is more properly described as a carboxylic acid–histidinate–zinc triad.

The carboxylate–histidine–zinc interaction is found in enzymes of different origins and markedly different functions, yet it may serve a unified function in catalysis. Indeed, this triad may distinguish catalytic zinc from structural zinc in native metalloprotein structures. In addition to promoting metal ion complexation, one possible role for the carboxylate–histidine–zinc interaction (or the weaker carbonyl–histidine–zinc interaction) is the modulation of the nucleophilicity of zinc-bound water. Nature can "fine tune" the nucleophilicity of zinc-bound water not only by suitable selection of direct metal ligands such as the side chains of histidine, cysteine, and glutamate but also by modulating the contacts of these side chains with neighboring enzyme residues. Histidine, because of its potent, dual Lewis acid/base character, is most susceptible to such modulation. Although cysteine or glutamate can make hydrogen-bond contacts while coordinated to metal ions, these contacts are not favorable due to electronic or stereochemical reasons: sulfur is not preferred in hydrogen bonding, and a carboxylate in syn coordination to a metal ion can receive a hydrogen bond only in the less favorable anti orientation. Hence, histidine is most susceptible to wide-range modulation by the protein as to the strength and "hardness" of metal ion complexation.

We conclude our discussion of carboxylate–histidine–zinc interactions by returning to the exemplar of the zinc finger. Although the zinc of TFIIIA presumably does not interact directly with the DNA molecule, it might do so indirectly. For instance, a phosphate–histidine–zinc interaction, rather than the analogous carboxylate–histidine–zinc interaction proposed earlier, may be important in the zinc-dependent DNA binding behavior of the postulated zinc fingers. Either possibility might be consistent with the structure of an isolated zinc finger recently proposed from a two-dimensional NMR study,[43] and our continuing studies of phosphate–Lewis acid stereochemistry may allow us to evaluate these possibilities.

Summary

We have identified a recurring structural feature in the extended coordination polyhedron of biological zinc, the carboxylate–histidine–zinc interaction. This triad is usually found as Asp^----His $\rightarrow Zn^{2+}$, and we find that syn stereochemistry is preferred between the carboxylate and histidine. All interacting atoms of the triad are held firmly in the plane of histidine's imidazole ring, and we find this recurring triad in all zinc enzymes of known three-dimensional structure. Importantly, the carboxylate–histidine–zinc interaction, as so far observed, may distinguish catalytic zinc from structural zinc in native metalloprotein structures. We currently account for 36 examples of this triad in proteins of known structure and their homologues with sequence identities as low as 20%. Interestingly, the Asp^----His $\rightarrow Zn^{2+}$ triad of the zinc protease may bear an evolutionary resemblance to the Asp^----His couple of the serine protease. Additionally, a carboxylate–histidine couple, where unoccupied by metal ions in protein structures (on average, about one per unique protein structure deposited in the PDB), may signal a regulatory location for the binding of transition metal ions.

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Registry No. His, 71-00-1; Asp, 56-84-8; Zn, 7440-66-6.

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Determination of the Microscopic Rate Constants for the Base-Catalyzed Conjugation of 5-Androstene-3,17-dione

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Abstract: The hydroxide ion catalyzed isomerization of 5-androstene-3,17-dione (**1**) to 4-androstene-3,17-dione (**2**) proceeds through the formation of an intermediate dienolate ion ($\mathbf{1}^-$). This dienolate ion has been observed in the ultraviolet spectrum (λ_{max} ca. 256 nm) during the isomerization reaction. Rate constants for the formation of the dienolate ion and both its reversion to reactant (**1**) and its conversion to product (**2**) in aqueous solution were measured. In addition, the rate of exchange of the C-6 protons of **2** in D_2O/MeOD was determined. These results enable a complete description of the reaction profile to be made, including all rate constants and the pK_a values for **1** (12.7) and **2** (16.1). The possible relevance of these results to the mechanism of action of the enzyme 3-oxo-Δ^5-steroid isomerase is briefly discussed.

The isomerization of β,γ-unsaturated ketones to their α,β-unsaturated isomers is a simple example of a class of reactions involving 1,3 proton shifts.[2] This reaction has proven to be a useful vehicle for the examination of several general phenomena, including stereoelectronic effects on proton transfer,[3] electrostatic catalysis,[3b] substituent effects on double bond protonation,[4] and nucleophilic catalysis.[5] In addition, much effort has been expended to try to elucidate the mechanism of the enzymatic reaction of 5-androstene-3,17-dione (**1**) to 4-androstene-3,17-dione (**2**) catalyzed by 3-oxo-Δ^5-steroid isomerase.[6]

(1) Current address: Crystallography Laboratory, FRRF-NCI, BRI Basic Research Program, P.O. Box B, Frederick, MD 21701.

