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Kinetic Competence of an Externally Generated Dienol Intermediate with Steroid Isomerase†

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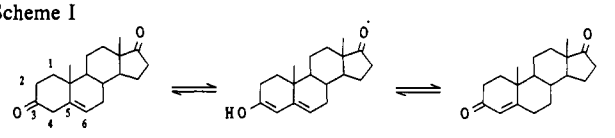
ABSTRACT: The putative intermediate dienol (**2**) in the steroid isomerase (KSI) catalyzed conversion of 5-androstene-3,17-dione (**1**) to 4-androstene-3,17-dione (**3**) has been independently generated and tested as a substrate for KSI. At pH 7, dienol **2** is converted by KSI to a mixture of **1** (46%) and **3** (54%). The apparent second-order rate constant for reaction of **2** with KSI to produce **3** ($k_{cat}/K_m = 2.3 \times 10^8$ M^{-1} s^{-1}) is similar to that for reaction of **1** with KSI ($k_{cat}/K_m = 2.1 \times 10^8$ M^{-1} s^{-1}), demonstrating that **2** is kinetically competent. Isomerization of **1** by KSI in D_2O gives only 5% of solvent deuterium incorporated into the product **3**. When **2** reacts with KSI in D_2O, and the product **3** is isolated (from direct reaction of **2** and from subsequent conversion of the **1** initially formed), ca. 80 atom % deuterium is located at C-6β, confirming that protonation of the dienol by KSI occurs at the same face as the proton transfer in the KSI catalyzed reaction of **1** to **3**.

The 3-oxo-Δ^5-steroid isomerase (KSI)[1] from *Pseudomonas testosteroni* catalyzes the isomerization of a variety of Δ^5-3-oxosteroids to their conjugated isomers [see Pollack et al. (1989b) for a review]. This reaction serves as a prototype for a variety of enzyme-catalyzed allylic rearrangements that involve a 1,3-hydrogen shift between carbon atoms (Schwab & Henderson, 1990). KSI is an extremely efficient enzyme, with k_{cat}/K_m values approaching the diffusion-controlled limit for 5-androstene-3,17-dione and 5-pregnene-3,20-dione as substrates. Early studies (Wang et al., 1963; Malhotra & Ringold, 1965) suggested that the enzymatic isomerization

Scheme I



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[1] Abbreviations: KSI, 3-oxo-Δ^5-steroid isomerase; HSD, 3α-hydroxysteroid dehydrogenase; PAGE, polyacrylamide gel electrophoresis; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

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proceeds through the intermediate formation of a dienol (Scheme I), although no direct evidence was presented until 1986, when it was shown that a trienol analogue of the dienol intermediate could serve as a substrate for KSI at pH 4.5 (Bantia & Pollack, 1986). Subsequently, we demonstrated that the dienol 3-hydroxy-3,5-androstadiene-17-one (**2**) is converted to a mixture of **1** and **3** by KSI at pH 5.0 (Eames et al., 1990).

Since the optimal pH range for the KSI reaction is 7–9 and the enzymatic rate depends upon the ionization of a group with a pK_a of about 4.6 (Pollack et al., 1986), the relevance of these findings to the mechanism of action of KSI may be questioned. Thus, at pH $\leq$ 5 a significant fraction of the enzyme is in the normally unreactive protonic state. For example, at pH 4.5, approximately 50% of the catalytically important functional group (Asp-38) is protonated, and the reactions of the dienol and trienol intermediates may be catalyzed by the substantial proportion of the enzyme in the "wrong" protonation state.

We now report that the dienol **2** reacts rapidly with KSI at pH values of $\geq$7, where KSI is maximally active, and where the predominant protonation state of the enzyme is the one that catalyzes the reaction of **1** to **3**. Furthermore, **2** reacts with KSI *faster* than the β,γ-unsaturated ketone substrate (**1**) and is thus kinetically competent, even though only 1 part in 10^5 of the enzyme is in the ionization state predicted to catalyze the ketonization of **2**. Direct measurement of the partitioning of the putative intermediate **2** at pH 7 shows that reaction of **2** with KSI gives approximately equal amounts of **1** and **3**. We discuss the mechanism of the reaction of **2**, the kinetic competence of **2**, and the apparent discrepancy between our results and the conclusions reached by Xue et al. (1990) concerning the nature of the rate-limiting step for the KSI-catalyzed conversion of **1** to **3**.

Materials and Methods

Materials. KSI was isolated and purified as described previously (Eames et al., 1989). 5-Androstene-3,17-dione (**1**) was prepared by B. Zeng or G. Blotny by isomerization of 4-androstene-3,17-dione (**3**) (Pollack et al., 1989a). Water used for kinetic measurements was double distilled in glass; all other reagents were reagent grade or better. Deuterated phosphate buffers were prepared by adding 10 g of phosphoric acid-d_3 (Aldrich, 85 wt % in D_2O, 99+ atom % D) to 100 g of D_2O (Aldrich, 99.9 atom % D). About 15 g of a 40 wt % solution of NaOD/D_2O (Aldrich, 99 atom % D) was then added. The total phosphate concentration of this solution was calculated to be about 800 mM. Phosphate-*d* buffers of lower concentration were prepared by diluting this stock solution with D_2O. pD values of the solutions were adjusted with either DCl or NaOD and are reported as the measured pH value on a Radiometer pHM 85 pH meter plus 0.4 (Glasoe and Long, 1960). Tris buffers were prepared in deuterium oxide by dissolving 0.138 g of Tris and 1.173 g of Tris-HCl in ca. 8 mL of D_2O. This solution was frozen and lyophilized. After a second lyophilization from D_2O, the resultant powder was dissolved in 250 mL of D_2O to give a 34 mM solution of Tris-*d* with pD 7.81.

The bis(cyclohexylammonium) salt of the dienol phosphate of Scheme II (**2P**) was prepared by G. Blotny from the corresponding dimethyl ester (Blotny & Pollack, 1988) by trimethylsilyl bromide cleavage by the method of Dzingeleski et al. (1990): mp 173–175 °C; ^1H NMR (D_2O, DDS) δ 5.37 (m, 1 H) and 5.69 (s, 1 H); UV (H_2O) λ_{max} 238.5, ϵ 21 000); one spot tlc (silica Merck 60, 1:1 methanol/chloroform, R_f 0.39). Anal. Calcd for $C_{31}H_{52}N_2PO_5$: C, 65.92; H, 9.45; N, 4.96; P, 5.48. Found: C, 65.93; H, 9.44; N, 5.05; P, 5.55.

