pH Dependence of the Kinetic Parameters for 3-Oxo-Δ^5-steroid Isomerase. Substrate Catalysis and Inhibition by (3*S*)-Spiro[5α-androstane-3,2′-oxiran]-17-one†

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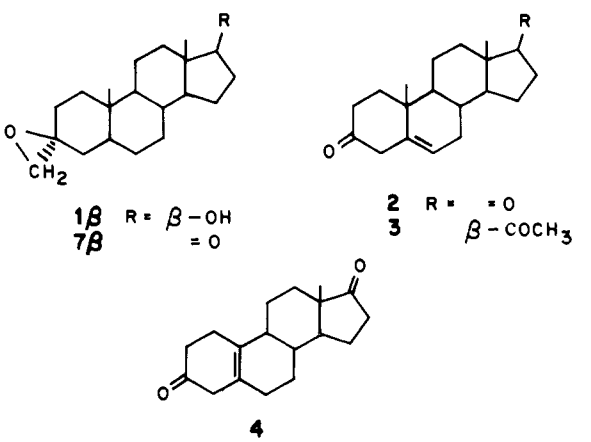
ABSTRACT: The pH–rate profiles for k_{cat}^{obsd} and $(k_{cat}/K_M)^{obsd}$ at 25.0 °C have been measured for 3-oxo-Δ^5-steroid isomerase by using 5-androstene-3,17-dione (**2**), 5-pregnene-3,20-dione (**3**), and 5(10)-estrene-3,17-dione (**4**) as substrates. Results from the nonsticky substrate **4** suggest values for the pK of a catalytically important group on the free enzyme (pK_E) of 4.57 and the pK of the same group in the enzyme–substrate complex of 4.74. For the sticky substrates **2** and **3**, pK_{ES} is ca. 4.75 and 5.5, respectively. Analysis of the $(k_{cat}/K_M)^{obsd}$ vs. pH profile for **2** reveals that the intermediate E·S complex decomposes to products at a rate similar to its reversion to E + S. The pH–rate profile for inhibition of the isomerase by (3*S*)-spiro-[5α-androstane-3,2′-oxiran]-17-one (**7**β) shows values for pK_E of 4.75 and pK_{EI} of 4.90. The similarity of the pH–rate profiles for isomerization of **4** and inhibition by **7**β suggests that both reactions may be governed by the ionization state of the same carboxyl group of the enzyme.

Enzymatic catalysis of the isomerization of 3-oxo-Δ^5-steroids to their conjugated Δ^4-isomers has been detected in extracts from a variety of sources, both bacterial (Talalay & Wang, 1955; Smith et al., 1980a,b; Smith & Benisek, 1980) and animal (Samuels et al., 1951; Ewald et al., 1964; Murota et al., 1971). The most thoroughly characterized of these enzymes is the 3-oxo-Δ^5-steroid isomerase (EC 5.3.3.1, steroid Δ-isomerase) from the soil bacterium *Pseudomonas testosteroni* (Talalay & Benson, 1972; Batzold et al., 1976). The isomerase exists as a dimer of identical subunits with a monomeric molecular weight of 13 394 (Benson et al., 1971; Tivol et al., 1975; Benson et al., 1975). The amino acid sequence has been determined (Benson et al., 1971), and the X-ray structure has been solved to 6-Å resolution (Westbrook et al., 1984).

A mechanism involving a dienol intermediate was postulated (Malhotra & Ringold, 1965) on the basis of model studies, but further work has failed to provide information concerning functions of particular amino acid residues. Only two specific amino acids have been shown to be present at the enzyme active site, Asn-57 (Penning et al., 1981, 1982; Penning & Talalay, 1981) and Asp-38 (Martyr & Benisek, 1975; Ogez et al., 1977; Benisek et al., 1980; Kayser et al., 1983), although there is still some uncertainty as to whether residue 38 is Asp or Asn (Benson et al., 1971; Ogez et al., 1977). In addition, there is evidence for the participation of histidine in the catalytic process (Batzold et al., 1976); however, more recent results have cast doubt on a catalytic role for histidine (Benisek & Ogez, 1982).

We have recently proposed a catalytic function (electrostatic stabilization) for an active site carboxylate (possibly Asp-38), upon the basis of studies with the active-site-directed irreversible inhibitor (3*S*)-spiro[5α-androstane-3,2′-oxiran]-17β-ol (**1**β; see Chart I) (Pollack et al., 1979; Bevins et al., 1984). In order to further assess the relationship of the inhibition and catalytic reactions, we have examined the rate of inactivation of the isomerase at varying pH by (3*S*)-spiro[5α-androstane-3,2′-oxiran]-17-one (**7**β), a compound very similar to **1**β, but whose kinetic parameters are more amenable to this type of study. In addition, we have studied the pH–rate profile for the enzyme activity toward 5-androstene-3,17-dione (**2**), 5-pregnene-3,20-dione (**3**), and 5(10)-estrene-3,17-dione (**4**).