(2) For recent reviews of relevant material see: (a) Pollack, R. M.; Bounds, P. L.; Bevins, C. L. In *The Chemistry of Functional Groups, Enones*; Patai, S.; Rappoport, Z., Eds.; Wiley: New York, 1989; p 559. (b) Capon, B., *Ibid.* **1989**; p 1063.

(3) (a) Malhotra, S. K.; Ringold, H. J. *J. Am. Chem. Soc.* **1965**, *87*, 3228. (b) Whalen, D. L.; Weimaster, J. F.; Ross, A. M.; Radhe, R. *J. Am. Chem. Soc.* **1976**, *98*, 7319. (c) Pollack, R. M. *Tetrahedron*, in press.

(4) (a) Noyce, D. S.; Evett, M. *J. Am. Chem. Soc.* **1972**, *87*, 394. (b) Noyce, D. S.; Evett, M. *J. Am. Chem. Soc.* **1972**, *87*, 397.

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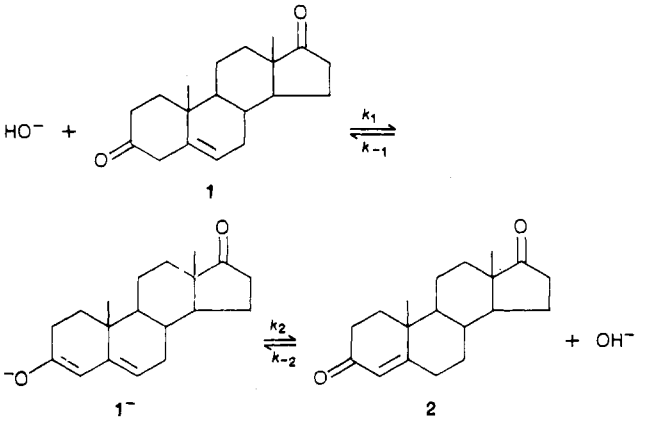
Our long-standing interest in the mechanism of the isomerase[7] has prompted us to carry out a detailed investigation into the energetics of the hydroxide ion catalyzed isomerization of **1** in order to better understand the solution chemistry of this reaction. The generally accepted mechanism involves abstraction of a proton from C-4 to generate a dienolate ion intermediate that is then protonated on C-6 (eq 1). We report here the measurement of all the microscopic rate constants for this interconversion, thus allowing us to determine the overall equilibrium constant, as well as the pK_a's of both the unconjugated ketone (**1**) and the conjugated isomer (**2**) in aqueous solution.[8] In addition, we discuss the possible relevance of our results to the mechanism of the reaction catalyzed by steroid isomerase.



(1)

Results

The rate of isomerization of **1** to **2** in sodium hydroxide solutions (5% MeOH, 25.0 °C, μ = 1.0 with KCl) was monitored by ultraviolet spectroscopy at 248 nm. Although the pseudo-first-order rate constants for this reaction are linearly correlated with the hydroxide ion concentration at the lowest base concentrations used (ca. 0.001–0.01 M), at concentrations approaching 0.1 M, the rate constant begins to level off, suggesting the formation of relatively large quantities of an intermediate. Steady-state kinetic analysis of the mechanism of eq 1 yields the rate expression of eq 2, where K_a^E is the ionization constant of the intermediate dienol. Since under our conditions (>0.001 N NaOH) the dienol should be almost totally ionized ($K_a^E[OH^-] \gg K_w$),[9] eq 2 simplifies to eq 3.[10] Least-squares fitting of the observed kinetic constants (k^{obsd}) to this equation gives a good fit to the data with $k_1/(k_{-1} + k_2) = 12 \pm 2\ M^{-1}$ and $k_2 = 0.122 \pm 0.007\ s^{-1}$.

$$k^{obsd} = k_1k_2K_a^E[OH^-]/\{(k_{-1} + k_2 + k_1[OH^-])K_a^E + k_1K_w\} \quad (2)$$

