Microscopic Rate Constants for the Acetate Ion Catalyzed Isomerization of 5-Androstene-3,17-dione to 4-Androstene-3,17-dione: A Model for Steroid Isomerase

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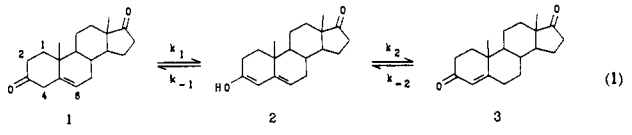
Abstract: The intermediate dienol 3-hydroxy-3,5-androstadien-17-one (**2**) in the isomerization of 5-androstene-3,17-dione (**1**) to 4-androstene-3,17-dione (**3**) has been generated in acetic acid/acetate buffers. Under these conditions, this dienol partitions almost exclusively to **1** rather than **3**. The rate constants for ketonization of **2**, acetate-catalyzed deuterium exchange of the C-4 hydrogens of **1** in D_2O, and isomerization of **1** to **3** have been used to calculate the microscopic rate constants for the individual steps in the acetate ion catalyzed isomerization. These rate constants provide values for the keto–enol equilibrium constants for **1** ($pK_E^1 = 2.7$) and **3** ($pK_E^3 = 6.0$) and the pK_a of **2** ($pK_a^E = 10.0$). The relevance of the rate and equilibrium constants to the mechanism of the corresponding reaction catalyzed by steroid isomerase is discussed.

The determination of free energy profiles from the microscopic rate constants for the individual steps of enzymatic reactions[1,2] has provided the experimental basis for general theories of enzyme action.[1g-i,m,n,3] The basic principles of enzyme mechanisms elucidated from these results have also been used to address evolutionary questions concerning the development of reaction stereospecificity,[4] metabolic pathway efficiency,[5] and organism survival.[4] Comparisons of free energy profiles for wild-type and mutant enzymes have proven quite fruitful in determining the contribution of individual amino acid residues to catalysis.[6] Similar detailed comparisons with the corresponding nonenzymatic reactions can also be valuable in quantitating enzymatic catalysis.[7]

We report here the detailed mechanism, along with the microscopic rate constants for the individual steps, for acetate ion catalysis of the isomerization of 5-androstene-3,17-dione (**1**) to 4-androstene-3,17-dione (**3**) as a model for the enzyme 3-oxo-Δ^5-steroid isomerase (also known as ketosteroid isomerase, KSI). Catalysis of this reaction by KSI is thought to involve the action of aspartic acid-38 as a base with concurrent hydrogen bond (or proton) donation by tyrosine-14, through the intermediacy of a dienol or dienolate (Scheme I).[8] The use of acetate as the base mimics the carboxylate group of aspartic acid-38 and provides base line rate constants for comparison with those of the enzymatic reaction.

Results

The generally accepted mechanism for the isomerization of β,γ-unsaturated ketones to their α,β-unsaturated isomers involves the formation of an intermediate dienol in acid or a dienolate in base (eq 1).[8] Although overall rate constants and partitioning coefficients (k_{-1}/k_2) for reactions of this type can be readily determined, absolute values of the rate constants for reaction of



(1)

1 2 3

$K_E^1 = k_1/k_{-1}$ $K_E^3 = k_{-2}/k_2$

the dienol(ate)s are difficult to obtain. We have approached this problem by generating the intermediate dienol in situ and observing its rate of ketonization, a strategy we previously used to determine the microscopic rate constants for the hydroxide ion catalyzed isomerization of **1** to **3**.[9]

Determination of k_{-1}. The intermediate dienol **2** was formed by reacting **1** with aqueous hydroxide and rapidly quenching this solution with acetate buffer after ca. 0.5 s of incubation in a stopped-flow spectrophotometer equipped with sequential mixing capability (eq 2). Treatment of **1** with 0.05 M sodium hydroxide