Chart I



Materials and Methods

The isomerase (specific activity, 43 800 units/mg) was isolated as described before (Kayser et al., 1983). 5(10)-Estrene-3,17-dione (**4**) was purchased from Steraloids and recrystallized from ethyl acetate/hexanes. Only one spot was apparent on thin-layer chromatography (silica, ethyl acetate/hexanes).

5-Androstene-3,17-dione (**2**) *and 5-pregnene-3,20-dione* (**3**) were prepared by pyridinium chlorochromate oxidation of the corresponding 3β-alcohols (Corey & Suggs, 1975). Both compounds were purified by high-performance liquid chromatography (HPLC) and showed negligible absorbance at 248 nm in water. Each, however, had an extinction coefficient of ca. 17 000 at 248 nm in the presence of added base, due to isomerization to the α,β-unsaturated ketone. Although thin-

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layer chromatography of **2** showed only one spot, **3** gave faint traces of two impurities that could not be removed.

(3S)-Spiro[5α-androstane-3,2′-oxiran]-17-one (**7β**) was synthesized by pyridinium chlorochromate oxidation of (3S)-spiro[5α-androstane-3,2′-oxiran]-17β-ol (**1β**) (Pollack et al., 1979) with added sodium acetate to neutralize the slightly acidic character of the reaction mixture. The product was chromatographed on silica gel with methylene chloride/hexane (4:1) to give an 80% yield. Recrystallization from hexane gave white needles: mp 163.5–165 °C; NMR ($CDCl_3$) δ 0.88 (3 H, s), 0.90 (3 H, s), 2.56 (2 H, s, oxirane CH_2); IR ($CDCl_3$) 1730 cm^{-1}; mass spectrum *m/e* 302. Anal. ($C_{20}H_{30}O_2$) C, H.

Kinetic Methods. (A) Isomerization of **2**, **3**, *and* **4**. Initial rates for conversion of the Δ^5-ketones to the corresponding Δ^4-isomers were determined as a function of pH at 25.0 ± 0.2 °C. Buffers used over the following pH ranges were acetate, pH 3.6–5.5 (0.034 M), and phosphate, pH 5.2–8.8 (0.034 M). The salts used to keep the ionic strength constant (μ = 0.1) were sodium chloride for acetate buffer and potassium chloride for phosphate buffer. The enzyme was stored in 0.25% bovine serum albumin (BSA) (pH 7) and was stable over the time used. The reaction cell contained 3.00 mL of buffer, methanol (3.2%), and various amounts of substrate (30–180 μM for **2**, 1–20 μM for **3**, and 10–86 μM for **4**). The reaction was initiated by addition of 20 μL of enzyme solution to give a final enzyme concentration of 3.6×10^{-5} μM (**2**), 1.3×10^{-5} to 7.8×10^{-5} μM (**3**), and 2.1×10^{-3} or 4.2×10^{-3} μM (**4**).

Initial rates (v) were determined from the plots of absorbance vs. time at 248 nm for the first 10% of the reaction by using either a Gilford 2400 or Gilford Response spectrophotometer. The limited solubility and low K_M values for **3** made it necessary to use low concentrations of **3** and to observe very small absorbance changes. For some concentrations of **3**, the total absorbance change monitored was only ca. 2×10^{-3}. The rate constants for these runs were obtained by using the Response and were reproducible to 10–20%. Reciprocal plots ($1/v$ vs. $1/[S]$) were fitted to a weighted least-squares program (assuming constant percentage error in rate) to obtain relative k'^{obsd}_{cat} and $(k'_{cat}/K_M)^{obsd}$. The true k^{obsd}_{cat} and $(k_{cat}/K_M)^{obsd}$ were obtained by dividing k'^{obsd}_{cat} and $(k'_{cat}/K_M)^{obsd}$ by the enzyme concentration and extinction coefficient of the products; extinction coefficients of 16 300 for **2** and **4** and 17 600 for **3** were used. Spontaneous isomerization was found to be negligible at all pH values for each substrate.

(B) Stability of the Enzyme at Extreme pH Values. Three milliliters of 0.034 M buffer (pH 3.6, acetate buffer; pH 8.8, phosphate buffer; μ = 0.1) was equilibrated at 25 °C for 15 min. Twenty microliters of enzyme (5.4×10^{-3} μM, stored in 0.25% BSA) was added, and the reaction was initiated by addition of 100 μL of 5-androstene-3,17-dione in methanol to give a final concentration of 60 μM. The rate of formation of product was obtained by measuring the increase in absorbance with time. A similar experiment showed negligible decrease in rate when the enzyme was incubated for 1 min (normal assay time) at these pH values before the addition of substrate.