3α-Hydroxysteroid dehydrogenase (HSD) was isolated from dried cells of *P. testosteroni* (Sigma H7127) by J. P. G. Mack. This enzyme elutes before KSI during the DEAE step of the KSI preparation (Kayser et al., 1983). The enzyme was stored frozen (−70 °C) in the DEAE eluting buffer. Analysis by SDS–PAGE showed a major band at 28 kDa and two minor bands. Activities were assayed by observing the change in absorbance at 260 nm due to NADH → NAD for reduction of 5α-androstane-3,17-dione (Sigma) at pH 5.0 or the change in absorbance at 340 nm at pH 9.0 due to NAD → NADH for oxidation of 3α-hydroxy-5α-androstane-17-one (Sigma) with 30 μM steroid and 200 μM NAD(H) (Boyer et al., 1965). A unit of activity was defined as that amount of enzyme that catalyzes the conversion of 1 μmol of substrate to products in 1 min under these conditions. No 3β-hydroxysteroid activity (<0.1% of total activity) was found with 3β-hydroxy-5α-androstane-17-one as a substrate. The HSD activity toward reduction of **3** was approximately 0.1% of that toward reduction of **1**. No KSI activity was apparent in the preparation of HSD.

Partitioning Ratio by Product Studies. To 240 μL of 2.1 mg/mL sweet potato acid phosphatase (Type X, Sigma) in 20 mM acetate (pH 5.0) were added 15 μL of 4 mM NADH (1.0 mM TES, pH 7.0), 0–40 μL of HSD (48 units/mL), 1.4 or 4.1 μL of 1.9 μM KSI (34 mM phosphate, pH 7.0), and enough water to bring the total volume to 300 μL (final pH 5.1). Five microliters of a methanol solution of the cyclohexylammonium salt of 17-oxo-3,5-androstadien-3-yl phosphate (**2P**) was then added to give a final steroid concentration of either 3 or 30 mM. After a 70-s incubation period, the amount of 4-androstene-3,17-dione (**3**) present in the reaction mixture was determined by HPLC analysis with an electronic integrator (Waters Nova-Pak C_{18} column, 65% MeOH, UV detection at 248 nm, retention time of **3** = ca. 6.7 min). Peak integration counts for individual runs were divided by the average of the integration counts for runs with no HSD present to give values of the fraction of **3** present (f_3).

As a control, identical experiments were performed with samples of **3** instead of **2P** in the original incubation mixture. These experiments showed that at high concentrations of HSD, some of the **3** is reduced at the 17-position to yield small amounts of testosterone. The relatively small correction factors calculated from these experiments were then applied to the values of f_3 obtained at high concentrations of HSD.

Kinetics. Solutions of **1** were used within 2 h of preparation to avoid excessive formation of **3** by isomerization of **1**. Enzyme solutions were made up immediately before use. The dienolate ion (2^-) was prepared by mixing a 5×10^{-4} M solution of **1** in 20% methanol/water with an equal volume of aqueous 1.0 M sodium hydroxide with the two syringes in drive 1 of a HiTech PQ/SF-53 stopped-flow spectrophotometer. After a 0.5-s delay, the dienolate solution was rapidly mixed with buffer solution in a 1:5 ratio, with drive 2 of the spectrophotometer, to give the dienol (**2**). Kinetics of the conversion of **2** to a mixture of **1** and **3** at 25.0 °C in the presence of KSI were determined with buffer concentrations of 400 mM in the initial solutions (before mixing). Final buffer compositions (330 mM) were determined by calculation of the amount of buffer neutralized with the sodium hydroxide in the dienolate solution. The pH of the final solutions was obtained by measurements of independently mixed aliquots of the solutions. The kinetics were monitored at 243 nm, the isosbestic wavelength for **2** → **3**. Independent determinations of the rate constants for the KSI-catalyzed isomerization of **1** to **3** (enzyme assays) were obtained by following the same

procedure, except that water was substituted for the sodium hydroxide solution in drive 1. As no neutralization takes place upon mixing with the buffer solution in drive 2, this solution was prepared such that the final concentrations of buffer species are identical with those in the corresponding dienol partitioning experiment.

The data from each run were fit to a single exponential equation (enzyme assays and buffer rates for **2** → **1**) or a double-exponential equation (partitioning experiments) with the stopped-flow software. Fits in all cases were excellent, showing random deviations between observed and calculated values. The loss of absorbance at 243 nm (A_0) for complete conversion of **2** to **1** was obtained from the preexponential term of the single-exponential fit of the absorbance (A) vs time (t) curve ($A = A_0e^{-k_2t}$) for buffer catalysis. The data for the partitioning experiments were fit to a double exponential ($A = A_1e^{-(k_a'+k_c')t} + A_2e^{-k_b't}$). Normalization by division of this equation by A_0 gives eq 3 of the text, where $[\mathbf{1}]/[\mathbf{2}]_0 = A/A_0$, $B = A_1/A_0$ and $[\mathbf{1}]/[\mathbf{2}]_0 - B = A_2/A_0$. The value of k_a' was calculated from the expression $B = A_1/A_0 = k_a'/(k_a' - k_b' - k_c')$.

A_0 values are the average of 10–12 runs; agreement between runs was generally better than ±2%. Rate constants for the partitioning experiments are the average of 5–10 runs, with measurements made immediately after the determination of the corresponding A_0 values for each solution. Partitioning ratios were calculated for each run separately and then averaged. Enzyme decomposition was shown to be less than 10% during a series of runs, as judged from the variation of rate constants and from independent enzyme assays. Three independent sets of data (10–12 runs each) were obtained for each determination of rate constants. Values of k_a' were corrected by subtraction of the corresponding rate constants for buffer-catalyzed conversion of **2** to **1**, although these corrections are quite small.

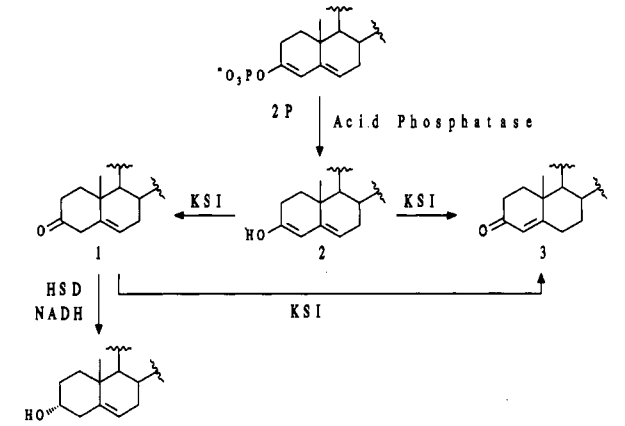
NMR Measurements. Alkaline phosphatase (Sigma, Type III-S, suspension in 2.5 M ammonium sulfate) was exchanged with deuterium by centrifugation (10 min at 13000 rpm), resuspension of the pellet in D_2O, and centrifugation of the D_2O suspension. The resulting alkaline phosphatase solution was decanted from the pellet and used immediately.

Eight hundred microliters of the alkaline phosphatase solution was added to 30 mL of 34 mM Tris-*d* buffer (pD 7.81, 1.2% MeOD) containing 0.4 μM KSI. Six hundred fifty microliters of a 5 mg/mL solution of **2P** in MeOD was then added with stirring. After 5 min of incubation at room temperature (sufficient time for complete hydrolysis of **2P**), the reaction mixture was passed through a C_8 SPICE cartridge (Analtech). The **3** retained in the cartridge was then eluted with methylene chloride. After being dried over anhydrous $MgSO_4$, the methylene chloride solution of **3** was evaporated to dryness. Mass spectral analysis of this sample showed incorporation of ca. 1.2 atom % D.

