enzyme requires a knowledge of the rate and equilibrium constants for the interconversion of all of the bound species on the enzyme surface. In favorable cases, the resulting free energy profile can then be compared with the corresponding free energy profiles for the uncatalyzed reaction and for appropriate model systems to quantitatively assess the enzyme's catalytic ability. Complete (or nearly complete) free energy profiles have been determined for reactions catalyzed by wild-type and mutant triosephosphate isomerases (Albery & Knowles, 1976a; Nickbarg & Knowles, 1988; Raines et al., 1986), *Escherichia coli* F_1-ATPase (Al-Shawi & Senior, 1988), EPSP synthase (Anderson et al., 1988a,b), dihydrofolate reductase (Fierke et al., 1987; Andrews et al., 1989), DNA polymerase I (Kuchta et al., 1987), various β-lactamases (Christensen et al., 1990), and the ATPases of dynein (Johnson, 1985; Holzbaur & Johnson, 1989) and myosin (Johnson, 1985). However, the only system for which the free energy profiles for *both* the enzymatic and nonenzymatic reactions could be obtained is triosephosphate isomerase (Hall & Knowles, 1975; Richard, 1984).

We report here the free energy profile for the isomerization of 5-androstene-3,17-dione (**1**) to 4-androstene-3,17-dione (**3**) catalyzed by the enzyme 3-oxo-Δ^5-steroid isomerase (steroid Δ-isomerase; EC 5.3.3.1) of *Pseudomonas testosteroni*, along with a comparison with the free energy profiles for the corresponding nonenzymatic reactions catalyzed by hydroxide ion at pH 7 (Pollack et al., 1989b) and acetate ion (Zeng & Pollack, 1991). This enzyme (KSI;[1] also called Δ^5-3-keto-

† This work was supported by Grant GM 38155 from the National Institute of General Medical Sciences, U.S. Public Health Service.
* Address correspondence to this author at the University of Maryland Baltimore County.

[1] Abbreviations: KSI, 3-oxo-Δ^5-steroid isomerase; TIM, triosephosphate isomerase; EDAC, *N*-ethyl-*N'*-[3-(dimethylamino)propyl]-carbodiimide; EM, effective molarity.

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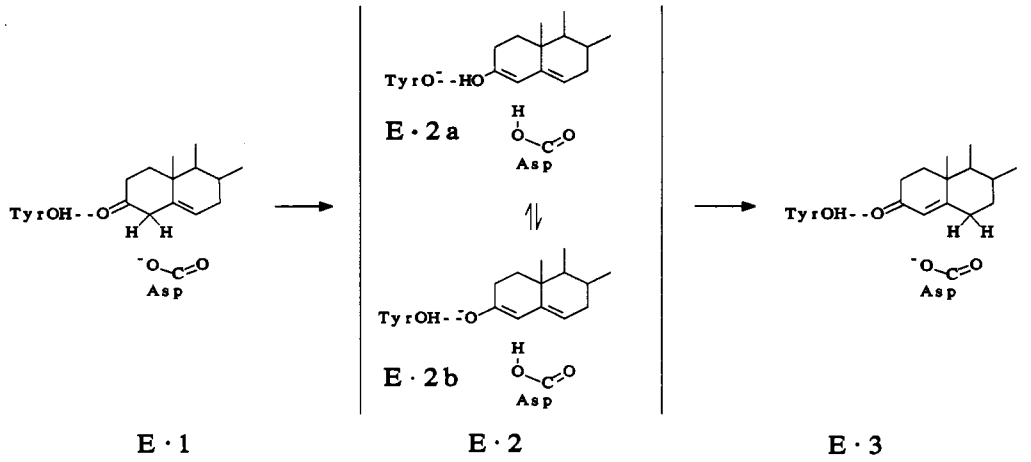
Scheme I



steroid isomerase) catalyzes the isomerization of a wide variety of $\Delta^{5(6)}$ and $\Delta^{5(10)}$ steroids through the formation of an intermediate enzyme-bound dienol(ate) (**2**) (Scheme I) [for recent reviews, see Pollack et al. (1989a) and Schwab and Henderson (1990)]. Both deprotonation of **1** at C-4 and subsequent protonation of **2** at C-6 occur primarily, if not exclusively, on the β face of the steroid with almost complete conservation of the transferring proton (Malhotra & Ringold, 1965; Viger & Marquet, 1977; Viger et al., 1981), suggesting a one-base mechanism. Substantial evidence has accumulated indicating that the amino acid residue that acts as the proton shuttle is Asp-38 (Benisek et al., 1980; Bounds & Pollack, 1987; Kuliopulos et al., 1989). Catalysis is also aided by Tyr-14, which functions as an electrophile to stabilize the transition states for deprotonation/protonation, either by proton donation or by hydrogen bonding to the oxygen at C-3 (Kuliopulos et al., 1989; Zeng & Pollack, 1991).

We have recently developed the methodology to generate the dienol derived from 5-androstene-3,17-dione in situ and shown it to be kinetically competent upon reaction with the enzyme (Eames et al., 1990; Hawkinson et al., 1991). The ability to generate the dienol intermediate and use it as a substrate for KSI allows the direct determination of the partitioning of the intermediate at the active site of the enzyme. We now describe the use of partitioning ratios of **2** with KSI in both water and deuterium oxide to generate the free energy profile for the KSI-catalyzed conversion of **1** to **3**.

Materials and Methods

Materials. KSI was isolated as described previously (Eames et al., 1989). 5-Androstene-3,17-dione (**1**) was prepared by G. Blotny or B. Zeng by deconjugation of 4-androstene-3,17-dione (**3**) (Pollack et al., 1989b). The cyclohexylammonium salt of 17-oxo-3,5-androstadien-3-yl phosphate (**2P**) was prepared by G. Blotny (Hawkinson et al., 1991). Water used for kinetic measurements was double distilled in glass. All other reagents were reagent grade or better. Deuterated acetate buffers were prepared by dissolving an appropriate amount of anhydrous sodium acetate in D_2O (99.9 atom % D) and titrating the solution to the required pD with DCl/D_2O (99 atom % D). pD values are reported as the measured pH value on a Radiometer pHM 85 pH meter plus 0.4 (Glasoe & Long, 1960). Deuterated phosphate buffers were prepared as previously described (Hawkinson et al., 1991).

Determination of Partitioning Ratios. Experiments to determine the partitioning ratio of the dienol (**2**) upon reaction with KSI, both by product studies and by kinetic measurements, were conducted as previously described (Hawkinson et al., 1991). For kinetic studies in deuterated solutions, the flow circuit of the HiTech PQ/SF-53 stopped-flow spectrophotometer was flushed well with D_2O just prior to performing the experiment.