(C) Inhibition by (3S)-Spiro[5-androstene-3,2′-oxiran]-17-one (**7β**). The pH dependence of inactivation of isomerase by **7β** was determined at constant ionic strength (μ = 0.1) and 21.0 ± 0.2 °C. Buffers used (0.034 M) were sodium acetate (pH 4.4–5.5) and potassium phosphate (pH 5.5–8.0). The salts used to keep the ionic strength constant were sodium chloride for acetate buffer and potassium chloride for phosphate buffer. Buffer (500 μL) was incubated at 21 °C in a polypropylene vessel equipped with a magnetic stirrer for 10 min. Twenty microliters of ca. 30 μM enzyme (1 μM in the reaction mixture) was added to the reaction cell and stirred for 5 s. Then, 20 μL of (3S)-spiro[5α-androstane-3,2′-oxiran]-17-one (9–25 μM in the reaction cell) in methanol was added, and the solution was again stirred for 5 s. At different time intervals, 20-μL aliquots were removed and diluted in 6 mL of phosphate buffer (0.034 M, pH 7, 0.25% BSA). From this solution, 50 μL was removed and assayed for enzyme activity with 5-androstene-3,17-dione as substrate (Talalay & Benson, 1972). Plots of log activity vs. time were fitted to a linear least-squares program to obtain pseudo-first-order rate constants of inactivation (k). The reciprocal plots ($1/k$ vs. $1/[I]$) were fitted to a weighted least-squares program (assuming constant percentage error in rate constant) to give k_3^{obsd} and $(k_3/K_I)^{obsd}$. The enzyme was found to be stable at all pH values during the time used for inactivation except at the lowest pH studied (4.48). The rate constant for denaturation of the enzyme at pH 4.48 was obtained by measuring the enzyme activity at different time intervals and fitting the data (log activity vs. time) to a linear least-squares program.

Stability of **7β** *at pH 4.0.* A solution of **7β** (25 μM) in 5 mL of acetate buffer (0.034 M, pH 4.0, μ = 0.1, 4% methanol) was incubated at 21.5 °C for 30 min and then extracted with ether (3 × 1 mL). The organic extract was dried by blowing nitrogen and then analyzed by thin-layer chromatography (silica, hexanes/ethyl acetate, 6:4). Only one spot with an R_f of 0.47, corresponding to the R_f value of **7β**, was observed.

RESULTS

pH Dependence of the Kinetic Parameters for the Inactivation of Isomerase by (3S)-Spiro[5α-androstane-3,2′-oxiran]-17-one (**7β**). Preliminary experiments at pH 7 (21 °C) with **7β** showed that it inhibits the isomerase in a time-dependent, concentration-dependent manner. Analysis of the kinetics by the method of Kitz and Wilson (1962) showed that the reaction exhibits saturation kinetics with values of k_3 = 1.0 min^{-1} and $k_3/K_I = 1.4 \times 10^4$ M^{-1} min^{-1} (eq 1). Com-

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} EI \xrightarrow{k_3} E\text{–}I \text{ (inactive enzyme)} \qquad (1)$$

parison of these values to those previously obtained (Pollack et al., 1979) for **1β** ($k_3 = 7 \times 10^{-2}$ min^{-1}, $k_3/K_I = 4 \times 10^3$ M^{-1} min^{-1}) showed that **7β** reacts more rapidly than **1β** under both saturating and nonsaturating conditions. This fact proved to be important when we examined the rates of inactivation at low pH values where correction has to be made for the rate of enzyme denaturation.

Determinations of k_3^{obsd} and $(k_3/K_I)^{obsd}$ for the inactivation of isomerase by **7β** were carried out over the pH range 4.48–8.02 at 21.0 °C and at constant ionic strength (μ = 0.1) with acetate and phosphate buffers. Both buffers were used at pH 5.8 to rule out specific buffer effects. Aliquots were removed at different intervals of time from a solution containing enzyme and **7β** and assayed for enzyme activity. The data were fitted to the logarithmic form of the first-order rate equation by using a linear least-squares program to obtain the rate constant (k). Plots of $1/k$ vs. $1/[7\beta]$ at each pH gave k_3^{obsd} and $(k_3/K_I)^{obsd}$. The stability of the enzyme was also determined at each pH. Although there was slight denaturation of enzyme at pH 4.48, denaturation at all other pH values was negligible over the time scale used. The rate of denaturation at pH 4.48 was measured at the same temperature, ionic strength, and enzyme concentration as the inactivation. The rate of denaturation, which was less than 10% of the rate of inactivation by **7β**, was subtracted from the