This sample of **3** was dissolved in 625 μL of 80% MeOD–D_2O and the NMR spectrum was obtained at 500 MHz (16 scans, 90° pulse width, 12-s delay time). NaOD/D_2O (40 wt %, 6.8 μL) was then added to the NMR tube ($[NaOD]_{final}$ = 0.15 M), and the NMR spectrum was obtained approximately every 5 min for 20–25 min. Comparison of these spectra with those for an unlabeled sample of **3** demonstrates that ca. 80% of the hydrogens at C-6β were deuterium.

Mass Spectral Measurements. The general procedure involved the addition of 5.3 μL of ca. 190 μM KSI to 20 mL of deuterated buffer (phosphate or Tris) containing 2.1% MeOD. The solution was stirred for 45–60 s, followed by the addition of 250 μL of a freshly prepared solution of steroid in MeOD. The reaction mixture was immediately poured into a separatory funnel with 5 mL of chloroform and extracted twice. The combined chloroform extracts were dried over anhydrous magnesium sulfate and concentrated to ca. 0.5 mL before analysis on a Hewlett Packard HP 5988A mass spectrometer. To enhance the sensitivity of the mass spectral measurements, peaks near the m/e of the molecular ion were selectively monitored. As a control, this procedure was repeated with **3** in both deuterated and nondeuterated buffers; reactions with **3** and KSI showed no incorporation of deuterium under these conditions.

Scheme II



The samples were spotted on silica tlc plates and developed with 1:1 hexane/ethyl acetate. All samples showed a major spot at R_f = 0.38–0.44, indicating the presence of **3**; some samples also showed a very faint spot at R_f = 0.8. Detection with iodine showed no additional spots, indicating no residual **1**.

The fraction of deuterium incorporated into **3** during isomerization (f_D) was calculated from the ratios of $(M + 1)/[M + (M + 1)]$ of the **3** produced by isomerization of **1** and from the **3** isolated from the control experiment in a nondeuterated buffer, assuming a maximum incorporation of one deuterium per molecule of **1**. Corrections were made for natural abundance of either one ^{13}C or one deuterium per molecule. The validity of the analysis was checked by comparison of calculated ratios of $(M + 1)/(M + 2)$ with observed values.

Results

Partitioning of the Dienol from Product Studies. Dienol **2** was generated by the action of acid phosphatase on the corresponding dienol phosphate (**2P**) in the presence of 0.01–0.03 μM KSI in acetate buffer (pH 5.1, 16 mM acetate, 1.7% methanol, ca. 22°C). This concentration of KSI is sufficient to trap >99% of the dienol before it is isomerized by buffer (vide infra). Upon reaction with KSI, dienol **2** can partition to form both the Δ^5-ketone (**1**) and the Δ^4-ketone (**3**). The Δ^5-ketone is then isomerized to the Δ^4-ketone by the action of KSI. In order to determine the relative amounts of the two isomers initially formed, 3α-hydroxysteroid dehydrogenase (HSD) and NADH were added to irreversibly trap **1** as the 3α-ol (Scheme II). The amount of **3** formed during the reaction was determined by HPLC analysis. This procedure was repeated at various concentrations of HSD (constant [KSI]).

The fraction of **3** formed (f_3) and concentrations of HSD were fit to eq 1 by nonlinear least-squares analysis to give c_1, which corresponds to extrapolation of f_3 to the limit of infinite concentration of HSD (Figure 1). The average of these results

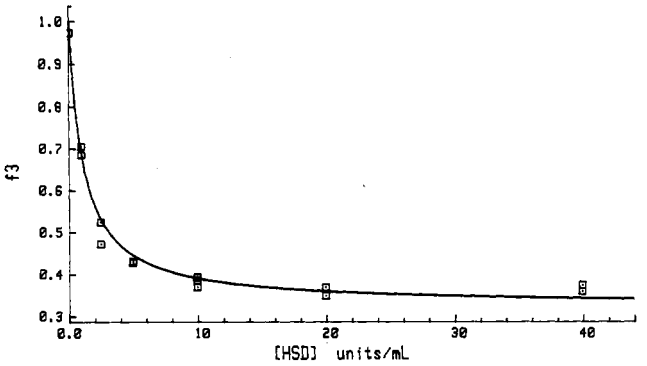


FIGURE 1: Plot of the fraction of **3** recovered from the reaction of dienol **2** with KSI in the presence of HSD (f_3) against the concentration of HSD. The dienol was generated by the action of sweet potato acid phosphatase on **2P** (30 μM) in the presence of KSI (0.03 μM), HSD, and NADH (200 μM) in an acetate solution (11 mM acetate, pH 5.0, 1.7% MeOH). The theoretical line (eq 1) is calculated from the following parameters: $c_1 = 0.324$ and $c_2 = 0.938$.

Table I: Percent Formation of 4-Androstene-3,7-dione (from Reaction of Dienol **2** with KSI)[a]

initial [2] (μM)	[KSI] (μM)	% 3 formed
3	0.01	39.1 ± 2.8
		37.7 ± 1.4
3	0.03	42.4 ± 1.7
30	0.01	32.5 ± 1.0
30	0.03	29.6 ± 2.3

[a] Calculated from eq 1 of the text.

obtained over a 3-fold range of [KSI] and a 10-fold range of [**2P**] (Table I) shows that 35 ± 5% of **3** is formed at pH 5.

$$f_3 = c_1 + (1 - c_1)/(1 + c_2[\mathrm{HSD}]) \qquad (1)$$

The parameter c_2 is related to the ratio of the rates of isomerization and reduction of **1** by KSI and HSD. A derivation of eq 1 is given in the Appendix.

Generation of the Dienol for Kinetic Studies. We have previously shown (Pollack et al., 1989a) that the isomerization of **1** to **3** catalyzed by sodium hydroxide occurs through the intermediacy of the anion $\mathbf{2}^-$ and that this anion is formed rapidly and reversibly with a half-life of ca. 35 ms in 0.5 M NaOH. Subsequent protonation of $\mathbf{2}^-$ on C-6 by water to give **3** is relatively slow ($t_{1/2} \approx 6$ s). Thus, **1** was mixed in a sequential mixing stopped-flow spectrophotometer with sodium hydroxide, followed by quenching with buffer after ca. 0.5 s to produce a solution of the dienol **2**. Calculations based upon the known rate constants for hydroxide ion catalyzed isomerization of **1** to **3** (Pollack et al., 1989a) indicate that the composition of the solution at the time of quenching is 12% **1**, 83% **2**, and 5% **3**.

When **2** is generated in aqueous acetate or phosphate buffer solutions, protonation occurs preferentially on C-4 to give almost exclusively the Δ^5-unsaturated ketone **1**, resulting in a pseudo-first-order loss of virtually all of the ultraviolet absorbance in the region near 240 nm. Rate constants for the disappearance of **2** were determined in the absence of KSI in acetate buffer solutions (333 mM, pH 5.1) and phosphate buffer solutions (333 mM, pH 7.0 and 7.7) with 3.3% methanol as a cosolvent. Rate constants are substantially higher in phosphate ($k^{obsd} = 1.39 \pm 0.05$ s^{-1} at pH 7.0 and 1.76 ± 0.02 s^{-1} at pH 7.7) than in acetate ($k^{obsd} = 0.155 \pm 0.004$ s^{-1}). In each case, however, the rate constants are sufficiently slow such that subsequent measurements of the reaction of **2** with KSI require only minimal corrections.