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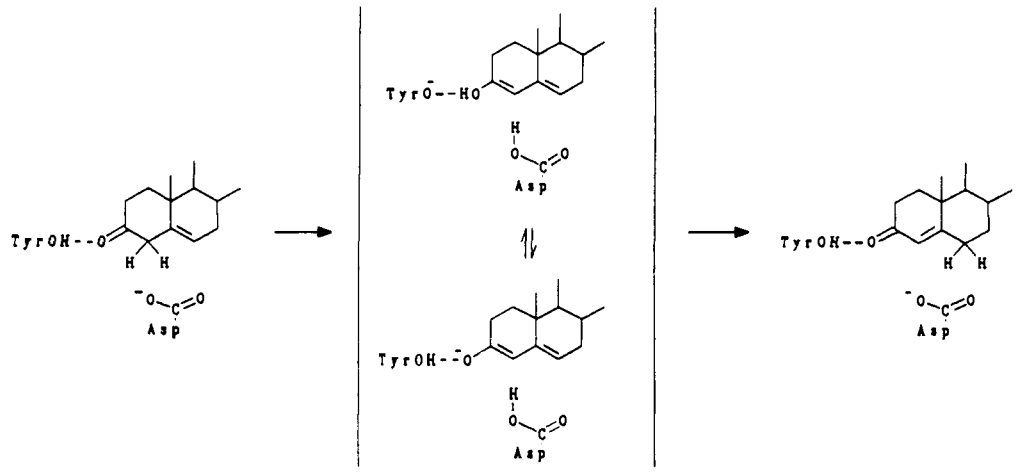
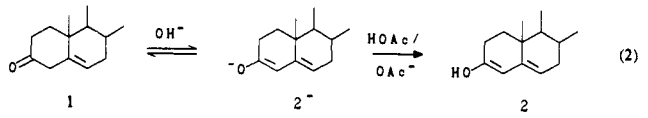
Scheme I



ion gives rapid ($t_{1/2} \approx 0.34$ s) equilibrium formation of the dienolate ion 2^-, followed by a slower ($t_{1/2} \approx 5.7$ s) conversion to **3**.[9] In 0.05 M sodium hydroxide, calculations based upon the



known rate constants show that ca. 35% of **1** ($pK_a = 12.7$) is converted to 2^- at 0.5 s, with less than 2% of **3** formed, and the remainder being residual **1**. Quenching this solution with acetate buffer (eq 2) converts 2^- to **2**; the decrease of absorbance of **2** can then be monitored at 238 nm (λ_{max} of **2**). Since the reaction of **1** in acetate buffer is much slower than that of **2** (vide infra), there is no interference from residual **1**.

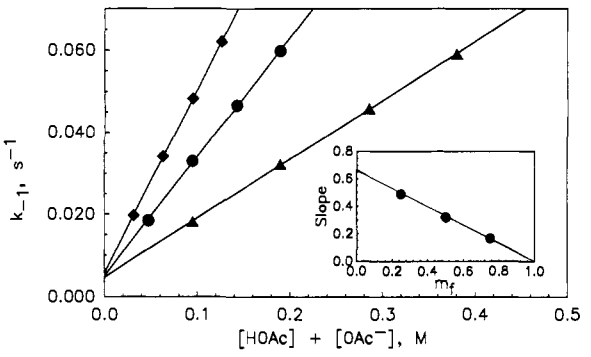


Figure 1. Rate constants for conversion of **2** to **1** (k_{-1}) as a function of the total acetate concentration (3.3% methanol, 25.0 °C, $\mu = 0.09$ with NaCl). Ratios of [OAc⁻]:[HOAc] are 2.74:1 (♦), 1:1.22 (●), 1:3.84 (▲). Inset is a plot of the slopes of these correlations against the mole fraction of [HOAc].

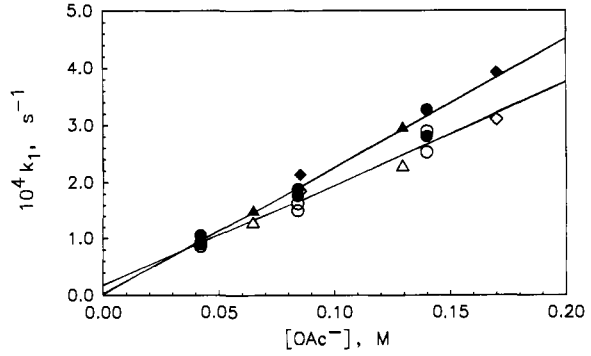


Figure 2. Rate constants for the exchange of the C-4α (solid symbols) and C-4β (open symbols) protons of **1** against the concentration of trideuterioacetate ion (80% CD_3OD/D_2O, 25 °C). Ratios of [CD_3COO^-]:[CD_3COOD] are 3:1 (♦); 1:1 (●); 1:3 (▲).