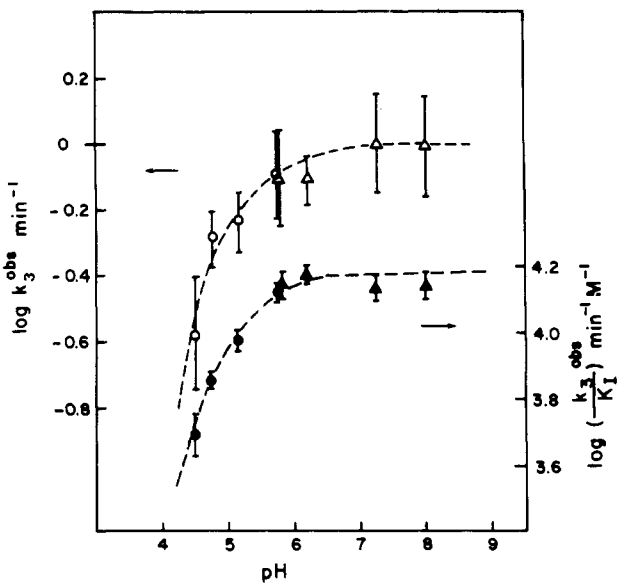


FIGURE 1: pH dependence of log $(k_3/K_I)^{obsd}$ and log k_3^{obsd} for the inactivation of 3-oxo-Δ^5-steroid isomerase by (3*S*)-spiro[5α-androstane-3,2′-oxiran]-17-one (7β) at 21.0 °C. The curves are theoretical, with the parameters in the text and eq 3 and 4. Circles are for acetate buffer; triangles are for phosphate buffer.

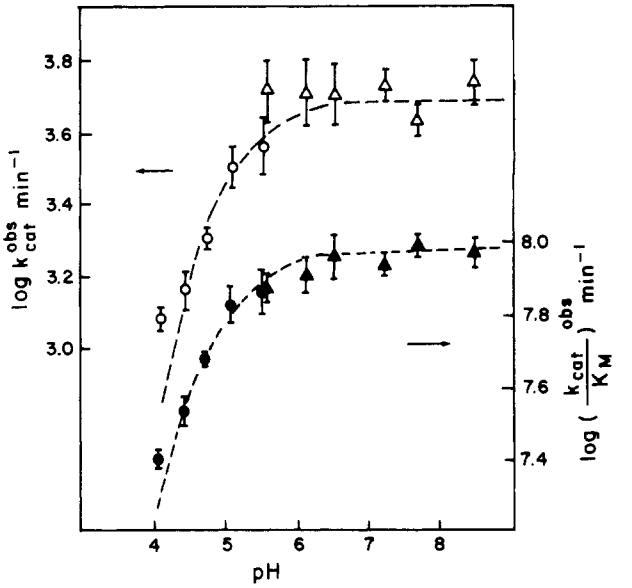
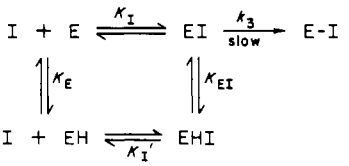


FIGURE 2: pH dependence of log $(k_{cat}/K_M)^{obsd}$ and log k_{cat}^{obsd} for the 3-oxo-Δ^5-steroid isomerase catalyzed isomerization of 5(10)-estrene-3,17-dione (**4**) at 25.0 °C. The curves are theoretical, with the parameters in the text and eq 6 and 7. Circles are for acetate buffer; triangles are for phosphate buffer.

apparent rate of inactivation to obtain the true rate of inactivation (Moore & Pearson, 1981).

The pH dependence of the inactivation of isomerase by 7β may be analyzed by using the model given in eq 2, where E



(2)

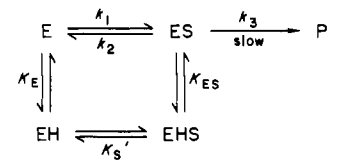
and EH are the unprotonated and protonated forms of the enzyme, respectively, K_E and K_{EI} are the ionization constants for EH and EHI, and K_I and K_I' are the dissociation constants of EI and EIH, respectively. The values for k_3^{obsd} and $(k_3/K_I)^{obsd}$ as a function of pH are related to the pH-independent values by eq 3 and 4, where H is [H$^+$]. Plots of log k_3^{obsd} vs.

$$k_3^{obsd} = k_3K_{EI}/(K_{EI} + H) \quad (3)$$

$$(k_3/K_I)^{obsd} = (k_3/K_I)[K_E/(K_E + H)] \quad (4)$$

pH and log $(k_3/K_I)^{obsd}$ vs. pH are shown in Figure 1. Analysis of reciprocal plots of $1/k_3^{obsd}$ vs. H and $(K_I/k_3)^{obsd}$ vs. H using a weighted least-squares program gives values of pK_E = 4.76 ± 0.05, pK_{EI} = 4.85 ± 0.10, k_3 = 1.0 ± 0.3 min^{-1}, and k_3/K_I = (13.8 ± 1.0) × 10^{-3} M^{-1} min^{-1}.