$$k^{obsd} = k_1k_2[OH^-]/(k_{-1} + k_2 + k_1[OH^-]) \quad (3)$$

Confirmation of the formation of significant quantities of the anionic intermediate (1^-) during the reaction was obtained from a series of rapid spectral scans (about 2 s) taken at 2–3-s intervals after addition of **1** to 1.0 N sodium hydroxide. These scans show the immediate (on that time scale) appearance of an intense (ϵ ca. 15 000) absorbance maximum at about 256 nm, followed by a slower disappearance of this peak and the appearance of a peak at 248 nm due to the product, **2**. A good isosbestic point was observed, although the value of the isosbestic point depends somewhat on the base concentration. The rate of formation of the intermediate at concentrations of hydroxide from 0.01 to 0.25 N was monitored by stopped-flow spectrophotometry. In order to make the second reaction invisible to the method of measurement, the absorbances were obtained at the isosbestic point for the conversion of 1^- to **2**. Although the rate expression for the formation of 1^- is complex, it simplifies to eq 4 with the assumptions that (1) all of the dienol is ionized and (2) $k_{-1} \gg k_2$. An excellent correlation of the observed pseudo-first-order rate constants with hydroxide ion concentration was obtained, giving $k_1 = 41.1 \pm 0.6\ M^{-1}\ s^{-1}$ and $k_{-1} = 3.03 \pm 0.05\ s^{-1}$. It should be noted here that the ratio k_{-1}/k_2 equals 25, in agreement with the assumption made in the kinetic analysis.

$$k^{obsd} = k_1[OH^-] + k_{-1} \quad (4)$$

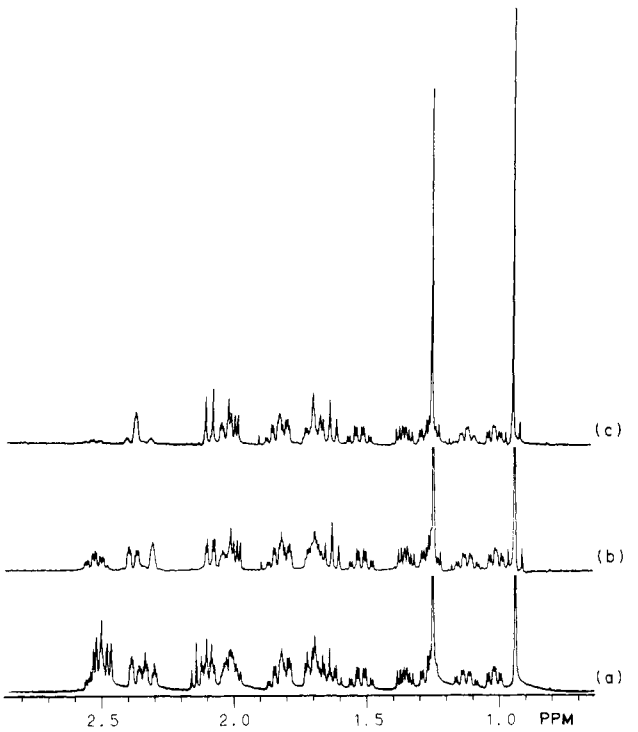


Figure 1. Partial ^1H NMR spectra for **2** in 80% CD_3OD/D_2O with (a) no added base, (b) 0.156 N NaOD after 5 min, and (c) 0.156 N NaOD after 66 min at 25.0 °C.

The last microscopic rate constant necessary to fully describe this system (k_{-2}) can, in principle, be determined directly, or indirectly by measuring the overall equilibrium constant K (= $k_1k_2/k_{-1}k_{-2}$). Attempts to measure the equilibrium concentration of **1** by HPLC analysis of equilibrated mixtures of **1** and **2** were unsuccessful due to the small amounts of the unconjugated isomer at equilibrium. The rate of exchange of the C-6 protons in NaOD/80% CD_3OD–D_2O, however, could be determined by ^1H NMR as a measure of k_{-2} in this solvent. Appropriate corrections were then made to convert this value to one for aqueous solution.

The hydrogens in the 500-MHz ^1H NMR spectrum of **2** have been assigned.[11] The C-6 protons show up as part of two multiplets: one at 2.45–2.57 ppm contains peaks due to the hydrogens at C-6β, C-2β, and C-16β; the other at 2.28–2.40 ppm has peaks due to protons at C-6α and C-2α. Figure 1 shows the NMR spectra in this region for **2** in 80% CD_3OD/D_2O with (a) no added base, (b) 0.156 N NaOD after 5 min, and (c) 0.156 N NaOD after 66 min at 25.0 °C. At 5 min two of the three protons above 2.45 ppm have been lost, while there is little change in the intensity of the peaks between 2.3 and 2.4 ppm. A ^{13}C–^1H correlation spectrum of a similar sample, coupled with an analysis of the splitting pattern, showed that the remaining peaks are due to protons at C-6β (2.53 ppm), C-6α (2.38 ppm), and C-2α (2.31

(7) (a) Kashino, S.; Katz, H.; Glusker, J.; Pollack, R. M.; Bounds, P. L. *J. Am. Chem. Soc.* **1987**, *109*, 6765. (b) Bounds, P. L.; Pollack, R. M. *Biochemistry* **1987**, *26*, 2263. (c) Bevins, C. L.; Pollack, R. M.; Kayser, R. H.; Bounds, P. L. *Biochemistry* **1986**, *25*, 5159. (d) Bantia, S.; Pollack, R. M. *J. Am. Chem. Soc.* **1986**, *108*, 3145. (e) Pollack, R. M.; Bantia, S.; Bounds, P. L.; Koffman, B. M. *Biochemistry* **1986**, *25*, 1905.