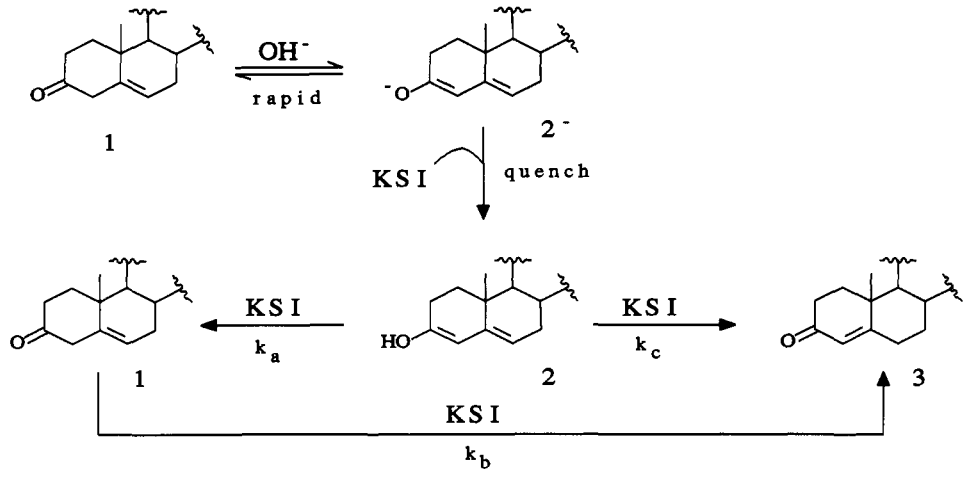
Determination of Steady-State Kinetic Constants. The steady-state kinetic parameters k_{cat} and K_m for the reaction of KSI with **1** under the conditions of the dienol partitioning experiments (330 mM phosphate buffer, pH 7.0, 3.3% MeOH) were obtained as previously described (Pollack et al., 1986).

The inhibition constant (K_i) for 4-androstene-3,17-dione (**3**) in 34 and 330 mM phosphate buffer (pH 7.0, 3.3% MeOH) was determined by the method of Pollack et al. (1979). Accordingly, a solution of **3** in methanol was added to a cuvette containing 3.0 mL of phosphate buffer ($[\mathbf{3}]_{cuvette}$ = ca. 10–90 μM). After temperature equilibration (25.0 °C), 10 μL of a stock solution of 5-androstene-3,17-dione (**1**) in methanol was added to the cuvette ($[\mathbf{1}]_{cuvette}$ = 8 μM) and the reaction initiated by addition of KSI ($[KSI]_{cuvette}$ = 0.12 nM). The change in absorbance at 248 nm due to enzymatic conversion of **1** to **3** was monitored for 5 min (>5 half-lives). Pseudo-first-order rate constants (k^{obs}) were obtained by a single-exponential fit of the absorbance data by nonlinear least-squares analysis. The inhibition constant for **3** was then determined from weighted least-squares analysis of a plot of $1/k^{obs}$ against [**3**].

Results

Partitioning of the Intermediate Dienol. The dienol **2** can be generated in situ by treatment of a solution of **1** with sodium hydroxide (ca. 0.5 N) for about 0.5 s, followed by rapid quenching into a buffer solution (Eames et al., 1990; Hawkinson et al., 1991). Since **1** is quite acidic for a ketone [pK_a 12.7; Pollack et al. (1989b)], 0.5 N sodium hydroxide converts most of **1** to the anion $\mathbf{2}^-$ ($t_{1/2} \approx 35$ ms). Subsequent conversion of $\mathbf{2}^-$ to **3** is much slower ($t_{1/2} \approx 6$ s). Quenching of $\mathbf{2}^-$ with buffer results in protonation on the oxygen to produce the dienol. When the quench solution contains KSI, the rate constants for enzyme-catalyzed conversion of **2** to **1** (k_a) and of **2** to **3** (k_c), along with the rate constant for enzyme-catalyzed conversion of **1** to **3** (k_b), can be determined by monitoring the UV absorbance at 243 nm, the isosbestic point for the conversion of **2** → **3** (Scheme II). An initial drop in absorbance is seen that is due to the reaction of **2** → **1**, followed by an absorbance rise from the subsequent conversion of **1** → **3** (Figure 1). An analysis of the double-exponential curve describing the variation of absorbance with time gives values for the apparent second-order rate constants k_a, k_b, and k_c.

Scheme II



Scheme III

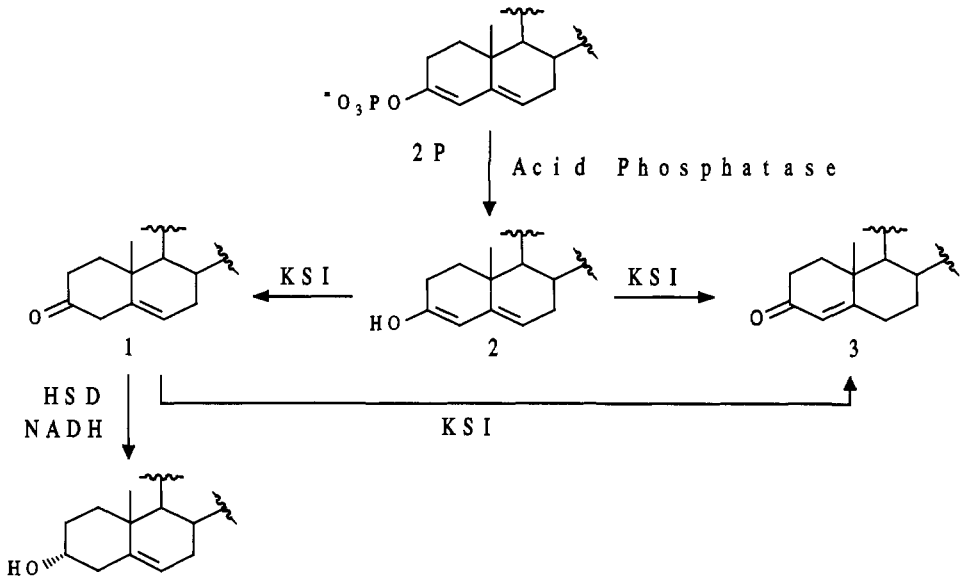


Table I: Rate Constants for the Reaction of **2** with KSI at 25 °C in 333 mM Phosphate, pH 7.0[a-d]

% MeOH	10^8k_a (M^{-1} s^{-1})	10^8k_b (M^{-1} s^{-1})	10^8k_c (M^{-1} s^{-1})	Q ($=k_a/k_c$)[e]
2.5	1.6 ± 0.4	2.1 ± 0.3	2.5 ± 0.8	0.64 ± 0.05
3.3	2.1 ± 0.2	2.1 ± 0.4	2.3 ± 0.3	0.88 ± 0.08
5.8	2.7 ± 0.3	1.5 ± 0.2	2.0 ± 0.2	1.4 ± 0.1
10.0	2.7 ± 0.3	0.67 ± 0.07	1.2 ± 0.1	2.2 ± 0.2
3.3[f] (D_2O)	2.4 ± 0.4	0.46 ± 0.07	1.2 ± 0.2	2.0 ± 0.1
3.3[g]	2.5 ± 0.3	2.5 ± 0.3	3.1 ± 0.4	0.80 ± 0.05

[a] Rate constants are defined in Scheme II and in Hawkinson et al. (1991), eq 2. Second-order rate constants were obtained from duplicate or triplicate runs, each consisting of 5–10 determinations, at three enzyme concentrations. Enzyme concentrations were calculated by using a specific activity of 52000 units/mg for the pure protein (Talalay & Benson, 1972). [b] Enzyme concentrations of 0.05, 0.1, and 0.2 μM. [c] pH values between 6.9 and 7.1 for all runs. [d] Rates of ketonization in the absence of KSI were on the order of 10-fold slower than the enzyme-catalyzed rates. Values of k_a were corrected for the rate of the buffer-catalyzed reaction. [e] Ratios were obtained from individual runs and then averaged. [f] pD values between 7.3 and 7.5. [g] pH 7.7.