pH Dependence of the Kinetic Parameters for the Isomerization of 5(10)-Estrene-3,17-dione (**4**). The values for k_{cat}^{obsd} and $(k_{cat}/K_M)^{obsd}$ were determined at 25.0 °C and constant ionic strength (μ = 0.1) with acetate and phosphate buffers over the pH range 4.0–8.5. Both buffers were used at pH 5.8 to rule out specific buffer effects. Since the limiting value for k_{cat}/K_M (ca. 9 × 10^7 M^{-1} min^{-1}) is less than that expected for association between an enzyme and substrate (Fersht, 1984), the reaction can be analyzed by using eq 5, where $K_M = (k_2 + k_3)/k_1$. Since $k_2 \gg k_3$, $K_M = K_S$ and $k_{cat} = k_3$. Confirmation of the fact that E and ES are in equilibrium for **4** comes from the ca. 100-fold higher value of k_{cat}/K_M for **2** than for **4** (Wientraub et al., 1980). The pH dependencies of k_{cat}^{obsd} and $(k_{cat}/K_M)^{obsd}$ are given by eq 6 and 7 (Cleland, 1977) where k_{cat} and k_{cat}/K_M are the pH-independent values.



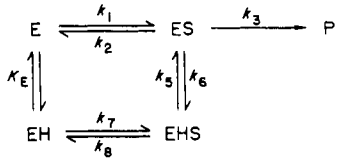
(5)

$$k_{cat}^{obsd} = k_{cat}K_{ES}/(K_{ES} + H) \quad (6)$$

$$(k_{cat}/K_M)^{obsd} = (k_{cat}/K_M)[K_E/(K_E + H)] \quad (7)$$

Plots of log $(k_{cat}/K_M)^{obsd}$ vs. pH and log k_{cat}^{obsd} vs. pH are shown in Figure 2. The data for the reciprocal plots $1/k_{cat}^{obsd}$ vs. H and $(K_M/k_{cat})^{obsd}$ vs. H were fitted to a weighted least-squares program to give pK_E = 4.57 ± 0.05, pK_{ES} = 4.74 ± 0.08, k_{cat} = (5.1 ± 0.6) × 10^3 min^{-1}, and k_{cat}/K_M = (8.8 ± 0.9) × 10^7 M^{-1} min^{-1}. The pH–rate profile was also measured at 21.0 °C, giving values of pK_E = 4.48, pK_{ES} = 4.66, k_{cat} = 1.61 × 10^3 min^{-1}, and k_{cat}/K_M = 3.78 × 10^7 min^{-1}. It should be noted here that if the values for both k_{cat} and k_{cat}/K_M at pH <4.2 are eliminated from consideration, the calculated pK_a's are 0.15–0.2 units higher and the fit is somewhat improved.

pH Dependence of the Kinetic Parameters for the Isomerization of 5-Androstene-3,17-dione (**2**) *and 5-Pregnene-3,20-dione* (**3**). k_{cat}^{obsd} and $(k_{cat}/K_M)^{obsd}$ for the isomerization of 5-androstene-3,17-dione as a function of pH were determined at 25.0 °C and constant ionic strength (μ = 0.1). Since k_{cat}/K_M is ca. 10^{10} M^{-1} min^{-1}, the reaction is likely to be at or near the diffusion limit (Fersht, 1984), and it is necessary to represent the mechanism by eq 8 (Cleland, 1977), in which



(8)

the only step considered to be at equilibrium is K_E, the protonation of enzyme (Cleland, 1977). The relative rates of the other steps are not specified. Expressions for $(k_{cat}/K_M)^{obsd}$ and k_{cat}^{obsd} for the above mechanism are given by eq 9 and 10

(Cleland, 1977),[1] where $K_{ES} = k_5/k_6$. Values of K_E and K_{ES}

$$(k_{cat}/K_M)^{obsd} = \frac{\frac{k_1k_3}{k_2 + k_3}\left[1 + \frac{k_5k_7H}{k_1(k_5 + k_8)K_E}\right]}{\left(1 + \frac{H}{K_E}\right)\left[1 + \frac{k_2k_5k_7H}{k_1(k_2 + k_3)(k_5 + k_8)K_E}\right]} \quad (9)$$

$$k_{cat}^{obsd} = \frac{k_3\left[1 + \frac{k_5k_8H}{k_2(k_5 + k_8)K_{ES}}\right]}{1 + \frac{(k_5k_8 + k_3k_8 + k_2k_8 + k_2k_5)\left(\frac{H}{K_{ES}}\right) + k_5k_8\left(\frac{H}{K_{ES}}\right)^2}{k_2(k_5 + k_8)}} \quad (10)$$