(8) A portion of these results has been reported previously in preliminary form: Pollack, R. M.; Mack, J. P. G.; Eldin, S. *J. Am. Chem. Soc.* **1987**, *109*, 5048.

(9) Pollack, R. M.; Zeng, B., unpublished results.

(10) In our preliminary communication[8] this rate equation was given as $k^{obsd} = k_1k_2[OH^-]/(k_{-1} + k_1[OH^-])$, which is a simplification of eq 3 for $k_{-1} \gg k_2$, as is the case here.

(11) Summers, M. F.; Pollack, R. M.; Zeng, B., submitted for publication.

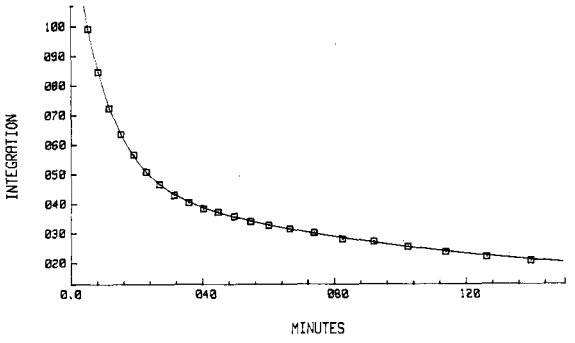


Figure 2. Plot of relative integration for the area corresponding to the C-2β, C-6β, and C-16β protons of **2** vs time at 25.0 °C in 0.0449 N NaOD (80% CD_3OD/20% D_2O).

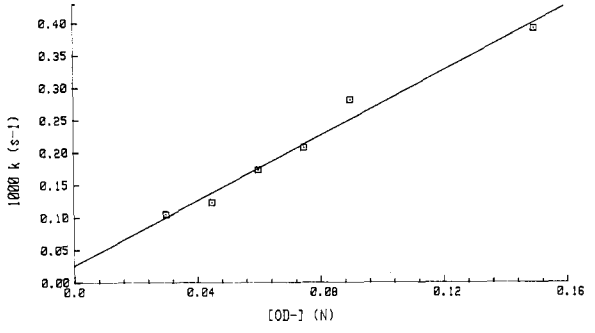


Figure 3. Plot of the observed rate constants for the exchange of the C-4 proton of **2** vs the concentration of deuteroxide ion.

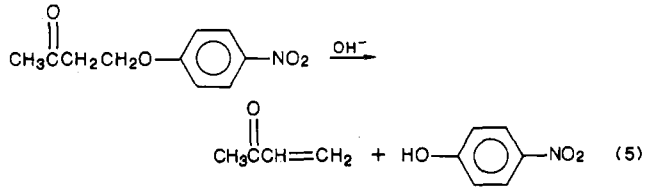
ppm). The C-2α proton has collapsed from a doublet of triplets to a broad singlet and has been shifted upfield about 0.03 ppm, as expected upon deuteration at C-2β.[12] At 66 min the C-2α and C-6β protons have nearly completely disappeared, with the C-6α proton collapsing to a broad singlet and shifting about 0.03 ppm upfield.

The rate of disappearance of the multiplet containing the C-6β proton at concentrations of deuteroxide from 0.03 to 0.09 N was monitored by integration of the area between 2.45 and 2.57 ppm. The data give a good fit to a double-exponential curve (Figure 2) that is consistent with two protons (C-16β and C-2β) exchanging rapidly with similar rate constants and one proton (C-6β) exchanging more slowly. Plots of these rate constants vs deuteroxide concentration are linear and give slopes of (3.5 ± 0.3) × 10^{-2} M^{-1} s^{-1} (C-16β and C-2β) and (2.1 ± 0.4) × 10^{-3} M^{-1} s^{-1} (C-6β) corresponding to the rates of exchange of these protons.

An independent determination of k_{-2} was obtained by monitoring the rate of disappearance of the C-4 vinyl proton at 5.76 ppm. Since $k_{-1} \gg k_2$, abstraction of a C-6 hydrogen from **2** and reprotonation by deuterium should lead to exchange at the C-4 position at the same rate as loss of the C-6 hydrogen. Loss of the C-4 proton at 0.03 N < [OD^-] < 0.15 N is pseudo first order. A plot of the observed rate constants vs the concentration of deuteroxide ion is linear with a slope corresponding to a second-order rate constant of (2.5 ± 0.2) × 10^{-3} M^{-1} s^{-1} (Figure 3), in good agreement with the rate constant obtained by direct measurement of the loss of the C-6β hydrogen. The y intercept from this plot [(2.3 ± 1.2) × 10^{-5} s^{-1}] is slightly greater than the expected value of zero, although the error is too large to conclude that it is significantly different than zero. If the plot is forced through the origin, a slightly higher rate constant [(2.9 ± 0.1) × 10^{-3} M^{-1} s^{-1}] is obtained. Since the measurement of the loss of the vinyl proton requires only analysis of a single-exponential fit, this rate constant (2.5 × 10^{-3} M^{-1} s^{-1}) is judged to be more accurate than the one determined from an analysis of the double-exponential fits and is used as the value for the exchange of the C-6β hydrogen.