Good exponential decay curves were observed for the disappearance of **2**, and the pseudo-first-order rate constants were determined in aqueous solution (3.3% methanol, 25.0 °C, $\mu = 0.09$) at three different ratios of acetic acid/sodium acetate (3.84:1, 1.22:1, and 1:2.75), with total acetate ion concentrations ranging from 0.022 to 0.088 M. The negligible absorbance of the final solution at 248 nm (λ_{max} of **3**) shows that protonation of **2** is almost exclusively at C-4 to generate the β,γ-unsaturated ketone **1**, rather than at C-6 to give **3**; thus, $k_{-1} \gg k_2$, and the observed rate constants correspond to conversion of **2** to **1** (k_{-1}). The values of k_{-1} are linear with the concentration of acetate ion and independent of the concentration of acetic acid (Figure 1); thus, $k_{-1} = k_{-1}^{OAc}[OAc^-] + k_{-1}^0$, where $k_{-1}^{OAc} = 0.64 \pm 0.01$ M^{-1} s^{-1} and k_{-1}^0 is approximately 5×10^{-3} s^{-1}.

Determination of k_1. Since $k_{-1} \gg k_2$, measurement of the rate of exchange of the C-4 protons of **1** for deuterium in deuterium oxide gives the rate constant k_1. The rates of disappearance of these protons were determined by ^1H NMR (500 MHz) in CD_3COOD/CD_3COONa buffers at 25 °C (CD_3COOD:CD_3COONa = 3:1, 1:1, and 1:3). Eighty percent CD_3OD/D_2O solutions were necessary to dissolve sufficient quantities of **1** to allow the observation of the spectra. The resonances corresponding to the 4α and 4β protons of **1** were assigned by COSY, NOESY, and HMQC spectroscopy via a procedure similar to one that we recently described[10] for the assignment of the proton resonances of **3**. The assignments for H-4α (2.82 ppm) and H-4β (3.45 ppm) are similar to those recently reported by Xue et al.[11]

At acetate concentrations ranging from 0.0419 to 0.170 M, the observed pseudo-first-order rate constants are proportional to acetate ion concentration and independent of acetic acid concentration for exchange of both the 4α and 4β protons. Plots of k_1 vs [OAc^-] are linear with intercepts indistinguishable from zero and slopes of $k_1^{OAc}(4\alpha) = (2.2 \pm 0.1) \times 10^{-3}$ M^{-1} s^{-1} for H-4α and $k_1^{OAc}(4\beta) = (1.7 \pm 0.1) \times 10^{-3}$ M^{-1} s^{-1} for H-4β (Figure 2). Since the exchange rate constants were determined in 80% CD_3OD/D_2O, it is necessary to convert them to values appropriate to the conditions used for determination of the other rate constants (3.3% methanol). A conversion factor for this reaction can be estimated from the ratio of rate constants for a similar reaction, the hydroxide ion catalyzed elimination of 4-nitrophenolate ion from 4-(4-nitrophenoxy)-2-butanone (**4**) to give methyl vinyl ketone in 80% CH_3OH/H_2O and 3.3% CH_3OH/H_2O.[12] Since this reaction involves a rate-determining proton transfer to form

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(11) Xue, L.; Talalay, P.; Mildvan, A. S. *Biochemistry* **1990**, *29*, 7491. They report values of 2.83 ppm (H-4α) and 3.28 ppm (H-4β) in deuterated chloroform.

(12) CH_3OH/H_2O (80%) is used instead of 80% CD_3OD/D_2O since we wish to eliminate any secondary isotope effect due to proton abstraction by OD^- instead of OH^-. Smaller secondary isotope effects from deuterated methanol or water are, thus, unavoidably uncorrected for.

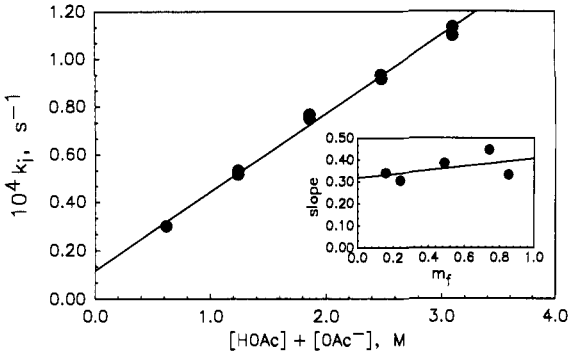


Figure 3. Rate constants for the isomerization to **1** to **3** (k_i) at [OAc⁻]/[HOAc] = 0.19 vs total acetate concentration (3.3% methanol, 25.0 °C, μ = 1.0 with NaCl). Inset is a plot of the slopes against the mole fraction of [HOAc].