can be obtained from these equations by using the high-pH and low-pH limits. A plot of log $(k_{cat}/K_M)^{obsd}$ vs. pH gives two asymptotes, given by eq 11 and 12. The crossover point

$$\lim_{H\to 0} \log (k_{cat}/K_M)^{obsd} = \log [k_1k_3/(k_2 + k_3)] \quad (11)$$

$$\lim_{H\to \infty} \log (k_{cat}/K_M)^{obsd} = \log (k_1k_3/k_2) - pK_E + pH \quad (12)$$

of these two asymptotes is pK_E^{app} and is given by eq 13. Similarly, a plot of log k_{cat}^{obsd} vs. pH has two asymptotes, which are given by eq 14 and 15. The pH value at the crossover of the asymptotes is pK_{ES}.

$$pK_E^{app} = pK_E - \log (1 + k_3/k_2) \quad (13)$$

$$\lim_{H\to 0} \log k_{cat}^{obsd} = \log k_3 \quad (14)$$

$$\lim_{H\to \infty} \log k_{cat}^{obsd} = pH - pK_{ES} + \log k_3 \quad (15)$$

log $(k_{cat}/K_M)^{obsd}$ vs. pH and log k_{cat}^{obsd} vs. pH are plotted in Figure 3. The crossover points of the asymptotes, pK_E^{app} [log $(k_{cat}/K_M)^{obsd}$ vs. pH] and pK_{ES} (log k_{cat}^{obsd} vs. pH), are difficult to determine accurately because of the presence of a hollow in the vicinity of the crossover of the asymptotes. The pK_E^{app} obtained from Figure 3 is approximately 4.3, and pK_{ES} is approximately 4.75. Values for k_{cat} $(=k_3)$ and k_{cat}/K_M $(=k_1k_3/k_2)$ are $(3.4 \pm 0.1) \times 10^6$ min^{-1} and $(1.4 \pm 0.1) \times 10^{10}$ M^{-1} min^{-1}, respectively.

The pH–rate profile for 5-pregnene-3,20-dione (**3**) was determined under the same conditions as for **2** and analyzed in a similar manner. Plots of log $(k_{cat}/K_M)^{obsd}$ and log k_{cat}^{obsd} vs. pH are shown in Figure 4. Since K_M for **3** is quite low (ca. 1 μM at low pH values), it is difficult to obtain good values for $(k_{cat}/K_M)^{obsd}$, and thus there is a lot of scatter in these results. These graphs allow estimates of $pK_E^{app} = 4.7$ and $pK_{ES} = 5.5$ to be made. Values of $k_{cat} = (1.6 \pm 0.2) \times 10^5$ min^{-1} and $k_{cat}/K_M = (2.0 \pm 0.5) \times 10^{10}$ M^{-1} min^{-1} were calculated from the data.

Discussion

The information that can be obtained from the variation of enzymatic rate constants with pH has been discussed in great detail by several authors (Knowles, 1976; Cleland, 1977; Dixon & Webb, 1980). The conclusions that are relevant to the present work may be briefly summarized as follows: For a reaction in which only one protonic state of the enzyme is active, decomposition of the enzyme–substrate complex is rate-limiting, and all intermediates are in protonic equilibrium