(12) Lambert, J. B.; Shurvell, H. F.; Verbit, L.; Cooks, R. G.; Stout, G. H. *Organic Structural Analysis*; Macmillan, New York, 1976; p 46.

In order to convert this value to one that is appropriate for aqueous solution, it is necessary to correct for both the difference in solvent and the isotope effect due to the change in base from OD^- to OH^-. The isotope effect may be estimated from the solvent isotope effect in the same solvent system for the conversion of 4-(4-nitrophenoxy)-2-butanone to methyl vinyl ketone (eq 5). This reaction is known to involve a rate-limiting proton transfer[13] and thus should provide a good model for the isotope effect on the loss of the C-6 proton of **2**. The reaction of eq 5 was monitored by stopped-flow spectrophotometry at 0.021 N base in 80% MeOH/H_2O and 80% MeOD/D_2O, giving rate constants of 0.45 ± 0.02 s^{-1} and 0.91 ± 0.04 s^{-1}, respectively, leading to a solvent isotope effect of k_{H_2O}/k_{D_2O} = 0.49 ± 0.03. Thus, the corrected value of k_{-2} in 80% MeOH is (1.3 ± 0.1) × 10^{-3} M^{-1} s^{-1}.



(5)

The equilibrium constant for the isomerization of **1** to **2** was determined in 80% MeOH by dividing the rate of the forward reaction by the rate of the reverse reaction (k_{-2}). The rate of isomerization of **1** to **2** was measured in this solvent at hydroxide concentrations of 0.008–0.2 N by observing the change in absorbance at 248 nm. A good linear correlation was observed between the pseudo-first-order rate constants and the hydroxide ion concentration, giving a second-order rate constant (k_1k_2/k_{-1}) of 3.17 ± 0.02 M^{-1} s^{-1}. Division of this value by k_{-2} yields a value for the overall equilibrium constant ($K = k_1k_2/k_{-1}k_{-2}$) of (2.4 ± 0.2) × 10^3. With the assumption that the equilibrium constant is invariant with added methanol and with a knowledge of the observed values of k_1, k_{-1}, and k_2 in aqueous solution, k_{-2} can be calculated to be (6.9 ± 0.6) × 10^{-4} M^{-1} s^{-1} in water.

pK_a values for both ketones (12.7 for **1** and 16.1 for **2**) were calculated as concentration equilibrium constants, on the basis of a value of 1.69 × 10^{-14} M^2 for the ion product of water in 1.01 M KCl.[14] The pK_a for **1** was determined in three ways. First, a fit of the rate constants for the overall isomerization reaction to eq 1 gives pK_a = 12.69 ± 0.08; second, division of k_1 by k_{-1} gives pK_a = 12.64 ± 0.02. Finally, extrapolation of the measured absorbances to time zero for addition of **1** to sodium hydroxide concentrations of 0.02–0.16 N (μ = 1.0 with KCl, 0.5% MeOH) gives a titration curve characteristic of an acid with pK_a = 12.61 ± 0.04. The pK_a of **2** was calculated from the pK_a of **1** and the equilibrium constant for the conversion of **1** and **2**.

We were also able to determine the rate constant for the exchange of the C-6α proton of **2** by ^1H NMR. Loss of this proton was monitored at relatively high deuteroxide concentrations (0.620 and 0.931 N). At these concentrations of base, both C-2 protons, the C-16 protons, and the C-6β proton exchange rapidly, leaving the C-6α proton as the sole proton in the region near 2.3–2.4 ppm. This proton, however, exchanges relatively slowly, even under these conditions, with a half-life of 7–10 h. Rate constants were obtained for the first 50–75% of reaction, giving a value of (2.8 ± 0.6) × 10^{-5} M^{-1} s^{-1}, about 100-fold slower than the rate constant for exchange of the C-6β hydrogen.