Table II: Rate Constants for the Reaction of **2** with KSI in 333 mM Acetate at 25.0 °C, pH 5.0[a-d]

% MeOH	10^8k_a (M^{-1} s^{-1})	10^7k_b (M^{-1} s^{-1})	10^7k_c (M^{-1} s^{-1})	Q ($=k_a/k_c$)[e]
2.5	1.4 ± 0.2	3.9 ± 0.5	6.8 ± 1.1	2.1 ± 0.2
3.3	1.4 ± 0.1	3.7 ± 0.4	6.5 ± 1.2	2.2 ± 0.2
10.0	0.94 ± 0.05	1.2 ± 0.1	3.1 ± 0.6	3.1 ± 0.5

[a] Rate constants are defined in Scheme II and in Hawkinson et al. (1991), eq 2. Second-order rate constants were obtained from duplicate or triplicate runs, each consisting of 5–10 determinations, at three enzyme concentrations. Enzyme concentrations were calculated by using a specific activity of 52000 units/mg for the pure protein (Talalay & Benson, 1972). [b] Enzyme concentrations of 0.1, 0.2, and 0.4 μM. [c] pH values between 5.0 and 5.1 for all runs. [d] Rates of ketonization in the absence of KSI were at least 100-fold slower than the enzyme-catalyzed rates. [e] Ratios were obtained from individual runs and then averaged.

Details of the method used to determine the rate constants have been published previously (Hawkinson et al., 1991).

The ratio Q ($=k_a/k_c$) gives a direct measurement of the partitioning of the KSI–**2** complex (E–**2**) to free **1** and free **3**. These partitioning values were determined for aqueous phosphate buffer solutions with varying concentrations of methanol cosolvent, and for deuterium oxide at pH (pD) values near neutrality, where KSI is maximally active (Table I). With D_2O as the solvent (phosphate buffer, 3.3% methanol), the partitioning toward **1** is more favorable (Q = 2.0) than in H_2O (Q = 0.88). Rate constants were also determined as a function of methanol concentration for acetate buffer at pH 5.0 (Table II). In both buffers, the fraction of **1** formed upon reaction of **2** with KSI increases as the percent methanol increases.

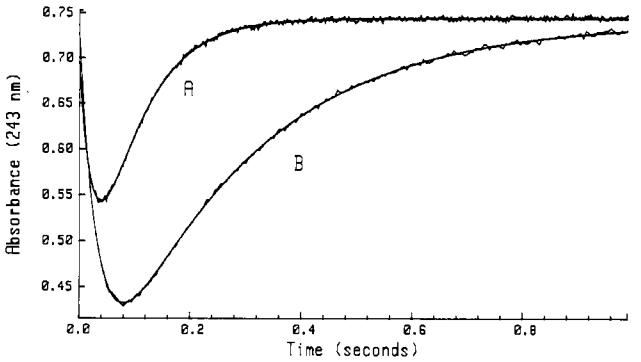


FIGURE 1: Absorbance change at 243 nm for the reaction of dienol **2** with KSI in water (A) and deuterium oxide (B). Solutions of **1** [ca. 5×10^{-4} M in 20% methanol(-*d*)] and 1.0 N NaOH(D) were mixed in a 1:1 ratio and allowed to age for about 0.5 s. This solution was then rapidly quenched with a phosphate buffer solution containing KSI in the observation chamber of a stopped-flow spectrophotometer. Final conditions: (A) 333 mM phosphate, 3.3% methanol, pH 7.3, 25 °C, 0.083 μM KSI; (B) 333 mM phosphate-*d*, 3.3% methanol-*d*, pD 7.4, 25 °C, 0.083 μM KSI. The theoretical lines are calculated from eq 3 of Hawkinson et al. (1991) using the following parameters: (A) $k_a' = 16.8\ s^{-1}$, $k_b' = 13.0\ s^{-1}$, $k_c' = 18.4\ s^{-1}$; (B) $k_a' = 18.1\ s^{-1}$, $k_b' = 3.7\ s^{-1}$, $k_c' = 11.0\ s^{-1}$.

An estimate of the enzymatic partitioning of the intermediate in deuterated acetate buffer was also obtained from generation of the dienol **2** by acid phosphatase catalyzed hydrolysis of the corresponding dienol phosphate in the presence of isomerase (Scheme III). Addition of 3α-hydroxysteroid dehydrogenase (HSD) and NADH allows the nonconjugated Δ^5-ketone (**1**) formed upon initial partitioning of the dienol to be irreversibly trapped as the 3α-alcohol. The relative amounts of Δ^5-3α-alcohol and Δ^4-ketone (**3**) in the reaction mixture were determined by HPLC analysis. Extrapolation of the results at various concentrations of HSD to infinite [HSD] yields the fraction of **3** formed upon the initial partitioning of the intermediate. The reaction of **2** with KSI in deuterium oxide (16 μM acetate-*d*, pD 5.0, 1.7% MeOD) gives 78 ± 1% of **1** ($Q = 3.5$), substantially higher than the 65 ± 5% of **1** ($Q = 1.9$) formed upon reaction of **2** with KSI in water under similar conditions (Hawkinson et al., 1991).

Determination of Steady-State Kinetic Constants and Binding Constants. Steady-state kinetic parameters for KSI-catalyzed conversion of **1** to **3** were determined in solutions of 330 mM phosphate buffer (pH 7.0, 3.3% methanol, $\mu = 0.66$). The value for k_{cat}/K_m ($3.0 \times 10^8\ M^{-1}\ s^{-1}$) is similar to that determined previously under conditions of lower buffer concentration [34 mM phosphate, 3.2% methanol, $\mu = 0.1$, $k_{cat}/K_m = 2.4 \times 10^8\ M^{-1}\ s^{-1}$ (Pollack et al., 1986)]. However, the individual k_{cat} and K_m values in 330 mM buffer ($3.8 \times 10^4\ s^{-1}$ and 118 μM, respectively) are substantially lower than those in 34 mM phosphate ($6.6 \times 10^4\ s^{-1}$ and 277 μM; Pollack et al., 1986). The value of K_i for **3** (148 μM) in 330 mM phosphate buffer (pH 7.0, 3.3% methanol) is similar to the value in 34 mM phosphate (162 μM).

Calculation of the Free Energy Profile. The relative partitioning ratios of the E–**2** complex in H_2O and D_2O, along with steady-state kinetic data, can be used to construct a nearly complete free energy profile for the reaction of **1** to **3** catalyzed by KSI in 3.3% methanol, pH 7, 25 °C (Figure 2). The calculations are based upon the following rationale, using the model of Scheme IV.