Determination of the Ultraviolet Spectrum of **2**. The ultraviolet spectrum of **2** was determined indirectly by examining

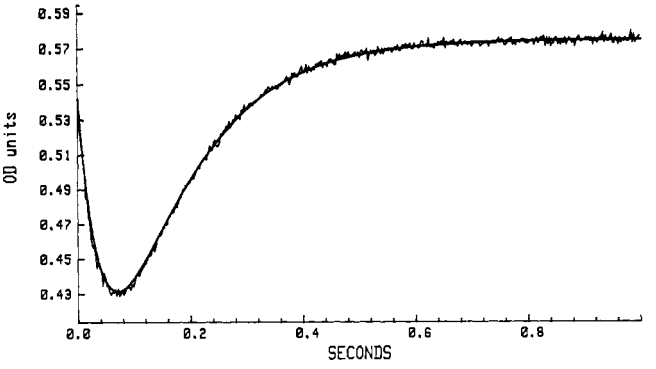
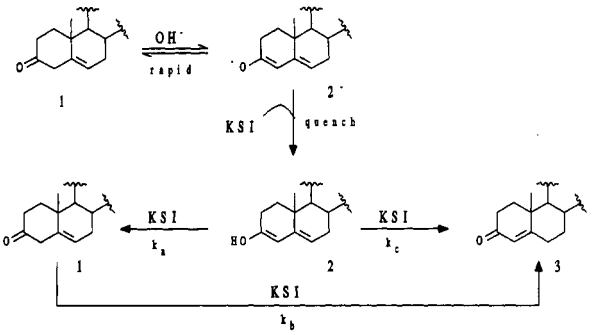


FIGURE 2: Absorbance change at 243 nm for the reaction of dienol **2** with KSI. Solutions of **2** (5×10^{-4} M in 20% MeOH) and 1.0 N NaOH were mixed in a 1:1 ratio and allowed to age for about 0.5 s. This solution was then rapidly quenched with 5 volumes of a pH 6.8 phosphate solution containing KSI (400 mM phosphate, 2.0% MeOH, and 0.050 μM KSI) in the observation chamber of a stopped-flow spectrophotometer (final pH 7.3, 3.3% MeOH, 25.0 °C). The theoretical line is calculated from the following parameters: $k_a' = 9.6$ s^{-1}, $k_b' = 7.7$ s^{-1}, and $k_c' = 11$ s^{-1}.

Scheme III



the variation in amplitude of the first-order loss of absorbance as a function of wavelength. Amplitudes for the conversion of **2** to **1** ($A_\lambda^{2\rightarrow1}$) were calculated for kinetic runs at 1-nm intervals from 230 to 255 nm in acetate buffers (230 mM, pH 5.1, and 333 mM, pH 5.1). Since **1** does not absorb appreciably in the ultraviolet, these amplitudes represent the absorbance of **2** at the wavelength used. The amplitude for the KSI-catalyzed conversion of **1** to **3** at 248 nm ($A_{248}^{1\rightarrow3}$) was measured under identical conditions. This value, along with the fraction of **2** present at the time of the quench (f_2) and the extinction coefficient of **3** at 248 nm ($\epsilon_{248}^3 = 16\,300$ M^{-1} s^{-1}) gave the concentration of **2**. Extinction coefficients for **2** at each wavelength (ϵ_λ^2) were then calculated from eq 2.

$$\epsilon_\lambda^2 = -(A_\lambda^{2\rightarrow1})(\epsilon_{248}^3)/(A_{248}^{1\rightarrow3})(f_2) \qquad (2)$$

The calculated absorbance spectrum of **2** ($\lambda_{max} = 236$ nm, $\epsilon = 17\,500$ M^{-1} cm^{-1}) is reasonable in light of the absorbance characteristics of the corresponding dienol phosphate **2P** ($\lambda_{max} = 237.5$ nm, $\epsilon = 12\,800$ M^{-1} cm^{-1}).

Partitioning of the Dienol from Kinetic Measurements. When **2** is generated in the presence of KSI in the buffer, the variation of absorbance with time is complex. An initial loss of absorbance near 240 nm is followed by a slower increase (Figure 2). Furthermore, the magnitude of the initial loss of absorbance and the subsequent increase varies with the wavelength. At lower wavelengths (ca. 230 nm), a relatively large initial drop in absorbance is followed by a smaller increase. At higher wavelengths, the initial drop is smaller and the subsequent increase is somewhat larger. We interpret this phenomenon in terms of a partitioning of the dienol **2** to the nonabsorbing Δ^5-ketone (**1**) and the absorbing ketone **3**, followed by enzymatic conversion of **1** to **3** (Scheme III).

Table II: Rate Constants for the Reaction of **2** with KSI (3.3% Methanol)[a]

pH	$10^{-8}k_a$ (M^{-1} s^{-1})	$10^{-8}k_b$ (M^{-1} s^{-1})	$10^{-8}k_c$ (M^{-1} s^{-1})	$k_c/(k_c + k_a)$[b] (%)
5.1[c]	1.4 ± 0.1	0.37 ± 0.04	0.65 ± 0.12	31.4 ± 3.1
7.0[d]	2.1 ± 0.2	2.1 ± 0.4	2.3 ± 0.3	53.2 ± 3.1
7.7[e]	2.5 ± 0.3	2.5 ± 0.3	3.1 ± 0.4	55.4 ± 2.0

[a] Rate constants are defined in eq 3. Apparent 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 52 000 units/mg for the pure protein. [b] Ratios were obtained from individual runs and then averaged. [c] Enzyme concentrations of 0.1, 0.2, and 0.4 μM. pH values between 5.0 and 5.1 for all runs. Rates of ketonization in the absence of KSI were at least 100-fold slower than the enzyme-catalyzed rates. [d] Enzyme concentrations of 0.05, 0.1, and 0.2 μM. pH values between 6.9 and 7.1 for all runs. 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 corrected for the rate of the buffer-catalyzed reaction. [e] Same as *d*, except pH 7.7.