Discussion

The general mechanism for the base-catalyzed isomerization of 5-androstene-3,17-dione in aqueous solution is well established. It has been known for a long time that deconjugation of α,β-unsaturated steroids can be effected by protonation at C-4 of the conjugate anion by dilute acid.[15] Jones and Wigfield[16] measured

(13) Hupe, D. J.; Wu, D. *J. Am. Chem. Soc.* **1977**, *99*, 7653.
(14) Harned, H. S.; Owens, B. B. *The Physical Chemistry of Electrolyte Solutions*, 3rd Ed.; Reinhold: New York, 1958; p 752. Values for these pK_a's given here differ slightly from those in our preliminary communication,[8] since they were based on a K_w of 1.59 × 10^{-14} M^2.
(15) Malhotra, S. K.; Ringold, H. J. *Tetrahedron Lett.* **1962**, 669.

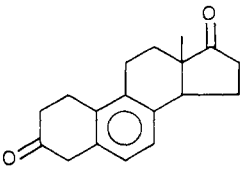
the rate of isomerization of **1** in aqueous solution at pH values from 10.6 to 11.7, and they observed a linear dependence of the pseudo-first-order rate constant on hydroxide ion concentration. However, their rate constants are approximately 7-fold larger than the values we obtain. These authors found curved first-order plots using the 4,4-dideuterio isomer and suggested that k_{-1} is competitive with k_2. Perera et al.[5e] found general-base catalysis of this reaction by tertiary amines and a solvent isotope effect of ca. 6, further substantiating the rate-limiting protonation of the dienolate ion. Where comparison can be made, our rate constants agree well with those of Perera et al. In addition, Okuyama and co-workers[5d] have measured rate constants for the isomerization of 17β-hydroxy-5-androsten-3-one, and these are similar to those we observe for **1**.

Equilibrium Constant. The equilibrium for the isomerization of 5-androstene-3,17-dione to 4-androstene-3,17-dione lies far toward the conjugated isomer (K = 2400). Similar results have been obtained for the equilibrium constants in aqueous solution for the isomerization of 3-cyclohexenone to 2-cyclohexenone (K = 275) and 3-cyclopentenone to 2-cyclopentenone ($K > 10{,}000$).[3b] Larger ring systems, however, show a more modest preference for the conjugated ketone and even may be more stable in the unconjugated form (K = 2.7 for 3- and 2-cycloheptenone, K = 0.25 for 3- and 2-cyclooctenones, and $K < 0.003$ for 3- and 2-cyclononenones in benzene solution, although the equilibrium constant for cyclohexenone isomerization is virtually unchanged in that solvent).[17] The preference of the larger ring systems for the unconjugated forms has been rationalized by recourse to unfavorable steric interactions in the conjugated isomers.[17]

Whalen and co-workers[3b] have suggested that the variation in equilibrium constants for the cyclopentenones and cyclohexenones is due to differences in planarity of the conjugated isomers of the two systems. A slight puckering of the 2-cyclohexenone ring causes some twisting of the bond between the carbonyl group and the double bond, decreasing the orbital overlap between the two groups. The large value of the equilibrium constant for the steroid system indicates that the π orbitals of the double bond and the carbonyl group are nearly coplanar. X-ray crystallographic results on 4-androstene-3,17-dione[18] confirm this interpretation, showing a dihedral angle of about 5° between the planes defined by the carbonyl group and the carbon–carbon double bond.

Partitioning of the Dienolate Ion. In common with other dienolate ions,[2b,3b,5e,15,17] $\mathbf{1}^-$ is protonated more rapidly on the α-carbon than the γ-carbon. As discussed by Whalen[3b] and by Capon,[2b] the discrimination in favor of the α-carbon is slight (<4-fold) if the dihedral angle between the double bonds is near zero. For systems in which the dihedral angle is significantly larger than zero, protonation at the α-carbon is greatly favored.[3b,17] These results generally parallel the equilibrium constants for isomerization: the larger the equilibrium constant the larger the tendency to protonate at the γ-carbon. Whalen and co-workers[3b] attributed the greater tendency for protonation at the γ-carbon of the dienolate of cyclopentenone relative to that of cyclohexenone to a greater twisting of the dienolate ion intermediate for the six-membered ring system, making it more difficult to delocalize the charge in the cyclohexadienolate ion than in the cyclopentadienolate. This rationale suggests that there may be some twisting of the single bond joining the two double bonds in the dienolate ion $\mathbf{1}^-$.

pK_a Values for 1 and 2. Both **1** (pK_a 12.7) and **2** (pK_a 16.1) are more acidic than typical saturated ketones such as acetone (19.16),[19] acetophenone (18.31),[20] and isobutyrophenone (18.26).[21] It appears that the effect of β,γ unsaturation on the K_a is about 10^6–10^7-fold, whereas the effect of α,β unsaturation is smaller, but still substantial (ca. 10^2–10^3-fold). Interestingly, the effect of the β,γ double bond is similar to that of a phenyl ring; the pK_a of the B-ring aromatic steroid **3** is 13.1,[9] very similar to that of **1**. Other benzyl ketones that show comparable acidity are 2-



3

indanone (pK_a 12.2)[22] and 2-tetralone (pK_a 12.9),[22a] although the analogous acyclic compound phenyl acetone is substantially less acidic (pK_a ca. 16).[23] The greater acidities of 2-indanone and 2-tetralone than of phenyl acetone can be attributed to steric interaction between the ortho hydrogen and the methyl group in the anion of the acyclic ketone phenyl acetone.[22a] Similar considerations should make 5-androstene-3,17-dione substantially more acidic than acyclic ketones with β,γ double bonds.