an anion,[13] its response to changes in solvent composition should be similar to that for the proton exchange of **1**. The ratio of the rate constants for the hydroxide-catalyzed elimination of **4** in 80% CH_3OH/H_2O (k = 20.8 ± 0.1 M^{-1} s^{-1}) and 3.3% CH_3OH/H_2O (k = 7.30 ± 0.03 M^{-1} s^{-1}) allows corrected values to be calculated for the proton exchange of **1** in 3.3% methanol ($k_1^{OAc}(4\alpha)$ = (7.7 ± 0.4) × 10^{-4} M^{-1} s^{-1} and $k_1^{OAc}(4\beta)$ = (6.0 ± 0.4) × 10^{-4} M^{-1} s^{-1}). The total rate constant for exchange (k_1^{OAc}) is equal to the sum of the exchange rates of the 4β- and 4α-hydrogens (k_1^{OAc} = $k_1^{OAc}(4\alpha)$ + $k_1^{OAc}(4\beta)$).

Determination of k_2. The rate of conversion of **1** to **3** in the presence of acetic acid/acetate buffers was monitored by observing the appearance of ultraviolet absorbance at the λ_{max} of the product (248 nm) at pH values from 3.8 to 5.6. Since the reaction is quite slow, high concentrations of buffer (0.24–4.1 M) were used (acetic acid:sodium acetate ratios of ca. 5:1 to 1:6; μ = 1.0). Good first-order kinetic behavior was observed over several half-lives, and buffer plots of the observed pseudo-first-order rate constants for isomerization (k_i) vs total buffer concentration are generally linear (Figure 3), although some downward curvature (5–15%) is apparent at high buffer concentrations in some cases. The k_i values show terms in both acetic acid and acetate ion as well as a small solvent term and may be expressed by eq 3, with k_i^{HOAc} = (3.9 ± 0.5) × 10^{-5} M^{-1} s^{-1}, k_i^{OAc} = (3.2 ± 0.5) × 10^{-5} M^{-1} s^{-1}, and k_i^0 = (7.8 ± 5.1) × 10^{-6} s^{-1}.

$$k_i = k_i^{HOAc}[HOAc] + k_i^{OAc}[OAc^-] + k_i^0 \quad (3)$$

Since the intermediate dienol partitions almost exclusively back to reactants rather than to products ($k_{-1} \gg k_2$), $k_i = k_1k_2/k_{-1} = k_2K_E^1$. Thus, values for k_2 may be calculated from a knowledge of the k_i, k_1, and k_{-1} terms. The k_2 step shows terms in acetic acid (k_2^{HOAc} = (1.8 ± 0.3) × 10^{-2} M^{-1} s^{-1}) and acetate ion (k_2^{OAc} = (1.5 ± 0.2) × 10^{-2} M^{-1} s^{-1}), as well as a buffer independent term (k_2^0 = (3.6 ± 2.3) × 10^{-3} s^{-1}).

$$k_2 = k_2^{HOAc}[HOAc] + k_2^{OAc}[OAc^-] + k_2^0 \quad (4)$$

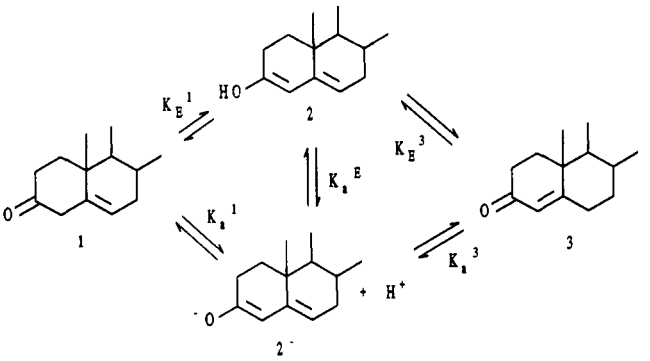
Determination of k_{-2}. In principle, k_{-2} can be measured by monitoring the rate of exchange of the C-6 protons of **3** in D_2O in the NMR, but the slow rate of this exchange with acetate makes this method impractical. However, k_{-2} can be readily calculated from a knowledge of the overall equilibrium constant and the other three rate constants ($K_{eq} = k_1k_2/k_{-1}k_{-2}$ = (2.4 ± 0.2) × 10^3).[9] The k_{-2} step has terms in acetate ion (k_{-2}^{OAc} = (1.3 ± 0.2) × 10^{-8} M^{-1} s^{-1}) and acetic acid (k_{-2}^{HOAc} = (1.6 ± 0.3 × 10^{-8} M^{-1} s^{-1}) and a buffer-independent term (k_{-2}^0 = (3.3 ± 2.1) × 10^{-9} s^{-1}).