In order to simplify the kinetic analysis, the reaction was monitored at the isosbestic point for the conversion of **2** to **3** (243 nm). Since both **2** and **3** have identical extinction coefficients at this wavelength and **1** is UV-transparent, the extent of loss of absorbance at the isosbestic wavelength is proportional to the concentration of **1**. The concentration of **1** at time t is given by eq 3.

$$[\mathbf{1}]/[\mathbf{2}]_0 = B\, e^{-(k_a'+k_c')t} + ([\mathbf{1}]_0/[\mathbf{2}]_0 - B)\, e^{-k_b't} \quad (3)$$

where $[\mathbf{X}]_0$ = initial concentration of **X**

$$B = k_a'/(k_b' - k_a' - k_c')$$

$$k_x' = k_x[\mathrm{E}] = (k_{cat}/K_m)_x[\mathrm{E}]$$

The experimental data were fit to a double-exponential curve by nonlinear regression analysis, giving values of k_a', k_b', and k_c' for each individual run. Rate constants were determined in acetate (pH 5.1) and phosphate (pH 7.0 and 7.7) buffers at 333 mM total buffer concentration (3.3% methanol, 25.0 °C), with KSI concentrations varying from 0.1 to 0.4 μM (acetate) and 0.05 to 0.2 μM (phosphate). Values for the apparent second-order rate constants (k_a, k_b, and k_c) are given in Table II. Rate constants for the reaction **1** to **3** in the presence of KSI were measured independently and were found to be indistinguishable from those determined from the double-exponential fit for the reaction of **2** with KSI (k_b).

Incorporation of Deuterium during Reaction of **1** *with KSI in* D_2O. The extent of proton exchange during the KSI-catalyzed isomerization of **1** to **3** was examined by isomerizing **1** to **3** with KSI in deuterium oxide. The amount of deuterium incorporated into the final product (**3**) was determined by mass spectrometry (Table III). The extent of incorporation of solvent deuterium is quite small (ca. 5%) at neutral pH in both phosphate and Tris buffers. If the substrate is allowed to incubate in phosphate buffer for a few minutes before addition of KSI, the amount of incorporation goes up dramatically (17% after 5 min of incubation, 44% after 20 min of incubation), presumably due to buffer-catalyzed exchange of **1** with solvent. Previous workers (Viger et al., 1981) have reported a larger amount of exchange with D_2O during the isomerization of **1** to **3** (22% at 25 °C, 51% at 30 °C). In that work, however, substrate was added to phosphate buffer before addition of KSI. It is not clear from the description of this work how long the substrate was allowed to incubate before KSI was added. Even a few minutes, however, might be enough to account for the discrepancy between the previous results and the present ones.

Table III: Amount of Deuterium Incorporation from Solvent D_2O in the KSI-Catalyzed Isomerization of **1** to **2**

conditions	mole fraction of D incorporated
34 mM phosphate (pD 7.78)	0.043 ± 0.010[a]
	0.054 ± 0.004[b]
	0.176 ± 0.006[c]
	0.440 ± 0.004[d]
800 mM phosphate (pD 7.78)	0.050 ± 0.003
34 mM Tris (pD 8.01)	0.060 ± 0.003
	0.055 ± 0.002[e]

[a] Average of three runs. [b] Stock solution of **1** in methanol aged 20 h before use. [c] Substrate incubated in buffer solution 5 min before addition of KSI. [d] Substrate incubated in buffer solution 20 min before addition of KSI. [e] Enzyme incubated in buffer solution 5 min before addition of KSI.

Stereochemistry of Protonation of **2** *by KSI.* In order to ascertain the stereochemistry of protonation of the dienol (4α vs 4β and 6α vs 6β), dienol **2** was produced by the action of alkaline phosphatase on the dienol phosphate **2P** in the presence of KSI in deuterium oxide (pD 7.9). Thus, enzymatic protonation of the dienol would occur with a deuterium. The resulting **3** was isolated and analyzed by mass spectrometry, showing the incorporation of about 1.2 atoms of deuterium per molecule.

Relative amounts of deuterium at C-4 and C-6 were obtained by ^{1}H NMR. There is substantial overlap of the C-2β, C-6β, and C-16β protons in the NMR spectra of **3** in both deuterated chloroform and deuterated methanol. However, the C-2β and C-16β protons exchange rapidly in basic methanol/water solutions relative to the protons at C-2α and C-6 (α and β) (Pollack et al., 1989a). Thus, the product (deuterated **3**) was dissolved in 0.15 M NaOD in 80% CD_3OD/D_2O, and the ^{1}H NMR spectrum was obtained at $t \approx 0$ and $t \approx 5$ min. Panels A and B of Figure 3 show the spectra at 0 and 5 min, respectively, while panel C shows the spectrum of an undeuterated sample of **3** after 5 min in the same solution. In both panels B and C the C-2β and C-16β protons have exchanged with deuterium, leaving peaks due to C-6β (2.53 ppm), C-6α (2.38 ppm), and C-2α (2.31 ppm). It is clear that there is only a small amount of C-6β H present in the product. Most, if not all, of the C-6α H is observable, however, although it has collapsed from a doublet to (primarily) a broad singlet due to the presence of deuterium on C-6β. Integration of these peaks indicates that ca. 80% of the C-6β hydrogens are deuteriums. The other 20% cannot be localized to either C-4 or C-6α by this method.

Discussion

Kinetic Competence of the Intermediate Dienol. Although in nonenzymatic reactions an intermediate along the reaction pathway must react to form products at least as rapidly as the reactant does, the situation for reactions of enzymes with externally generated intermediates is less clear-cut. A rigorous definition of kinetic competence for an intermediate in an enzyme-catalyzed reaction requires that the formation of an enzyme-bound intermediate and its reaction to products be sufficiently rapid to account for the conversion of substrate(s) to product(s) (Anderson & Johnson, 1990a,b). The classical view, however, based on steady-state kinetics, is that an intermediate is kinetically competent if it reacts as fast or faster than the substrate(s) when added to the enzyme (Cleland, 1990). A comparison of the apparent second-order rate constant for the reaction of the externally generated dienol (**2**) with KSI at pH 7.0 to form products ($k_c = 2.3 \times 10^8$ M^{-1} s^{-1}) to the rate constant for reaction of **1** at the same pH ($k_b = k_{cat}/K_m = 2.1 \times 10^8$ M^{-1} s^{-1}) shows that the dienol reacts

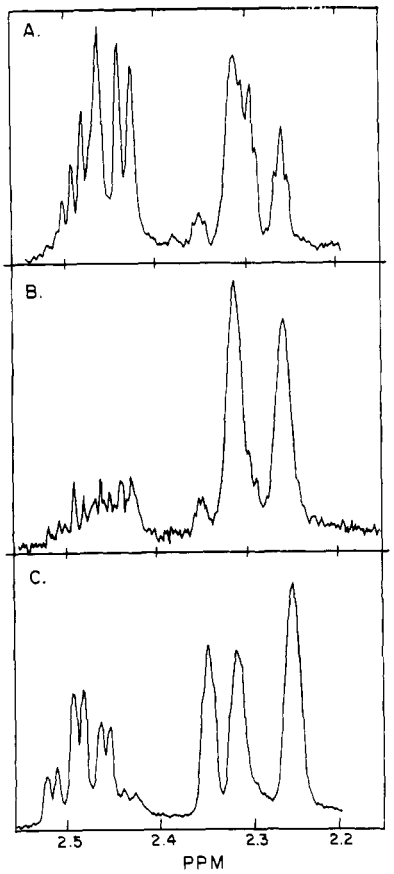


FIGURE 3: Partial ^1H NMR spectra for **3** in 80% CD_3OD/D_2O. (A) Isolated from the reaction of **2P** (190 μM) with alkaline phosphatase in the presence of KSI (0.4 μM) in D_2O buffer solution (34 mM Tris, pD 7.8, 3.3% MeOD). (B) Five minutes after the addition of 0.15 N NaOD (25 °C). (C) A separate sample of undeuterated **3** in 0.15 N NaOD/80% CD_3OD/D_2O after 5 min (25 °C).