Relative Rates of Proton Abstraction. In line with the greater equilibrium acidity of the C-4 hydrogens of **1** than of the C-6 hydrogens of **2**, the C-4 hydrogens of **1** are kinetically much more acidic than the C-6 hydrogens of **2** ($k_1/k_{-2} = 6.7 \times 10^4$). The hydrogen at C-2$\beta$ of **2**, which is not directly influenced by the presence of the double bond, is exchanged at an intermediate rate, about 10-fold faster than the C-6β hydrogen of **2**, in agreement with a previous report[24] that testosterone enolization in base preferentially results in exchange of the protons at C-2 rather than at C-6. The very slow rate of exchange of the C-6α hydrogen of **2** is consistent with the finding that 4-androstene-3,17-dione exchanges the β-hydrogen at that position ca. 50-fold more rapidly than the α-hydrogen in in *tert*-butoxide/*tert*-butyl alcohol.[25] This result is likely due to a better stereoelectronic orientation of the C-6β hydrogen.[26,27] Using the results of crystallographic studies[18] of **2**, we have calculated the dihedral angle between the bond from C-6 to the β-hydrogen and the π orbital of the double bond. This angle is approximately 20°, allowing good overlap of the incipient p orbital with the π orbital of the unsaturated system during enolization.[28] The bond between the α-hydrogen and C-6, on the other hand, shows a dihedral angle of about 77° with the π orbital of the unsaturated system,[27] inhibiting continuous orbital overlap as this hydrogen is abstracted.

Although we did not distinguish between abstraction of the α- and β-hydrogens at C-4 of **1** in the k_1 process, the fact that loss of the proton at C-4 of **2** shows only a single exponential suggests that $k_{-1} \gg k_2$ for both the hydrogens at C-4 of **1**. Since $k_{-1}/k_2 = 25$ for the more reactive hydrogen, the two hydrogens must be abstracted by hydroxide at rates that are within a factor of 10–20-fold of each other. Calculations using the crystallographic coordinates[29] of **1** show that the dihedral angles for the π orbital of the carbonyl with the bonds to the α- and β-hydrogens of C-4 are 57° and 8°, respectively. Thus, the more reactive hydrogen is likely to be the β-hydrogen. Although the dihedral angle to the C-4α hydrogen of **1** is substantial, it is not as unfavorable as the angle to the C-6α hydrogen of **2**. If one assumes a cosine dependence of the overlap energy on the dihedral angle,[30] then

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both the 4β-hydrogen of **1** and the 6β-hydrogen of **2** are well aligned for proton abstraction (cos 8° = 0.99; cos 20° = 0.94). However, the dihedral angle for the 6β-hydrogen of **2** (cos 77° = 0.22) is substantially worse than that for the 4β-hydrogen of **1** (cos 58° = 0.53), and it is not unreasonable to expect that the stereoelectronic discrimination between the two C-4 hydrogens of **1** might be less than it is for the C-6 hydrogens of **2**.

In agreement with this result, the reaction catalyzed by steroid isomerase shows competitive abstraction of both of the C-4 hydrogens, although the β-proton is abstracted preferentially.[3a,31] However, the enzymatic reaction shows protonation at C-6β exclusively in preference to that at C-6α.[3a,31]

Possible Relevance to the Mechanism of Steroid Isomerase. The steroid isomerase from *Pseudomaonas testosteroni* that catalyzes the isomerization of **1** → **2** is one of the most active enzymes known.[6] The k_{cat} of 7×10^4 s^{-1} is over 10^{11}-fold greater than the rate constant for the corresponding hydroxide ion catalyzed reaction extrapolated to pH 7. Most proposals for the mechanism of action of this enzyme have postulated an obligatory proton transfer from an enzyme acid to the carbonyl oxygen at C-3 of the substrate, either prior to or concurrent with proton abstraction from C-4β.[2,6] We,[32] and subsequently others,[33] have also suggested that a cationic intermediate might be stabilized by electrostatic interaction with an anionic side chain[32] or the negative end of an α helix.[33] The observation that the pK_a of 5-androstene-3,17-dione (12.7) is so low, however, suggests that the corresponding reaction catalyzed by steroid isomerase may involve an *anionic* intermediate, rather than a cationic intermediate.