Scheme IV

$$E + 1 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{\cdot}1 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E{\cdot}2 \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E{\cdot}3 \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E + 3$$

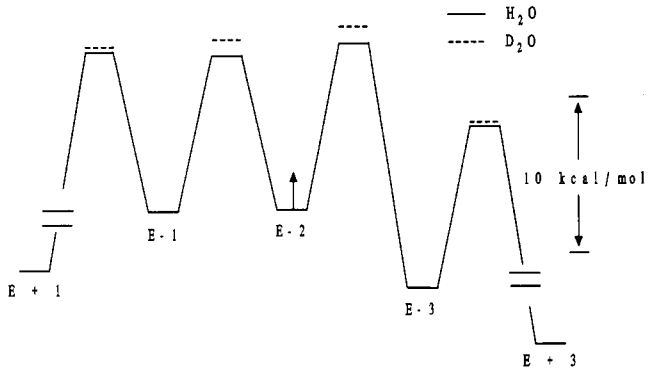


FIGURE 2: Free energy profile for the reaction of 5-androstene-3,17-dione (**1**) with 3-oxo-Δ^5-steroid isomerase to produce 4-androstene-3,17-dione (**3**) in H_2O (solid line) and D_2O (dashed line). The energy of the enzyme–dienol complex (E–**2**) is a lower limit. Relative energies of bound and unbound species depend on concentrations and thus are not specified.

(1) The rate constants for the association of **1** and **3** with KSI are assumed to be diffusion controlled and equal to each other ($k_1 = k_8$).

(2) The fact that the equilibrium constant in solution for **1** ⇄ **3** (K_{ext}) is equal to 2400 (Pollack et al., 1989b), coupled with the assumption that $k_1 = k_8$, requires that the energy of the transition state for dissociation of E–**3** (k_7) be much lower than that for the dissociation of E–**1** (k_2).

(3) The nearly 1:1 ratio of [**1**] to [**3**] formed upon reaction of **2** with KSI results from partitioning of a common intermediate E–**2**. The approximately equal barriers to formation of free **1** and free **3** each consist of two processes, protonation (either at C-4 or at C-6) and diffusion of the product (**1** or **3**) away from the enzyme. The ratio of [**1**] to [**3**] (Q = [**1**]/[**3**] = k_a/k_c) from the partitioning of E–**2** is given by

$$Q = k_r/k_f = [k_2k_4/(k_2 + k_3)]/[k_5k_7/(k_6 + k_7)] \quad (1)$$

where k_r and k_f are the relative rate constants for the formation of free **1** and free **3**, respectively, from E–**2**.

(4) If the diffusion steps were rapid ($k_2 \gg k_3$ and $k_7 \gg k_6$), then the observed product ratio would give k_4/k_5 directly, and thus the rate-limiting step for the chemical process. However, the high value of k_{cat}/K_m for the reaction of KSI with **1** is consistent with the height of the diffusion barrier for KSI + **1** being similar to or greater than the barrier for at least one of the chemical steps; thus k_2 cannot be assumed to be much less than k_3. In contrast, since the barrier height for k_2 is much higher than that for k_7, and the partitioning k_r/k_f is near 1:1, k_6 is almost certainly much less than k_7. Thus, eq 1 can be simplified to

$$Q = k_2k_4/k_5(k_2 + k_3) \quad (2)$$

(5) With the assumption that $k_6 \ll k_7$, the primary isotope effect on k_{cat}/K_m is given by eq 3; the superscript D indicates

$$^{H/D}(k_{cat}/K_m) = \frac{k_1k_3k_5}{^Dk_1{}^Dk_3{}^Dk_5} \frac{^Dk_2{}^Dk_4 + {}^Dk_2{}^Dk_5 + {}^Dk_3{}^Dk_5}{k_2k_4 + k_2k_5 + k_3k_5} \quad (3)$$

the rate constant for reaction in D_2O and a superscript H/D indicates a ratio of reactions in H_2O and D_2O. Combining eqs 2 and 3 gives an expression for the ratio of k_{cat}/K_m for H and D in terms of the partitioning ratio Q (eq 4). Since the

$$^{H/D}(k_{cat}/K_m) = \frac{k_1k_3(^DQ + 1)(^Dk_2 + {}^Dk_3)}{^Dk_1{}^Dk_3(Q + 1)(k_2 + k_3)} \quad (4)$$

comparison that is being made is between protonation of the intermediate in water and deuteration of the intermediate in

deuterium oxide, the appropriate ratio of k_{cat}/K_m is the ratio for *proton* transfer for **1** in H_2O with *deuterium* transfer in D_2O [$^{H/D}(k_{cat}/K_m) = k_b/^Dk_b = 4.6$].

(6) The ratio $k_1/^Dk_1$ may be estimated from the relative rates of diffusion in water and deuterium oxide ($k_1/^Dk_1 \approx 1.2$; Albery, 1975). This ratio, along with the experimentally determined partitioning ratios in water and deuterium oxide, allows eq 4 to be simplified to

$$b = 2.4a + 1.4 \quad (5)$$

where

$$a = k_2/k_3$$

$$b = {}^Dk_2/{}^Dk_3$$

(7) With the assumption that the primary isotope effects on protonation of **2** at C-4 and C-6 are equal ($k_4/^Dk_4 = k_5/^Dk_5$), the observed partitioning ratios in water and deuterium oxide may be expressed as

$$Q/^DQ = (1 + b^{-1})/(1 + a^{-1}) \quad (6)$$

Solving eqs 5 and 6 gives values for the ratio k_2/k_3 in water and deuterium oxide:

$$a = k_2/k_3 = 0.50$$

$$b = {}^Dk_2/{}^Dk_3 = 2.6$$

The ratios for k_4/k_5 in water and deuterium oxide can then be calculated from eq 2:

$$k_4/k_5 = {}^Dk_4/{}^Dk_5 = 2.7$$

(8) The kinetic isotope effect for the k_3 step can be calculated using a value for $k_2/^Dk_2$, which is obtained from the ratio of $k_1/^Dk_1$ and an estimate of $K_s/^DK_s$ ($=^Dk_1k_2/k_1{}^Dk_2$). We assume that the relative ratio of K_s ($=k_2/k_1$) in water and deuterium oxide is the same as the ratio of K_m values for reaction of the 4β-deuterated analogue of **1** ([4β-^{2}H]-**1**) with KSI in water and deuterium oxide ($K_m/^DK_m = 1.28$; Xue et al., 1990). Since the reaction of [4β-^{2}H]-**1** with isomerase shows a substantial kinetic isotope effect on k_{cat}/K_m, this substrate does not react at the diffusion limit. Thus, the ratio of $K_m/^DK_m$ should provide a good estimate of $K_s/^DK_s$ for **1**. Coupled with the fact that diffusion in water is faster than in deuterium oxide ($k_1/^Dk_1 \approx 1.2$; Albery, 1975), this result allows an estimate to be made for the kinetic isotope effect on k_2 ($k_2/^Dk_2 = 1.5$). This ratio, combined with the calculated values for k_2/k_3 and $^Dk_2/^Dk_3$, gives a calculated primary isotope effect of $k_3/^Dk_3 = 7.8$.

(9) A value for k_1 can be calculated from the experimentally determined k_{cat}/K_m and eq 7 ($k_1 = 8.6 \times 10^8$ M^{-1} s^{-1}). The

$$k_{cat}/K_m = \frac{k_1k_3k_5}{k_2k_4 + k_2k_5 + k_3k_5} \quad (7)$$

rate constant k_2 (8.6×10^4 s^{-1}) can be calculated from the ratio K_s ($=k_2/k_1 \approx 100$ μM).[2] From the previously calculated ratio of $k_2/k_3 = 0.50$, k_3 can be determined ($k_3 = 1.7 \times 10^5$ s^{-1}).