Scheme IV

$$\begin{array}{ccc} E + 1 & \overset{K_s}{\rightleftharpoons} & E \cdot 1 \\ \Big\| K_{ext} & & \Big\| K_{int} \\ E + 2 & \overset{K_D}{\rightleftharpoons} & E \cdot 2 \end{array}$$

$K_s = [E][1]/[E \cdot 1]$

$K_D = [E][2]/[E \cdot 2]$

$K_{ext} = [2]/[1]$

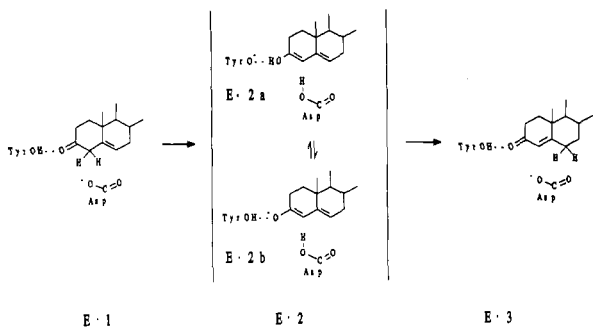
$K_{int} = [E \cdot 2]/[E \cdot 1]$

at about the same rate as the unconjugated ketone and is kinetically competent by the steady-state definition.

The magnitude of the apparent second-order rate constant for reaction of KSI and **2** suggests that this reaction is near the diffusion-controlled limit (Fersht, 1985); thus, the intermediate E·**2** should react to form products, or revert to reactants, faster than it dissociates to E + **2**. An estimate of this partitioning can be obtained from simple thermodynamic considerations (Scheme IV) (Cleland, 1990). The equilibrium constant for formation of **2** in aqueous solution is relatively large for formation of an enol (K_{ext} = 2 × 10^{-3}; Zeng & Pollack, 1991). Although the corresponding equilibrium constant at the active site is unknown, arguments based upon enzyme efficiency suggest that an internal equilibrium constant, K_{int}, of approximately unity would be reasonable and result in an efficient enzyme (Burbaum et al., 1989; Burbaum & Knowles, 1989). A value of about 100 μM for K_s for **1** under the experimental conditions may be estimated (D. C. Hawkinson, T. C. M. Eames, and R. M. Pollack, unpublished results), and the equilibrium constants on and off the enzyme for **1** ⇄ **2** are related by eq 4. The values for K_{ext} and K_s,

$$K_D = K_s(K_{ext}/K_{int}) \quad (4)$$

Scheme V



along with an estimate of 1.0 for K_{int}, allow calculation of a binding constant for the intermediate dienol (**2**) K_D of about 0.2 μM. Since the rate constant for association of **2** with KSI (k_{on}) is ca. 4 × 10^8 $M^{-1}s^{-1}$, the dissociation rate constant for E·**2** → **2** ($k_{off} = k_{on}K_D$) should be about 80 s^{-1}.

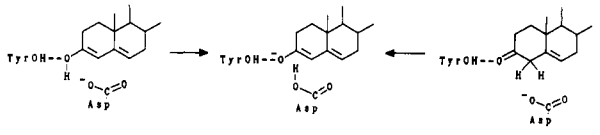
The rate constant for reaction of **2** → **3** (k_5) at the active site must be at least as fast as k_{cat} (under the experimental conditions k_{cat} = 3.8 × 10^4 s^{-1}; D. C. Hawkinson, T. C. M. Eames, and R. M. Pollack, unpublished results); thus, a comparison with k_{off} shows that less than 1 in 500 molecules of the enzyme–dienol complex should dissociate to free dienol. If the internal equilibrium constant (K_{int}) is greater than unity, then **2** would be bound more tightly to KSI. The dissociation rate constant would then be <80 s^{-1} and the ratio k_{off}/k_5 would be less than 1/500. Conversely, if K_{int} is less than unity, k_{off} would be >80 s^{-1}. In this case, however, k_5 would be increased over k_{cat} by a similar amount, and the overall ratio between k_{off} and k_5 would remain about 1/500.

Thus, the binding forces that are applied by KSI to stabilize the intermediate are sufficient to keep the rate of dissociation of the E·**2** complex low relative to further reaction to products, even for a diffusion-controlled rate of association of **2** with the enzyme. The binding of the intermediate to the enzyme is not strong by enzymatic standards, but the rapid reaction of **2** to products on the enzyme surface allows a relatively high rate of dissociation of the intermediate to be tolerated, leading to the observation of steady-state kinetic competence for **2**.

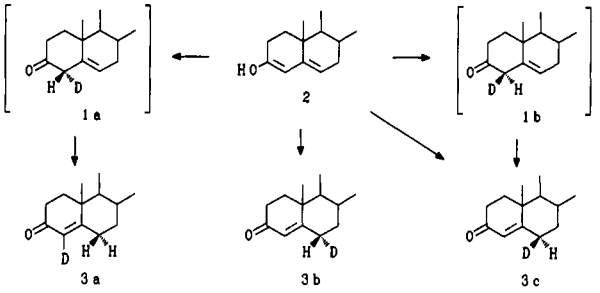
Mechanism of Reaction of **2** *with KSI.* The currently accepted mechanism for the KSI-catalyzed isomerization of **1** to **3** involves abstraction of the C-4β proton by a base (Asp-38), while the carbonyl oxygen is polarized by an acidic group (Tyr-14), either by hydrogen bonding or proton transfer (Scheme V) (Pollack et al., 1989b). We have previously suggested that the enzyme–intermediate complex is best represented by structure E·**2b** (a dienolate ion), instead of E·**2a** (a dienol) (Pollack et al., 1989b; Eames et al., 1989; Zeng & Pollack, 1991), and that proton transfer from Tyr-14 to O-3 of the steroid is not part of the reaction coordinate. Rather, the function of Tyr-14 in catalysis is likely to be hydrogen-bonding stabilization of the transition state leading to the intermediate dienolate ion (E·**2b**).

It has been argued (Xue et al., 1990) that in order for **2** to produce a catalytically active complex, it must react with enzyme that has its Tyr-14 ionized and Asp-38 protonated (E·**2a**). From the pK_a values of >10.9 for Tyr-14 (Kuliopulos et al., 1991) and 4.7 for Asp-38 (Pollack et al., 1986), it can be calculated that this ionic form of the enzyme is present in solution at pH 7 at less than 1 part in 10^6. This result suggests that the reaction of **2** with the "correct" ionic form of KSI (Tyr-O^-, Asp-COOH) will occur with a maximum rate constant of 10^{-6} times the rate constant for diffusion (10^{-6} × 10^9 = 10^3 $M^{-1}s^{-1}$). Yet the observed rate constant for reaction of **2** with KSI is greater than 10^8 $M^{-1}s^{-1}$.