It is of interest then to explore the acid/base properties of the putative dienol intermediate. A preliminary estimate of the pK_a for the dienol from 5-androstene-3,17-dione is about 10.[9] An independent estimate may be obtained from the corresponding pK_a of the enol of 2-indanone (pK_a = 8.4),[22c] a ketone with an unconjugated phenyl group rather than a double bond β to the carbonyl. This value should be a reasonable estimate since the pK_a of 2-indanone (12.2)[22] is similar to that of **1** and phenyl groups have been shown to be good models for the electronic character of double bonds.[34]

Site-directed modification of the isomerase has provided evidence that the acidic group of the isomerase that interacts with the carbonyl group of the substrate is a tyrosine.[35] If this interpretation is correct, then there would be little driving force for transfer of a proton from the phenolic side chain of tyrosine (pK_a = 10.1) to the oxygen of an enolate ion (pK_a ca. 10). The function of the tyrosine might simply be to stabilize the dienolate ion by hydrogen bonding. Consistent with this interpretation is the fact that the spectral properties (UV and/or fluorescence) of estradiol, equilenin, and dihydroequilenin when bound to the active site of the isomerase show characteristics of the ionized form.[7c,35b,36] These molecules can be envisioned as analogues of the intermediate dienol.

In this context, it is noteworthy that Wang et al.[36] and Kuliopulos et al.[35b] observed a marked shift in the ultraviolet absorbance spectrum of 19-nortestosterone when it is bound to the active site of the isomerase. 19-Nortestosterone shows a λ_{max} of 248 nm in aqueous solution, but in the presence of the isomerase the λ_{max} is shifted to 258 nm. Kuliopulos et al.[35b] speculated on the identity of the bound species on the basis of the ultraviolet spectra of 19-nortestosterone in acidic solutions. They ascribed the spectral shift to protonation of the carbonyl oxygen by Tyr-14 at the active site. Alternatively, this shift may be simply a solvent effect due to the better ability of tyrosine to hydrogen bond relative to water. Interestingly, the dienolate ion observed in the present work has a λ_{max} quite close to that observed for the isomerase-bound 19-nortestosterone, suggesting that the bound species may be the dienolate ion of 19-nortestosterone.

Experimental Section

Materials. 5-Androstene-3,17-dione was prepared by G. Blotny according to the procedure of Ringold et al.[15] and purified by column chromatography (silica gel Merck 60, methylene chloride), followed by recrystallization from ether (80% yield). The melting point was variable in the range 127–145 °C, depending on the rate of heating. This phenomenon has been previously reported[37] and was attributed to isomerization to **2** during heating. 4-Androstene-3,17-dione was purchased from Sigma and purified by column chromatography (silica, chloroform/methanol, 100:1), followed by recrystallization from ethyl acetate. A single spot was observed on TLC (silica, chloroform/methanol, 100:1), mp 171.5–173.5 °C (lit.[38] mp 168–170 °C). 4-(4-Nitrophenoxy)-2-butanone was synthesized according to published procedures[39] and purified by column chromatography (silica, ethyl acetate/hexane, 1:1), followed by recrystallization from ethyl acetate, mp 69.5–70.5 °C (lit.[39] mp 69.5–70.0 °C). The ^{1}H NMR spectrum was identical with the published spectrum.[13] Tetradeuteriomethanol (99.5 atom % D) was purchased from Aldrich. Water used for kinetic runs was redistilled from glass.

Kinetic Measurements. All kinetic experiments were performed at 25.0 ± 0.2 °C. Ultraviolet measurements were made on either a Gilford Response spectrophotometer (equipped with a Hi-Tech SFA-11 stopped-flow accessory for the more rapid runs) or a Hi-Tech PQ/SF-53 stopped-flow spectrophotometer. The concentration of **1** was generally 30–50 μM, and the concentration of 4-(4-nitrophenoxy)-2-butanone was about 40 μM. Reactions were generally monitored for 3–10 half-lives. First-order rate constants were calculated with a nonlinear least-squares regression program. Except for occassional runs with **1** at very low methanol concentration (0.5%), all reactions showed excellent first-order behavior. NMR kinetics were performed on a General Electric GN-500 spectrometer (500.11 MHz, ^{1}H) equipped with a variable-temperature probe set at 25.0 °C. The concentration of **2** was 8.8 mM. Integration was carried out automatically at approximately 20 time intervals corresponding to several half-lives, except in the case of the slowest reactions, which were monitored through one to two half-lives. Second-order rate constants were calculated according to a weighted least-squares analysis of plots of the observed rate constants vs hydroxide ion concentration. This program assumes constant percent error in the observed rate constants.

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