(10) The rate constant for dissociation of E–**3** ($k_7 = 1.3 \times 10^5$ s^{-1}) can be obtained from a knowledge of the inhibition constant for **3** ($K_i = 148$ μM $= k_7/k_8$) and the assumption that $k_8 = k_1$. In principle, it should then be possible to calculate k_4 and k_5 from the kinetic expression for k_{cat} (eq 8),

$$k_{cat} = \frac{k_3k_5k_7}{(k_3 + k_4)k_7 + k_5(k_3 + k_7)} \quad (8)$$

since all the other rate constants have been evaluated and the ratio of k_4/k_5 is known. In practice, however, this calculation involves a small difference between large numbers, and only lower limits of about 10^5 s^{-1} for k_5 and 3×10^5 s^{-1} for k_4 can be obtained in this way.

(11) The last remaining rate constant (k_6) is obtained from the fact that k_{cat}/K_m for the reverse reaction (**3** → **1**) is $[k_a/(k_a + k_c)](k_6k_8/k_7)$, and the ratio of k_{cat}/K_m for the forward and reverse reactions is equal to the equilibrium constant in solution [$K_{ext} = 2400$ (Pollack et al., 1989b)]. Thus, $k_6 = [(k_7/k_8)(k_a + k_c)/k_a](k_{cat}/K_m)/K_{ext} = 40$ s^{-1}, where k_{cat}/K_m is the value for the reaction of **1** to **3**. A summary of the calculated rate constants is given in Table III.

Table III: Calculated Rate Constants for the 3-Oxo-Δ^5-steroid Isomerase Catalyzed Conversion of 5-Androstene-3,17-dione (**1**) to 4-Androstene-3,17-dione (**3**) at 25.0 °C[a]

rate constant[b]	$\Delta G^\ddagger$ (kcal/mol)	rate constant[b,c]	$\Delta G^\ddagger$ (kcal/mol)
$k_1 = 8.6 \times 10^8$ M^{-1} s^{-1}		$^Dk_1 = 7.1 \times 10^8$ M^{-1} s^{-1}	
$k_2 = 8.6 \times 10^4$ s^{-1}	10.7	$^Dk_2 = 5.9 \times 10^4$ s^{-1}	10.9
$k_3 = 1.7 \times 10^5$ s^{-1}	10.3	$^Dk_3 = 2.3 \times 10^4$ s^{-1}	11.5
$k_4 > 3 \times 10^5$ s^{-1}	<10.0	$^Dk_4 > 4 \times 10^4$ s^{-1}	<11.2
$k_5 > 1 \times 10^5$ s^{-1}	<10.6	$^Dk_5 > 1 \times 10^4$ s^{-1}	<12.0
$k_6 = 40$ s^{-1}	15.3	$^Dk_6 = 5.0$ s^{-1}	16.5
$k_7 = 1.3 \times 10^5$ s^{-1}	10.5	$^Dk_7 = 8.6 \times 10^4$ s^{-1}	10.7
$k_8 = 8.6 \times 10^8$ M^{-1} s^{-1}		$^Dk_8 = 7.1 \times 10^8$ M^{-1} s^{-1}	

[a] pH 7, 3.3% methanol, 330 mM phosphate buffer. The details of the calculations are given in the text. [b] Calculated as described in the text. [c] Isotope effects for the k_4, k_5, and k_6 steps were assumed to be equal to the isotope effect on the k_3 step.

Discussion

Possible Sources of Error. Before the free energy profile obtained from the calculated rate constants (Figure 2) can be discussed, it is important to consider sources of error in the derived rate constants. A possible source of error in the partitioning ratios comes from the fact that some (or all) of the enzyme-bound intermediate from binding of **2** by KSI might be different from the intermediate generated from **1** and KSI. We have recently considered this question and concluded that there is no compelling evidence that these intermediates are not identical (Hawkinson et al., 1991). Of particular importance is the fact that the stereochemistry of protonation of the enzyme-bound intermediate generated from **2** and KSI is similar to the stereochemistry of protonation during the reaction of **1** → **3** with KSI. When **2** is allowed to react with KSI in deuterium oxide, and the product (**3**) is isolated, ca. 80% of the hydrogens at C-6β are deuterium (Hawkinson et al., 1991). This product comes from (1) direct partitioning of E–**2** to **3** and (2) reaction of E–**2** to **1** followed by isomerization of **1** → **3**. Although some labilization of the 4α hydrogen has been reported for **1** → **3**, hydrogen transfer is primarily 4β to 6β (Viger et al., 1981), so that the predominant stereochemistry of protonation of E–**2** is consistent with the formation of identical intermediates from KSI with **1** and with **2**.

We must also consider the possibility that the 20% of α protonation is a side reaction through a complex *not* on the

[2] Xue et al. (1990) have measured values of K_m for the reaction of [4β-^{2}H]-**1** with KSI in both H_2O (139 μM) and D_2O (109 μM). Since $k_2/k_{cat} \approx 5$–10 for the deuterated substrate, K_m [$=(k_2 + k_{cat})/k_1$] should be only slightly higher than K_s ($=k_2/k_1$). Thus, we use 100 μM as an estimate of K_s. Although the determinations of Xue et al. were done at lower buffer concentrations, there should be only a slight effect of ionic strength since K_i for **3** is virtually identical in 34 and 330 mM phosphate solutions.

reaction pathway from **1** → **3**. Thus, a portion of the reaction may result from the binding of **2** "upside down" to KSI (with Asp-38 at the α side of the steroid), resulting in protonation at C-4α or C-6α. In this case, it would be necessary to correct the observed partitioning for the amount of 4α or 6α protonation. Calculations based on the assumption that all of the α-side protonation is a side reaction at 4α give new values for k_2/k_3 (=0.28) and k_4/k_5 (=2.1). These ratios differ only slightly from the values calculated using the observed partitioning ratios ($k_2/k_3 = 0.5$; $k_4/k_5 = 2.7$), suggesting that there is no substantive error in the free energy profile from a side reaction at C-4α. The other possibility, protonation at 6α, is much less likely since no transfer of proton to this position has ever been reported with KSI. However, if 6α protonation is a side reaction, calculations show that k_2/k_3 could drop to as low as 0.05 and k_4/k_5 could increase to as much as 33. Although these values differ from those calculated using the observed partitioning ratios, the major effect on the free energy profile would be to lower the barrier for interconversion of E–**1** and E–**2** by 1.5 kcal/mol or less. The qualitative nature of the free energy profile would not be affected.

Finally, Xue et al. (1990) have reported secondary hydrogen isotope effects on the KSI-catalyzed reaction of **1** to **3**. On the basis of their results, they state that the rate-determining step is "enolization of the bound substrate" and that "reketonization of the dienol intermediate is a more rapid step". This interpretation, which is based on kinetic isotope effects for **1** substituted with deuterium at the 4α- or 6-position, is clearly inconsistent with the observed partitioning of **2** by KSI. In reevaluating the interpretation of Xue et al. of these isotope effects, we have concluded that their results do not allow for any reliable inference to be made concerning the nature of the rate-determining step (Hawkinson et al., 1991). Briefly, the *predicted* isotope effects for a mechanism with a free energy profile similar to that of Figure 2 and for rate-determining enolization are sufficiently close so that the precision needed to make this distinction is unattainable by the methods of Xue et al. (1990).