Scheme VI



Scheme VII

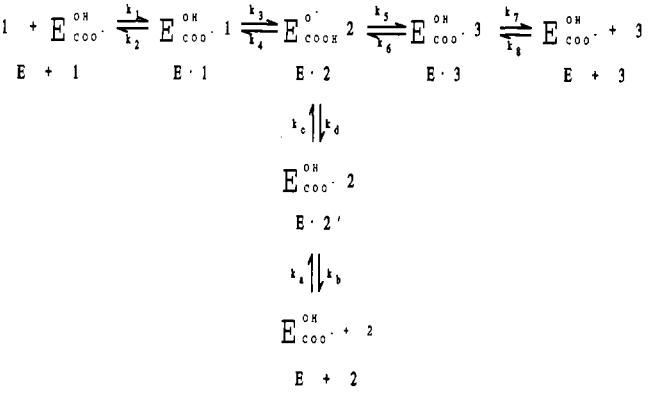


This apparent inconsistency can be understood if proton transfer within the active site from the dienol hydroxyl group to Asp-38 in enzyme-bound **2** is fast (Scheme VI). Since both the proton donor and acceptor are oxygen atoms, this reaction should be quite rapid (Eigen, 1964) and result in a catalytically competent intermediate complex. The overall mechanism for ketonization of the dienol then would involve deprotonation of the hydroxyl group by Asp-38 to produce the same complex that is produced by abstraction of the 4β proton of **1**. Protonation at C-6β would complete the reaction with both **1** and **2**. A similar mechanism has been proposed by Nickbarg et al. (1988) for the action of the H95Q mutant of triosephosphate isomerase. Alternatively, a proton transfer between Asp-38 and Tyr-14 within an initially formed encounter complex might occur, followed by binding of the dienol to KSI in the correct ionization state.

A further argument for the enzyme–dienol complex generated from KSI plus **2** being identical with the complex formed from KSI and **1** comes from a consideration of the ramifications of different complexes being formed. Consider an enzyme–dienol complex formed directly from **2** that can partition to both **1** and **3**, but that is not along the reaction pathway for KSI-catalyzed conversion of **1** to **3**. If this complex is able to form both reactants and products, by microscopic reversibility it will be an intermediate along *a* pathway for the reaction. Thus, there would have to be *two* different pathways for the enzymatic conversion of **1** to **3**, each having a dienol intermediate. Although this scenario is possible, it seems more reasonable that the initially formed enzyme–dienol complex rapidly is converted to one that is along the reaction pathway for **1** → **3**.

Stereochemistry of Protonation of **2** *by KSI.* The stereochemistry of protonation (α or β) at C-4 and C-6 can be determined from an analysis of the deuterium distribution in the product **3** when the reaction is run in deuterium oxide. If one assumes predominantly conserved proton transfer from 4β to 6β during the KSI-catalyzed isomerization of **1** to **3**,[2]

Scheme VIII



then the possible products are **3a**, **3b**, and **3c** (Scheme VII). Ketone **3a** would come from protonation of **2** at C-4α to give **1a**, which subsequently is isomerized to **3a**. Ketone **3b** comes from direct protonation of **2** at C-6α, and **3c** may be formed by protonation either at C-6β or at C-4β, followed by isomerization. Since approximately 80% of the final product is **3c**, it is clear that protonation at the β-face of the steroid predominates (either 4β or 6β). This result agrees with the predominant 4β to 6β proton transfer in the KSI-catalyzed isomerization of **1** to **3** and is consistent with the mechanism of reaction of **2** with KSI being similar to that of **1** with KSI. The lack of complete deuteration of the C-6β position agrees with the enzymatic results observed by Viger et al. (1981), who observed some labilization of the C-4α protons during catalysis.

There are two possible mechanisms by which protonation may occur at both the α-face and the β-face of C-4 of **2**. The first requires a two-base mechanism, that is, protonation at the β-face by one base (presumably Asp-38) and protonation at the α-face by a different base located below the plane of the steroid. The second possibility is that the steroid can bind "upside down" so that Asp-38 has access to both faces of the molecule. This explanation would account for the observation that 3β-oxiranyl steroids covalently modify Asp-38 by attack of Asp-38 at the α-side methylene (Bounds & Pollack, 1987). The epimerization at C-4 observed by Viger et al. (1981) in the KSI-catalyzed isomerization of **1** can be explained by abstraction of either the C-4α or C-4β proton by Asp-38, followed by dissociation of the E·**2** complex to give a loosely bound encounter complex E·**2**′ (Scheme VIII). Rebinding of **2** to the active site "upside down" would then allow reprotonation at the β face of C-4 or protonation at C-6β to give **3**. Dissociation of E·**2** to E·**2**′ is postulated since there is unlikely to be sufficient mobility of **2** within the active site to allow for rotation about the steroid axis (Eames et al., 1989). Complete dissociation of E·**2** to free KSI and **2** cannot occur; since the reaction of free KSI plus **2** is diffusion-controlled, dissociation of E·**2**′ to free KSI plus **2** must be slower than reaction to **1** or **3**.

Partitioning of the KSI–Dienol Complex. The nearly 1:1 ratio of products ([**1**]:[**3**]) formed from reaction of **2** with KSI may be analyzed in terms of Scheme VIII. The observed product ratio results from partitioning of E·**2**. The approximately equal kinetic barriers to formation of free **1** and **3** each consist of two processes, protonation (either on C-4 or C-6) and diffusion of the product away from the enzyme. If the diffusion steps were rapid ($k_2 \gg k_3$ and $k_7 \gg k_6$), the observed product ratio would give the ratio of k_4/k_5 directly, and thus the rate-limiting step for the chemical process. The high value of k_{cat}/K_m for the reaction of KSI with **1**, however, is consistent with the diffusion barrier for KSI + **1** being similar to or

[2] Several groups have investigated the stereospecificity of the proton transfer in the KSI reaction with **1**. Talalay et al. (1955) and Malhotra and Ringold (1965) have reported that the transfer is exclusively 4β to 6β, with a small amount of exchange with solvent. More recently, Viger *et al. (1981) found* that the 4α and 4β hydrogens are competitively abstracted, but they concluded that "The 4β → 6β proton transfer, accompanied by some exchange during the reprotonation of the enol..., may remain the exclusive route for the conjugation if it occurs on a substrate partially epimerized in the active site...".

Table IV: Predicted and Observed Secondary Kinetic Isotope Effects for KSI

	kinetic isotope effect predicted		
rate-limiting step	4αD	4,4D$_2$	6D
enolization[a]	>1.03	1.05	1.00
enolization/ketonization (50:50)[a]	>1.05	1.08	>0.98
observed[b]	1.11	1.06	1.00

[a] Calculated as described in the text. [b] From Xue et al. (1990).

greater than the barrier for at least one of the chemical steps, so that the observed partitioning can only be *roughly* correlated with the k_4/k_5 ratio.