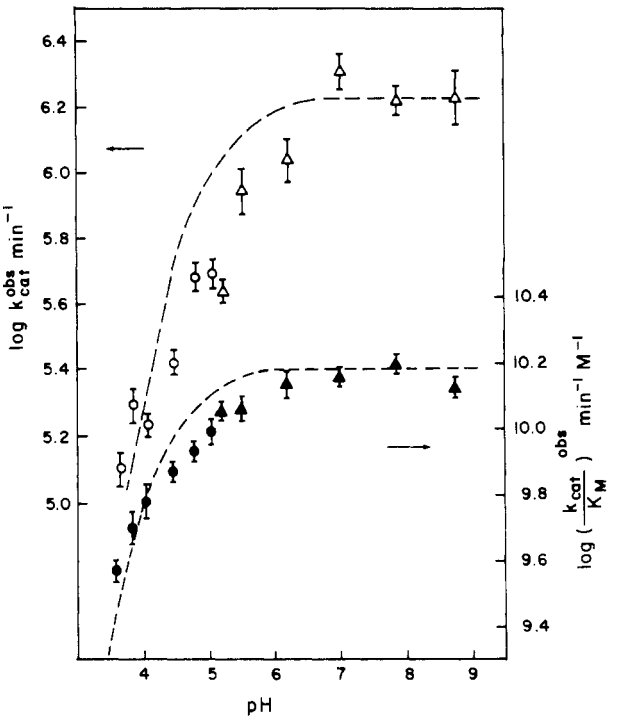


FIGURE 3: pH dependence of log $(k_{cat}/K_M)^{obsd}$ and log k_{cat}^{obsd} for the 3-oxo-Δ^5-steroid isomerase catalyzed isomerization of 5-androstene-3,17-dione (**2**) at 25.0 °C. The curves are theoretical for a simple titration curve (e.g., eq 6 and 7), with limiting values for $k_{cat}^{obsd} = 3.4 \times 10^6$ min^{-1}, $(k_{cat}/K_M)^{obsd} = 1.4 \times 10^{10}$ M^{-1} min^{-1}, $pK_E^{app} = 4.3$, and $pK_{ES} = 4.75$. Circles are for acetate buffer; triangles are for phosphate buffer.

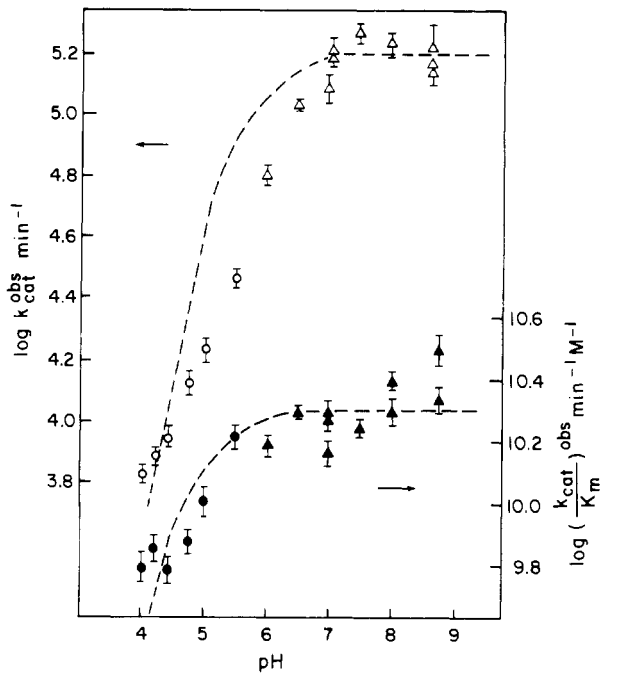


FIGURE 4: pH dependence of log (k_{cat}/K_M) and log k_{cat}^{obsd} for the 3-oxo-Δ^5-steroid isomerase catalyzed isomerization of 5-pregnene-3,20-dione (**3**) at 25.0 °C. The curves are theoretical for a simple titration curve (e.g., eq 6 and 7), using limiting values of $k_{cat} = 1.6 \times 10^5$ min^{-1}, $k_{cat}/K_M = 2.0 \times 10^{10}$ M^{-1} min^{-1}, $pK_E^{app} = 4.7$, $pK_{ES} = 5.5$. Circles are for acetate buffer; triangles are for phosphate buffer.

[1] Cleland's equation for k_{cat}^{obsd} includes a k_9 term for the release of a second product. Since there is only one product for this reaction, we have set $k_9 = \infty$ to derive eq 10.

(represented by eq 5), plots of $(k_{cat}/K_M)^{obsd}$ vs. pH will yield the pK values for free enzyme and/or substrate. On the other hand, plots of k_{cat}^{obsd} vs. pH will provide pK values of the enzyme–substrate complex whose decomposition to product is rate-limiting.[2]

If, on the other hand, the assumptions that all intermediates are in protonic equilibrium and the decomposition of enzyme–substrate complex is rate-limiting do not hold (eq 8), the mechanism may be represented by eq 2. For this mechanism, expressions for $(k_{cat}/K_M)^{obsd}$ and k_{cat}^{obsd} are quite complicated (eq 9 and 10). Plots of log $(k_{cat}/K_M)^{obsd}$ vs. pH may take the form of simple ionization curves if $k_7/k_1 = 1 + k_8/k_5$, or they may show a hollow ($k_7/k_1 < 1 + k_8/k_5$) or a hump ($k_7/k_1 > 1 + k_8/k_5$) (Cleland, 1977). In all three cases, however, the point where the high-pH and low-pH asymptotes cross gives pK_E^{app} (eq 13). This value is not the true pK of the enzyme but, rather, is shifted to a lower value by log $(1 + k_3/k_2)$. Thus for a sticky substrate ($k_3 > k_2$), one cannot obtain pK_E from the pH–rate profile alone. When $k_2 \gg k_3$, i.e., for a nonsticky substrate, then eq 13 reduces to eq 7 and the crossover point of the asymptotes, pK_E^{app}, is equal to the true pK_E.