Free Energy Profile of KSI. The free energy profile for the KSI-catalyzed conversion of **1** to **3** shows four energy barriers, all of which are kinetically significant (Figure 2, solid line). The transition states for two of the steps are at approximately equal energy (those due to k_1 and k_5 differ by ca. 0.1 kcal/mol), while the energy of the transition state for the k_3 step is only 0.5 kcal/mol lower. Although the transition state for dissociation of product from KSI (k_7) is substantially lower in energy than the three previous transition states, the rate constant for this step is comparable to those in the conversion of E–**1** to E–**3**. The apparent second-order rate constant under subsaturating conditions (k_{cat}/K_m) depends on the rate constants for the first three steps (eq 7). The first of these steps is diffusion (k_1), whereas the second and third are chemical steps (deprotonation, k_3, and protonation, k_5). Since the transition-state energies of each of these steps are comparable, no one step is cleanly rate-limiting, and both chemical steps and the diffusion of **1** and KSI make significant contributions to the rate constant. A comparison of k_1 (8.6×10^8 M^{-1} s^{-1}) with k_{cat}/K_m (3.0×10^8 M^{-1} s^{-1}) shows that the overall rate of reaction with subsaturating levels of substrate is about 35% of the rate of diffusion. For saturating levels of substrate the apparent first-order rate constant (k_{cat}) also depends on three kinetic barriers (eq 8), the two chemical steps (k_3 and k_5) and the rate of dissociation of **3** from KSI (k_7). Since k_7 is comparable in magnitude to k_3 and k_5, both the chemical steps and the loss of product are kinetically important.

Partitioning of E + 2 in D_2O. Dienol **2** reacts with KSI in water at pH 7 to produce primarily **3** (55%), but in deuterium oxide **1** is formed preferentially (66%). At pH 5, **1** is the predominant product in both H_2O and D_2O, but the partitioning toward **1** is greater in D_2O (78% vs 65% in H_2O). This observation can be explained by a consideration of the free energy diagrams for the reaction in both water and deuterium oxide (Figure 2). In water the interconversion of E–**1** and E–**2** is somewhat faster than either the rate of dissociation of E–**1** to E + **1** (k_2) or the rate of protonation at C-6 (k_5) to give E–**3** (which irreversibly dissociates to E + **3**). Thus, in H_2O the partitioning of E + **2** to free **1** and free **3** is controlled primarily by the relative heights of the barriers for k_2 and k_5. The rate-determining step for the formation of free **1** is a diffusion step (k_2), whereas the rate-determining step for the formation of free **3** is a chemical one. (Even though the diffusion step, k_7, makes a contribution to the *rate* of formation of **3** from E–**2**, the *commitment* to forming **3** instead of **1** occurs at the k_5 stage, since $k_6 \ll k_7$.)

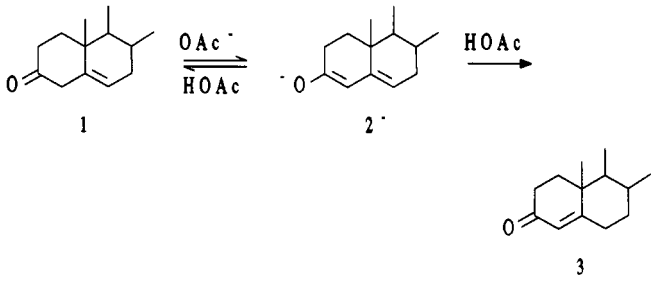
In deuterium oxide (relative to water), there is a significant increase in the heights of the barriers for k_3 and k_5 (the chemical steps), but only a slight increase for k_2 (a diffusion step). Since the rate-determining step for conversion of E–**2** to E + **3** in water is a proton transfer, its rate will be significantly decreased (ca. 8-fold) in D_2O. In constrast, the rate-determining step for the conversion to E + **1** in water is primarily a diffusion step, which will be affected less by the change to deuterium oxide. The rate of the chemical step k_3, however, is decreased sufficiently to become rate-determining for formation of **1**, but only a portion of that effect is seen in the partitioning. Thus, in D_2O, the partitioning of the enzyme-bound dienol is due primarily to the relative heights of the barriers for protonation at C-4 and C-6 (the chemical steps).

It should be noted that the *overall* rate constant ($k_a + k_c$) for reaction of KSI + **2** in water is only 20–25% faster than the reaction in deuterium oxide, even though there are significant isotope effects on the rate constants for reaction of E–**2**. This result suggests that the rate-determining step for the reaction of KSI + **2** is diffusion to give the initial Michaelis complex, consistent with our previous interpretation (Hawkinson et al., 1991).

Effect of Methanol Concentration on the Partitioning of E + 2. As the concentration of methanol is increased, the ratio of [**1**]/[**3**] (=Q) formed from the partitioning of **2** with KSI increases (Tables I and II). This result can be explained by again noting that the rate of formation of free **1** from E–**2** is controlled primarily by a diffusion process, while the rate of formation of free **3** is controlled by a chemical step. The near invariance of the rate constants for diffusion of E + **2** ($k_a + k_c$) with methanol composition of the solvent (Table I) suggests that the rate of diffusion of E + **1** (k_1) does not depend significantly on methanol concentration in this range.[3] Furthermore, the addition of methanol to the solution disfavors binding of steroids (e.g., **1**) to KSI (Falcoz-Kelly et al., 1968). Since k_1 should not change appreciably with solvent composition, the less favorable binding of **1** to KSI should show up primarily as an increase in k_2 as the methanol concentration is increased. Thus, the rate of dissociation of E–**1** (k_2), which is the rate-determining step for the formation of free **1**, should increase, whereas the rate of the chemical step that controls the formation of free **3** (k_5) should not change appreciably,

[3] The viscosity of methanol/water solutions varies by ca. 25% from 2.5% methanol to 10% methanol (Weast & Astle, 1981).

Scheme V



increasing the ratio of [1]/[3].[4]

The above rationale predicts that as the methanol concentration is increased, the ratio k_2/k_3 increases and k_{cat}/K_m for the isomerization of **1** becomes less diffusion controlled. A corollary is that the primary kinetic isotope effect on k_{cat}/K_m for [4β-^{2}H]-**1** should increase in solutions of higher methanol concentration. Weintraub et al. (1980) have indeed shown that to be the case; the primary kinetic isotope effect (k_H/k_D) on k_{cat}/K_m increases from 2.9 in 3.3% methanol to 3.6 in 10% methanol.[5]

Comparison with the Free Energy Profile for the Acetate-Catalyzed Isomerization. We have recently reported the microscopic rate constants for the reaction of **1** → **3** catalyzed by acetate ion, as a model for the active site aspartic acid residue (Asp-38) of KSI (Zeng & Pollack, 1991). This reaction proceeds through the intermediate formation of the dienolate ion **2**⁻ (Scheme V), analogous to the abstraction and readdition of a proton by the aspartate of KSI. The energies of activation for these steps with acetate ion as a catalyst can be superimposed on the corresponding free energy profile for the KSI reaction, with the assumption that acetate associates with steroid with a dissociation constant of 10 M and a rate constant similar to that with the enzyme (Figure 3).[6]

Two points of interest emerge from a comparison of the free energy profiles: (1) The relative barrier heights for protonation at C-4 (k_4) and at C-6 (k_5) are similar for catalysis by acetate ion (k_4/k_5 = 19 for β-side protonation) and by KSI (k_4/k_5 = 3). Although KSI favors protonation at C-6 relative to C-4 compared to catalysis by acetate in solution, the difference is not large (ca. 1 kcal/mol). (2) The equilibrium constant for **1** ⇄ **3** is similar on the enzyme surface (K_{int}) and in solution (K_{ext}). Knowles and co-workers have suggested that the "internal equilibrium constant" should be perturbed in the direction of unity for reversible enzymes, those that operate near equilibrium (Albery & Knowles, 1976b; Knowles, 1987; Burbaum et al., 1989; Burbaum & Knowles, 1989). However, it is not clear whether KSI should be viewed as a reversible enzyme, since its physiological role has yet to be defined.