Nevertheless, the approximately equal partitioning of **2** upon reaction with KSI to give **1** and **3** conflicts with the conclusion of Xue et al. (1990) that the rate-limiting step for KSI catalysis of **1** to **3** is enolization (k_3). If, as they propose, k_3 were rate-limiting, then E·**2** should partition to give **3** in much larger amounts than **1**. Xue et al. (1990) base their conclusion on their interpretation of secondary deuterium isotope effects for the reaction of KSI with **1** labeled with deuterium at C-4α (**1a**), C-4α and C-4β (**1b**), and C-6 (**1c**). The k_{cat}/K_m values for these compounds are compared with those for unlabeled **1** for **1a** and **1c** and with that for **1** deuterated at C-4β for **1b**. Since proton transfer is predominantly 4β to 6β, these comparisons give secondary isotope effects for the nontransferring protons at C-4 and C-6. Observed isotope effects for **1a** (1.11), **1b** (1.06), and **1c** (1.00) are interpreted in terms of a change in hybridization at C-4 and not at C-6, consistent with rate-limiting enolization.

Although this interpretation is attractive on the surface, calculations of *predicted* isotope effects for a mechanism with enolization rate-limiting and for a mechanism with equal barriers to enolization and ketonization show that these results are incapable of distinguishing between the two scenarios. In Table IV are listed predicted kinetic isotope effects, calculated according to the method of Northrup (1982), for k_{cat}/K_m with (1) enolization entirely rate-limiting for k_{cat} and (2) 50:50 mixture of enolization/ketonization rate-limiting for k_{cat}. In each case, correction was made for a commitment factor of 1.6 as reported by Xue et al. (1990). With a value for the equilibrium isotope effect of 1.127 (Cook et al., 1980) and the assumption of a half-transferred proton in the transition state, the prediced kinetic isotope effect for rate-limiting enolization for **1a** is 1.03; for **1b**, 1.05; and for **1c**, 1.00. If, on the other hand, we assume (using a commitment factor of 1.6) that the transition states for enolization and ketonization are equal in energy, and a half-transferred proton in the transition state, the following predicted values are calculated: **1a**, 1.05; **1b**, 1.08; and **1c**, 0.98. A comparison of the observed isotope effects and the predicted isotope effects for the two cases clearly shows that it is impossible to differentiate between these two possibilities. Further complicating the interpretation of these isotope effects is the fact that there is some labilization of the 4α proton during the reaction (Viger et al., 1981).

Xue et al. (1990) have suggested that the partitioning ratio that we observed (Eames et al., 1990) for the reaction of **2** with KSI is skewed due to "an unusually facile proton transfer to C-4 [which] 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..." This mechanism, however, involves a concerted 1,3 sigmatropic rearrangement. A suprafacial rearrangement of this type is forbidden by orbital symmetry considerations (Woodward & Hoffmann, 1970), and an antarafacial rearrangement would require an extremely strained transition state. Thus, a direct transfer of the proton from the 3-hydroxyl to C-4 can be confidently ruled out.

In summary, all of the evidence available at this time is consistent with approximately equal barriers to breakdown of the intermediate enzyme–dienol complex to **1** and to **3**. The possibility that the partitioning of **2** with KSI that we observe does not reflect the partitioning of the E·**2** complex formed from E and **1** cannot be rigorously ruled out, but on the basis of current data, there is no reason to suppose that this is the case.

Acknowledgments

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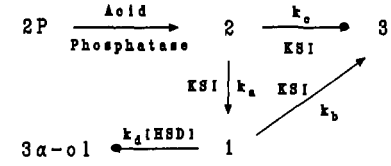
Appendix

Derivation of Equation 1. The reaction of Scheme II may be rewritten as



The fraction of **3** formed (f_3) may be expressed as

$$f_3 = \frac{k_c}{k_a + k_c} + \frac{k_b}{k_b + k_d[\mathrm{HSD}]}\frac{k_a}{k_a + k_c}$$
$$= c_1 + 1/(1 + c_2[\mathrm{HSD}])(1 - c_1) \qquad (5)$$

where c_1 is equal to the initial fraction of **2** that partitions to **3** [$= k_c/(k_a + k_c)$] and c_2 is the fraction of **3** formed from the **1** initially formed ($= k_d/k_b$). Extrapolation of [HSD] to ∞ gives $f_3 = c_1$.

Registry No. **1**, 571-36-8; **2**, 1229-13-6; **3**, 63-05-8; KSI, 9031-36-1; deuterium, 7782-39-0.

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Purification and Characterization of a Human Recombinant T-Cell Protein-Tyrosine-Phosphatase from a Baculovirus Expression System†

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ABSTRACT: A 48-kDa human T-cell protein-tyrosine-phosphatase (TC.PTPase) and a truncated form missing an 11-kDa C-terminal segment (TCΔC11.PTPase) were expressed by using the baculovirus system and characterized after extensive purification. The full-length PTPase was restricted to the particulate fraction of the cells from which it could be released by a combination of salt and detergent. The enzyme was entirely specific for phosphotyrosine residues. It displayed a low level of activity toward phosphorylated, reduced, carboxamidomethylated, and maleylated lysozyme (RCML), but was 12 times more active toward phosphorylated myelin basic protein (MBP). By contrast, the 37-kDa form localized in the soluble fraction, and its activity toward RCML was 5 times higher than that observed with MBP. The autophosphorylated cytoplasmic domain of the EGF receptor served as substrate for both enzymes. Limited proteolysis of either protein gave rise to a 33-kDa fragment displaying the substrate specificity of the truncated form. These data lend further support to the view that the C-terminal segment of the T-cell PTPase serves a regulatory function, playing an important role in the localization and substrate specificity of the enzyme.

Phosphorylation of proteins on tyrosyl residues has been implicated in signal transduction and the control of cell growth, proliferation, differentiation, and transformation (Hunter & Cooper, 1985; Yarden & Ullrich, 1988). Obviously, this process is regulated by the interplay of numerous protein-tyrosine-kinases and -phosphatases [reviewed in Tonks and Charbonneau (1989) and Hunter (1989)]. A protein-tyrosine-phosphatase (PTPase 1B) of ca. 35 kDa was previously purified to homogeneity from human placenta and characterized (Tonks et al., 1988a,b). PTPase 1B was cloned from human placenta cDNA libraries (Brown-Shimer et al., 1990; Chernoff et al., 1990); the open reading frame predicts a protein of 50 kDa, indicating that the 35-kDa enzyme isolated was in fact a truncated form of the original molecule. Related PTPases of ca. 50 kDa were cloned from human T-cells (Cool et al., 1989) and rat brain (Guan et al., 1990), and a growing number of such molecules are being identified [for a review, see Fischer et al. (1991)]. The leukocyte common antigen, CD 45 [reviewed by Thomas (1989)], was the first transmembrane molecule found to possess PTPase activity (Charbonneau et al., 1988, 1989; Tonks et al., 1988c, 1990). Since then, a number of receptor-linked PTPases displaying a wide variety of external domains were cloned (Streuli et al., 1988; Krueger et al., 1990; Kaplan et al., 1990; Mathews et al., 1990; Sap et al., 1990). These enzymes contain two tandem domains in their intracellular segments, each homologous to the low molecular weight PTPases (Charbonneau et al., 1989).

The T-cell PTPase was recently expressed in baby hamster kidney (BHK)[1] cells and its function investigated. In contrast

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