$$k_{-2} = k_{-2}^{HOAc}[HOAc] + k_{-2}^{OAc}[OAc^-] + k_{-2}^0 \quad (5)$$

Discussion

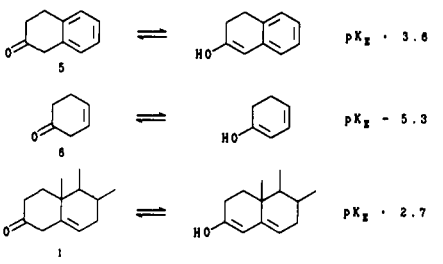
Keto–Enol Equilibrium Constants and pK_a of the Dienol. The equilibrium constant for the conversion of **1** to **2** (pK_E^1) can be readily calculated from the ratio of the forward and reverse rate

Scheme II



constants for the interconversion ($K_E^1 = k_1^{OAc}/k_{-1}^{OAc} = [k_1^{OAc}(4\alpha) + k_1^{OAc}(4\beta)]/k_{-1}^{OAc}$ = (2.1 ± 0.2) × 10^{-3}, pK_E^1 = 2.7 ± 0.1). Since the equilibrium constant for **1** ⇄ **3** (K_{eq} = 2400 ± 200) and the acidity of **1** (pK_a^1 = 12.65 ± 0.07) are known,[9] the keto–enol equilibrium constant for **3** (K_E^3 = (8.9 ± 1.2) × 10^{-7}, pK_E^3 = (6.0 ± 0.1) and the acidity of the dienol (K_a^E = (1.0 ± 0.2) × 10^{-10}, pK_a^E = 10.0 ± 0.1) can be calculated to go with the previously calculated value of pK_a^3 = 16.1,[9] completing the thermodynamic cycle. Scheme II summarizes the relationships between the equilibrium constants.

The equilibrium constant for formation of the dienol from **1** (pK_E^1 = 2.7) is significantly greater than the corresponding equilibrium constants for enol formation from simple ketones, such as acetone (pK_E = 8.3)[14] and cyclohexanone (pK_E = 6.4).[15] Other β,γ-unsaturated ketones that form conjugated enols, such as 2-tetralone (**5**; pK_E = 3.6)[16] and 3-cyclohexanone (**6**; pK_E = 5.3),[17] are also more enolized at equilibrium than saturated ketones.



Partitioning of the Intermediate Dienol. Both acetic acid and acetate ion catalyze protonation of the dienol at C-6 (k_2), whereas there is no detectable term in acetic acid for protonation at C-4 (k_{-1}). The most reasonable interpretation of these kinetic terms is that protonation at C-6 may occur either by direct protonation of the dienol **2** by acetic acid (k_2^{HOAc}) or by prior ionization of the dienol to the dienolate ion followed by protonation of the dienolate ion **2⁻** by acetic acid (k_2^{OAc}). The k_2^{OAc} term then is equal to $k_2'^{HOAc}K_a^E/K_a^{HOAc} = 5.75 \times 10^{-6}$ $k_2'^{HOAc}$, where $k_2'^{HOAc}$ is the second-order rate constant for protonation of **2⁻** by acetic acid at C-6 and K_a^{HOAc} is the ionization constant for acetic acid. Thus, the rate constant for protonation of the dienolate ion at C-6 by acetic acid ($k_2'^{HOAc}$) is (2.6 ± 0.4) × 10^3 M^{-1} s^{-1}. A similar calculation of the rate constant for protonation of the dienolate ion at C-4 by acetic acid yields the rate constants $k_{-1}'^{HOAc}(4\alpha)$ = (6.3 ± 1.3) × 10^4 M^{-1} s^{-1} and $k_{-1}'^{HOAc}(4\beta)$ = (4.9 ± 1.0) × 10^4 M^{-1} s^{-1}, giving a k_{-1}/k_2 ratio of 43 ± 12 for protonation of **2⁻** by acetic acid. A summary of the rate and equilibrium constants is given in Table I.

A comparison of the rate constant for protonation of the dienolate ion at C-6 by acetic acid with that for protonation of

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(15) (a) Keeffe, J. R.; Kresge, A. J.; Schepp, N. P. *J. Am. Chem. Soc.* **1988**, *110*, 1993. (b) *Ibid.* **1990**, *112*, 4862.

(16) Eldin, S.; Pollack, R. M.; Whalen, D. L. *J. Am. Chem. Soc.* **1991**, *113*, 1344.