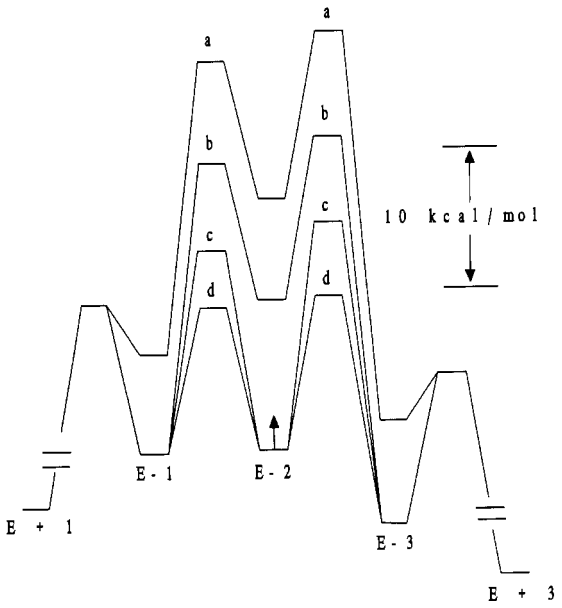


FIGURE 3: Free energy profiles for the reaction of 5-androstene-3,17-dione (**1**) to 4-androstene-3,17-dione (**3**): (a) catalyzed by acetate ion, assuming formation of an encounter complex of **1** and acetate ion with a dissociation constant of 10 M; (b) with uniform binding optimized; (c) with uniform binding and differential binding optimized; and (d) catalyzed by steroid isomerase. Relative energies of bound and unbound species depend on concentrations and thus are not specified.

The catalytic ability of KSI can be dissected into contributions from a combination of uniform binding, differential binding, and catalysis of elementary steps, as proposed by Albery and Knowles for triosephosphate isomerase (Albery & Knowles, 1976a,b, 1977). We consider, in turn, how each of these can lower the activation barrier for the acetate-catalyzed reaction (Figure 3a) and how they can be rationalized in mechanistic terms. The hydrophobic binding pocket provided by KSI undoubtedly provides the interactions necessary to bind steroids and is unlikely to be able to effectively distinguish one steroid from another (Falcoz-Kelly et al., 1968). Since the K_s for **1** is ca. 100 μM, this *uniform binding* would lower the energy of all of the bound species (**1**–**3** and the transition states) by ca. 7 kcal/mol relative to acetate ion (with an assumed K_s of 10 M), without changing the overall equilibria (Figure 3b). This effect alone would correspond to a rate increase in k_{cat}/K_m of about 10^5-fold, although there would be no increase in the reaction of the acetate–**1** complex (k_{cat}).

The next highest level of sophistication, *differential binding*, could be provided by Tyr-14, which is in position to hydrogen bond to the oxygen at C-3 (Kuliopulos et al., 1991). Since the stability of a hydrogen bond is much greater for a charged atom acceptor than a neutral atom acceptor (Weiner et al., 1984; Fersht, 1987, 1988), Tyr-14 of KSI would lower the energies of the intermediate dienolate, and the transition states leading to/from it, relative to the energies of the reactant and product. Stabilization of the intermediate dienolate ion by hydrogen bonding of Tyr-14 to the oxyanion at C-3 by 11 kcal/mol (relative to hydrogen bonding by water) would equalize the energies of E–**1** and E–**2** (Figure 3c).[7] The

[4] It should be noted that the variation of the individual rate constants (k_a and k_c) with solvent composition cannot be interpreted, since they represent partitioning that occurs *after* the rate-determining step (diffusion of **2** plus E) in the reaction of **2** with KSI.

[5] Weintraub et al. (1980) have also calculated values of k_1 for KSI plus **1** at various concentrations of methanol from the primary kinetic isotope effects on k_{cat} and K_m. Unfortunately, the method they used is only valid for systems in which k_3 is the rate-determining step for k_{cat}; thus, their calculation of a 4-fold variation in k_1 from 3.3% to 10% methanol is unreliable.

[6] In order to compare the two reactions, it is necessary to estimate the pseudo-first-order rate constant for the deprotonation and protonation steps for acetate catalysis in an encounter complex analogous to the E·S complex for KSI. We arbitrarily assume that the rate constant within this complex corresponds to that for an acetate concentration of 10 M. The qualitative nature of the analysis to follow is not appreciably changed by somewhat different estimates of the dissociation constant.

[7] Fersht (1987, 1988) has estimated from site-directed mutagenesis experiments that the energy of a hydrogen bond from an uncharged donor to a charged acceptor is about 3–6 kcal/mol. However, calculations by Weiner et al. (1984) suggest that this value might be substantially larger (up to 20 kcal/mol). Of course, there are other possible sources of differential binding of **2** relative to **1**, such as conformational differences between **2** and **1** that may lead to tighter binding of KSI to **2**.

corresponding stabilization of the transition states leading to/from the dienolate would be somewhat less, ca 5–6 kcal/mol, corresponding to an increase in rate of 10^4-fold. Finally, *catalysis of the chemical steps*, that is, deprotonation/protonation, by Asp-38 acting as a general base/acid could account for the balance of the difference in energy (4–5 kcal/mol) between the model system and KSI (Figure 3d). This difference corresponds to the difference between the catalytic abilities of acetate ion and Asp-38 of KSI, after the other factors have been factored out.

Although it is not entirely clear what the source of this "extra" catalytic power is, there are several reasonable explanations. Benisek et al. (1980) have found Asp-38 to be about 100-fold more reactive toward *N*-ethyl-*N'*-[3-(dimethylamino)propyl]carbodiimide (EDAC) catalyzed amidation by cystamine, compared to the other carboxylic acid residues of KSI. This difference, which may be due to desolvation of Asp-38 as the reactants bind in the hydrophobic active site, is likely to be at least as great for steroids as it is for cystamine and EDAC. An enhanced reactivity of this magnitude would account for 3 kcal/mol of the discrepancy between Asp-38 and acetate. It is also possible that the transition states for deprotonation of **1** and protonation of **2** are asymmetrical with the transferring proton closer to the steroid than to Asp-38, although, in model systems, the β value for general base catalysis of isomerization of β,γ-unsaturated ketones is about 0.5 (Whalen et al., 1976).