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Table I. Summary of the Rate and Equilibrium Constants for the Acetic Acid/Acetate Ion Catalyzed Interconversion of **1**, **2**, **2**$^-$, and **3** at 25.0 °C and 3.3% Methanol

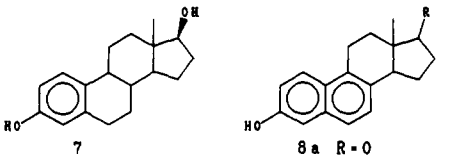
reaction	rate constant
$1 \xrightarrow{OAc^-} 2^-$	$k_1^{OAc} = 7.7 \times 10^{-4}$ M^{-1} s^{-1} (H-4α) 6.0×10^{-4} M^{-1} s^{-1} (H-4β)
$2^- \xrightarrow{HOAc} 1$	$k_{-1'}^{HOAc} = 6.3 \times 10^4$ M^{-1} s^{-1} (H-4α) 4.9×10^4 M^{-1} s^{-1} (H-4β)
$2 \xrightarrow{HOAc} 3$	$k_2^{HOAc} = 1.8 \times 10^{-2}$ M^{-1} s^{-1}
$2^- \xrightarrow{HOAc} 3$	$k_{2'}^{HOAc} = 2.6 \times 10^3$ M^{-1} s^{-1}
$3 \xrightarrow{HOAc} 2$	$k_{-2}^{HOAc} = 1.6 \times 10^{-8}$ M^{-1} s^{-1}
$3 \xrightarrow{OAc^-} 2^-$	$k_{-2}^{OAc} = 1.3 \times 10^{-8}$ M^{-1} s^{-1}
$1 \rightleftharpoons 2$	$K_E^1 = 2.1 \times 10^{-3}$, p$K_E^1 = 2.7$
$1 \rightleftharpoons H^+ + 2^-$	$K_a^1 = 2.2 \times 10^{-13}$ M, p$K_a^1 = 12.7$
$2 \rightleftharpoons H^+ + 2^-$	$K_a^E = 1.0 \times 10^{-10}$ M, p$K_a^E = 10.0$
$2 \rightleftharpoons 3$	$K_E^3 = 8.9 \times 10^{-7}$, p$K_E^3 = 6.0$
$3 \rightleftharpoons H^+ + 2^-$	$K_a^3 = 8.0 \times 10^{-17}$ M, p$K_a^3 = 16.1$
$1 \rightleftharpoons 3$	$K_{eq} = 2400$

the dienol shows that the dienolate ion is protonated ca. (1.5×10^5)-fold more rapidly than the dienol at C-6. The ratio for C-4 protonation for the dienolate vs the dienol must be at least 1 order of magnitude greater than the ratio for C-6 since no term in acetic acid is seen for k_{-1}. The greater contribution of protonation of the dienol relative to protonation of the dienolate at C-6 compared to C-4 is consistent with the preference for protonation of dienolates at the α-carbon and β-alkyl-substituted dienols at the γ-carbon.[8,18]

Relative Rates of Proton Abstraction. The greater lability of the C-4 hydrogens of **1** than that of the C-6 hydrogens of **3** ($k_1^{OAc}(4\alpha)/k_{-2}^{OAc} = 6 \times 10^4$ and $k_1^{OAc}(4\beta)/k_{-2}^{OAc} = 5 \times 10^4$) is expected in light of the greater equilibrium acidity of the protons at C-4 of **1**. A similar rate ratio for abstraction of the C-4 hydrogens (α and β) of **1** to the C-6 hydrogens of **3** was found for catalysis by hydroxide ion ($k_1^{OH}/k_{-2}^{OH} = 6.0 \times 10^4$).[9b] The rates of abstraction of the two C-4 protons of **1** by acetate ion are virtually identical (±30%). From the crystal structure determination of **1**,[19] the dihedral angles from the 4α-hydrogen to the π-orbitals of the carbonyl group and the $\Delta^{5,6}$ double bond are ca. 55 and 70°, respectively. Similarly, the corresponding dihedral angles for the 4β-hydrogen are both ca. 10°. Stereoelectronic considerations, based upon the requirement for continuous orbital overlap of the incipient p-orbital with the π-orbital of the carbonyl during enolate ion formation,[9,20] predict that the axial proton (4β) should be abstracted preferentially compared to the equatorial one (4α). However, relative rate ratios for abstraction of axial and equatorial protons in simple cyclohexanones are variable and generally on the order of 5-fold or less.[20] The small discriminations observed in these reactions can be explained in terms of a transition state that resembles the enolate ion.[21]

Although we were unable to determine the relative rates of abstraction of the 6α- and 6β-protons of **3** by acetate ion (k_{-2}^{OAc}), it is reasonable to assume that the 6β proton is kinetically much more acidic. The corresponding reactivities with *tert*-butoxide as the base in *tert*-butyl alcohol show that the 6β proton is abstracted ca. 50-fold faster than the 6α proton.[22] These results are consistent with the observation that KSI competitively abstracts both the 4α and 4β protons of 5-androstene-3,17-dione but protonates the intermediate at C-6β and not C-6α.[23]

Relevance to the Mechanism of Steroid Isomerase. The first step of the mechanism for isomerization of **1** catalyzed by KSI involves deprotonation by Asp-38 at the C-4β hydrogen of the substrate, leading to a dienolate ion stabilized by hydrogen bonding from Tyr-14 or by proton transfer from this group.[8] The isomerization is completed by protonation at C-6β by the conjugate acid of Asp-38. The nature of the intermediate, that is, whether it is a dienol or a dienolate ion, has received considerable attention recently.[8,11,24] Although UV and fluorescence spectra of the intermediate analogues estradiol (**7**),[25] equilenin (**8a**),[24] and dihydroequilenin (**8b**)[25,26] bound to KSI can be interpreted in terms of binding of the anion, Xue et al.[11] have recently postulated that the isomerase reaction involves a proton transfer from Tyr-14 as part of the reaction coordinate. However, since the pK_a of **2** (pK_a 10.0) in aqueous solution is similar to that of equilenin (p$K_a \approx$ 9),[27] **2** may also be more stable as the anion when bound to KSI.



7 **8a** R = O; **8b** R = α-O, β-OH

The similarity of the pK_a of Tyr-14 (pK_a = 9.7)[28] to the pK_a of **2** argues that there is little stabilization to be gained from a proton transfer from the tyrosine to **1** as the C-4 proton is being abstracted. Particularly relevant in this regard is that the pK_a of the C-3 oxygen in the transition state is almost certainly much lower than 10. If one assumes pK_a values of ca. −6 for the protonated ketone and 10 for the dienol and a transition state that is halfway toward products, then a pK_a of ca. 2 can be calculated for the oxygen in the transition state. Thus, in accordance with the *libido rule* of Jencks,[29] a proton transfer from Tyr-14 to the O-3 of the steroid would be unfavorable in the transition state, and the mechanism is probably best depicted as stabilization of the transition state by hydrogen bonding.

The arguments presented by Xue et al.[11] for a concerted enolization step are based upon the following observations: (1) the effect of mutations of Tyr-14 (Y14F) and Asp-38 (D38N) are additive, and (2) the solvent kinetic isotope effect ($^{H_2O}k_{cat}/^{D_2O}k_{cat}$ = 1.6) is identical for 4β-H and 4β-D 5-androstene-3,17-dione substrates. Although these results are consistent with a concerted mechanism, they are equally consistent with stabilization of an intermediate dienolate ion (**2**$^-$) by hydrogen bonding from Tyr-14. Xue et al.[11] interpret the first observation in terms of Tyr-14 and Asp-38 acting in the same step, and the second observation to require that the two deuterium isotope effects operate in the same step. Although other mechanisms can be envisioned that are consistent with these results, this interpretation is reasonable. However, it is not true that Tyr-14 must be donating a proton in this step. Hydrogen bonding from Tyr-14 to O-3 is also consistent with the results. The relatively small solvent hydrogen isotope effect may be rationalized as due to a change in stretching frequency of the O–H bond as the hydrogen bond acceptor becomes more basic (—O$^-$ vs =O). There is no evidence that the reaction involves proton donation from Tyr-14 as part of the reaction coordinate. The KSI reaction is "concerted" only in the fact that both Tyr-14 and Asp-38 act in the same step, not in the requirement for two simultaneous proton transfers.