Other possible contributing factors to the increase in catalytic ability of Asp-38 relative to acetate are proximity and orientation. These effects were implicitly taken into account in the assumption that acetate and **1** form a complex; our estimate of the dissociation constant implies that catalysis from this complex is the equivalent of catalysis of 10 M acetate. Although "effective molarities" (EM) of this magnitude are common for carboxylate-catalyzed enolizations, intramolecular reactions in which a carboxylate acts as a nucleophile commonly show effective molarities of 10^4–10^8 or more (Kirby, 1980). Gandour (1981) has discussed this discrepancy and has concluded that the low EM values for intramolecular general base catalysis by carboxylate in model systems are due to an inability to achieve optimal orientation for proton abstraction. He has estimated that the kinetic basicity of the syn orbitals of the carboxylate oxygens is 10- to 1000-fold greater than that of the anti orbitals. Since in model systems it is generally the anti orbitals that are correctly positioned to abstract a proton, these systems might give a lower effective molarity than could be obtained by a "correctly" oriented carboxylate. However, recent efforts to quantitate this effect have led to no firm conclusion about its magnitude (Tadayoni et al., 1989, 1991; Li & Houk, 1989).

Comparison with the Aqueous Reaction at pH 7. The rate constant for hydroxide ion catalyzed isomerization is ca. 100-fold greater than the rate constant for the hydronium ion catalyzed reaction (Perrera et al., 1980; Pollack et al., 1989b). This observation, coupled with the fact that the rate of enolization of **1** is proportional to $[OH^-]$ at pH values down to ca. 5–6 (B. Zeng and R. M. Pollack, unpublished observations), suggests that in the absence of buffer the predominant mechanism for isomerization of **1** in aqueous solution at pH 7 is hydroxide ion catalysis. We have recently determined the free energy profile for the isomerization of **1** to **3** catalyzed by hydroxide ion (Pollack et al., 1989b). As in the case of the acetate-catalyzed isomerization, the reaction proceeds through the intermediate formation of $\mathbf{2}^-$; the partitioning of the intermediate ($k_4/k_5 = 25$) is comparable for both acetate and hydroxide ion catalysis. A comparison of the k_{cat} value for KSI ($k_{cat} = 3.8 \times 10^4\ s^{-1}$) with the rate of isomerization at pH 7 due to hydroxide ion ($k = 1.7 \times 10^{-7}\ s^{-1}$) gives a rate enhancement due to KSI of ca 10^{11}-fold (15–16 kcal/mol) due to differential binding and catalysis of elementary steps. The analysis above indicates that ca. 10^4-fold of this rate increase is due to Tyr-14, with the rest (ca. 10^7-fold) being due to Asp-38. Kuliopulos et al. (1989) have constructed mutant KSI's with Tyr-14 replaced with Phe (Y14F) and Asp-38 replaced with Asn (D38N). Decreases in k_{cat} relative to the wild type of $10^{4.7}$-fold (Y14F) and $10^{5.7}$-fold (D38N) were observed, in agreement with our conclusions.

Is KSI a "Perfect Enzyme"? The evolutionary development of enzymes has been the subject of much discussion over the last two decades (Albery & Knowles, 1976b; Brocklehurst, 1976; Chin, 1983; Benner, 1989; Burbaum et al., 1989; Pettersson, 1989). Albery and Knowles (1976b) have noted that the maximum apparent second-order rate constant (k_{cat}/K_m) possible for an enzymatic reaction is the rate constant for diffusion of substrate to the enzyme active site in the thermodynamically favorable direction. They have discussed the evolution of enzymes in terms of an efficiency function that describes how nearly k_{cat}/K_m for an enzymatic reaction approaches the rate constant for diffusion, that is, kinetic "perfection". Although it may be argued (Benner, 1989; Pettersson, 1989) that this criterion is not sufficient for "catalytic perfection", enzymes that adhere to the definition of perfection of Albery and Knowles (1976) are clearly optimal catalysts for their size, under conditions of $[S] \ll K_m$.

It is clear from the free energy profile of Figure 2 that k_{cat}/K_m for KSI approaches the diffusion-controlled rate constant. A comparison of k_{cat}/K_m for **1** with k_1 shows that about 35% of the collisions between enzyme and substrate lead to product formation. All four of the transition states for the reaction are kinetically significant. Although the transition state for the dissociation of E–**3** is substantially lower than those for the other three transition states, the rate constant k_7 is of the same order of magnitude as k_3 and k_5 so that passage over this transition state is kinetically important. Thus, any further increase in the chemical steps or the rate of diffusion of the product could only lead to an increase in k_{cat}/K_m of a factor of 3.

The free energy profile for KSI can be compared to the corresponding free energy profile for triosephosphate isomerase (TIM) calculated by Albery and Knowles (1976a) on the basis of isotope exchange measurements.[8] Both enzymes catalyze reactions that involve a sequential enolization and ketonization involving a carboxylate base (Asp for KSI, Glu for TIM). The overall reaction is a 1,2-proton transfer for TIM; for KSI, it is a 1,3-proton transfer. In addition, both enzymes function near the diffusion limit under conditions of $[S] \ll K_m$. When the two free energy profiles are superimposed, using E–S as the reference state (Figure 4), the remarkable similarity of the energetic features of KSI and TIM can be appreciated. The relative differences in energy for all of the intermediates and transition states are within ±3 kcal/mol. For both enzymes, the rate-determining step is the diffusion together of the less stable substrate and the enzyme. Not only has nature made use of a similar mechanism for TIM and KSI, but the enzymes have evolved to have similar free energy profiles.

[8] Recently, however, Rose et al. (1990) have concluded that "... some changes may be needed in the interpretations..." of Albery and Knowles (1976a).

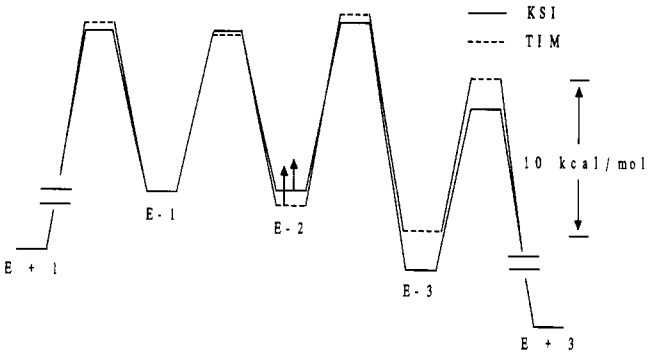


FIGURE 4: Comparison of the free energy profile for steroid isomerase (KSI) catalyzed conversion of 5-androstene-3,17-dione (**1**) to 4-androstene-3,17-dione (**3**) (solid line) and the free energy profile for triosephosphate (TIM) catalyzed conversion of glyceraldehyde 3-phosphate to dihydroxyacetone phosphate (dashed line). Relative energies of bound and unbound species depend on concentrations and thus are not specified.

ACKNOWLEDGMENTS

We thank Dr. G. Blotny and B. Zeng for preparation of **1** and **2P** and Professor Donald J. Creighton for several helpful discussions.

Registry No. **1**, 571-36-8; **2**, 1229-13-6; **3**, 63-05-8; D_2O, 7789-20-0; 3-oxo-Δ^5-steroid isomerase, 9031-36-1.

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

Liang Xue,‡ Paul Talalay,§ and Albert S. Mildvan*,‡
Department of Biological Chemistry and Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Received June 21, 1991; Revised Manuscript Received August 22, 1991

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

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

† This work was supported by NIH Grants DK 07422 (to P.T.) and DK 28616 (to A.S.M.).
‡ Department of Biological Chemistry.
§ Department of Pharmacology and Molecular Sciences.

0006-2960/91/0430-10858$02.50/0