The overall rate of isomerization of **1** → **3** in solutions of moderate concentrations of acetate ion is relatively slow ($t_{1/2}$ = 2.5 h at 1 M acetic acid and 1 M acetate ion). The corresponding value for the decomposition of the substrate complex with KSI is $t_{1/2} = 10^{-5}$ s,[30] leading to an enzymatic rate enhancement of

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ca. 10^9, equivalent to 12–13 kcal/mol. A substantial fraction of this rate acceleration is clearly due to hydrogen bonding of Tyr-14 to the developing negative charge. A mutant enzyme in which Tyr-14 has been changed to Phe shows a rate decrease of ca. 10^5-fold relative to the wild-type enzyme, demonstrating the importance of this group.[26] An additional source of the enzymatic rate acceleration is the decrease in molecularity in the KSI reaction, resulting in an entropic advantage for proton transfer.[31] Finally, Benisek's group has shown that Asp-38 may be intrinsically hyperreactive; thus, carbodiimide-catalyzed amidation of KSI with cystamine gives modification of Asp-38 at a rate about 100-fold faster than modification of other carboxyl groups of the enzyme.[32] Although the source of this enhanced reactivity is not apparent, it could be an important contributor to the impressive value of k_{cat}.

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Experimental Section

All materials have been described previously.[9b] Ultraviolet kinetic measurements, except for k_{-1}, were performed at 25.0 ± 0.4 °C with a Gilford 2400 spectrophotometer as described previously.[9b] Measurements of k_{-1} were made in the following way. 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 0.1 M sodium hydroxide with the two syringes in drive 1 of a HiTech PQ/SF-53 stopped-flow spectrophotometer at 25.0 °C. 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**. The loss of absorbance due to the dienol was monitored as a function of time at 238 nm for 5–9 half-lives of reaction.

Enolization rate constants were determined with a General Electric GN-500 spectrometer (500.11 MHz, ^{1}H), equipped with a variable-temperature probe set at 25 °C, with a concentration of **1** of 86 mM. Integration was carried out automatically at 20–25 time intervals corresponding to 2–8 half-lives of reaction. The 4α- and 4β-hydrogens of **1** were assigned by a combination of HMQC, COSY, and NOESY as described previously for **3**.[10]

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A CD Method for Determination of the Absolute Stereochemistry of Acyclic Glycols. 1. Application of the CD Exciton Chirality Method to Acyclic 1,3-Dibenzoate Systems

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Abstract: To determine the absolute configuration and conformation of chiral acyclic 1,3-glycols, the CD exciton chirality method has been applied to various acyclic 1,3-diesters with *p*-bromobenzoate, *p*-chlorobenzoate, or benzoate chromophore. Acyclic *anti*-1,3-dibenzoates exhibit typical exciton split CD Cotton effects, the sign of which agrees with the sign of the screw sense between two benzoate chromophores in the conformation expanded in a zigzag form. For example, the CD spectrum of bis(*p*-bromobenzoate) (2*S*,4*S*)-**2** shows first the positive and then secondly the negative Cotton effects: λ_{ext} 252.5 nm, $\Delta\epsilon$ +26.5 and λ_{ext} 236.0 nm, $\Delta\epsilon$ −9.1, A = +35.6. On the other hand, *syn*-1,3-dibenzoates exhibit *no* exciton split Cotton effects irrespective of the asymmetric structure. Instead the CD spectra of *syn*-dibenzoates show a weak single Cotton effect: e.g., (2*S*,4*R*)-**5**, λ_{ext} 238.0 nm, $\Delta\epsilon$ −1.7. Other 1,3-dibenzoates composed of primary and secondary alcoholic benzoates exhibit exciton split CD Cotton effects of half intensity in comparison with those of *anti*-1,3-dibenzoates: e.g., 1,3-bis(*p*-bromobenzoate) (*S*)-**1**, λ_{ext} 252.6 nm, $\Delta\epsilon$ +13.9 and λ_{ext} 235.9 nm, $\Delta\epsilon$ −3.9, A = +17.8. These results provided the CD method for determination of the absolute stereochemistry of acyclic 1,3-glycols.

The CD exciton chirality method for determination of absolute stereochemistry on the basis of the mechanism of a chiral exciton coupling between two or more chromophores has been extensively applied to various natural products and chiral synthetic organic compounds.[2] The exciton chirality method, however, has been mainly applied to cyclic compounds except for a few examples of acyclic dibenzoate, dibenzamide, and benzoate–benzamide systems.[3-5] Other examples[6] of acyclic systems are acyclic allylic benzoates, the absolute configuration of which has been determined by the application of the allylic benzoate method.[7] The main reason why the exciton chirality method has been rarely applied to acyclic compounds is that the conformation of acyclic systems is, in general, more complex than that of cyclic systems. On the other hand, much attention has been focussed on many natural products having a polyhydroxylated chain such as polyene macrolide antibiotics because of their important biological activity.[8-11] Furthermore, the asymmetric synthesis of acyclic compounds has

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