For an active-site-directed irreversible inhibitor, with E and EI in equilibrium (eq 2), true values for pK_E and pK_{EI} will be obtained, from plots of log $(k_3/K_I)^{obsd}$ and log k_3^{obsd}, respectively, vs. pH.

pH Dependence of the Isomerization of 5(10)-Estrene-3,17-dione (**4**). The variation of both $(k_{cat}/K_M)^{obsd}$ and k_{cat}^{obsd} with pH follows a theoretical curve for a single ionization constant, giving a pK_E of 4.57 and a pK_{ES} of 4.75. These represent the true values since **4** is a nonsticky substrate and the substrate has no ionizing groups in the pH range examined. The Michaelis constant, K_M (50–60 μM), is independent of pH, indicating that $K_M = K_S$ (Haldane, 1930; Cornish-Bowden, 1976), which is consistent with the above argument. The observed values of k_{cat}/K_M and k_{cat} at pH 7 are similar to those obtained previously by Weintraub et al. (1980).[3]

Wang et al. (1963) have examined the effect of pH on the kinetic parameters for the isomerization of 17β-hydroxy-5(10)-estren-3-one. They used two different concentrations of substrate (24.3 and 60 μM) for the pH studies. Although their results are qualitatively similar to ours, the concentrations chosen by Wang et al. (1963) are too high to yield accurate measures of $(k_{cat}/K_M)^{obsd}$ and too low to give k_{cat}^{obsd}.

Previous estimates of pK_E come from the work of Weintraub et al. (1977), who studied the reversible inhibition of the enzymatic isomerization by estrone and estradiol at varying pH values. The K_I^{obsd} for estradiol is constant between pH 6 and pH 7, whereas below pH 6, it increases. The pK_E value obtained from the variation of K_I^{obsd} with pH is 4.9. An identical pK_E value was obtained from the pH dependence of K_I^{obsd} for estrone. However, it must be noted that estrone inhibits the enzyme in a noncompetitive fashion (Weintraub et al., 1977) and hence may not bind at the active site.

pH Dependence of the Isomerization of 5-Androstene-3,17-dione (**2**) *and 5-Pregnene-3,20-dione* (**3**). Neither **2** nor **3** gives plots of k_{cat}^{obsd} or $(k_{cat}/K_M)^{obsd}$ vs. pH that correspond to simple titration curves, although the data for k_{cat}/K_M for **3** are not sufficient for detailed analysis (Figures 3 and 4). Both the k_{cat} and k_{cat}/K_M values are in good agreement with those obtained by others (Batzold et al., 1976). The k_{cat}/K_M for the isomerization of **2** is about 1.4×10^{10} M^{-1} min^{-1}, which suggests that the reaction is probably near the diffusion-controlled limit (eq 8) as has previously been suggested (Malhotra & Ringold, 1965; Weintraub et al., 1980). In such a case, the pK_E^{app} obtained from the log $(k_{cat}/K_M)^{obsd}$ vs. pH profile is displaced to a value lower than the true pK_E (Cleland, 1977). This diplacement depends upon the ratio k_3/k_2 (decomposition of E·S to products vs. dissociation of E·S to reactants). The pK_E^{app} for the catalysis of 5-androstrene-3,17-dione (**2**) isomerization is ca. 4.3. An estimate of the true pK_E obtained from the isomerization of **4** is 4.6. Substitution of these values into eq 13 gives a ratio for k_3/k_2 of ca. 1.

The ratio k_3/k_2 has been determined independently by two different groups (Malhotra & Ringold, 1965; Weintraub et al., 1980), by measuring the primary isotope effects on the kinetic parameters of [4β-^{2}H]-5-androstene-3,17-dione isomerization. The values of k_3/k_2 obtained by these two different groups were 2.1 (Malhotra and Ringold) and 1.3 (Weintraub and co-workers). Although the transfer of the proton during the isomerization process is primarily from 4β to 6β, there is some abstraction of the 4α proton (<25%; Viger et al., 1981). Because of the competitive abstraction of the 4α and 4β protons, it is difficult to determine the ratio k_3/k_2 accurately. However, the 4β proton abstraction is more than 75% (50% intramolecular transfer), and therefore the value of k_3/k_2 obtained is probably a good approximation.

The presence of a hollow in the vicinity of the crossover of asymptotes in the plots of log $(k_{cat}/K_M)^{obsd}$ vs. pH and log k_{cat}^{obsd} vs. pH for the catalysis of 5-androstene-3,17-dione indicates that $1 + k_8/k_5 > k_7/k_1$ (Cleland, 1977). This situation could result from shielding of the proton in EHS from solvent by bound steroid, making it necessary for steroid dissociation (k_8) to occur before dissociation of the proton. The rate constant for loss of proton from EHS (k_5) would then be very small relative to k_8.

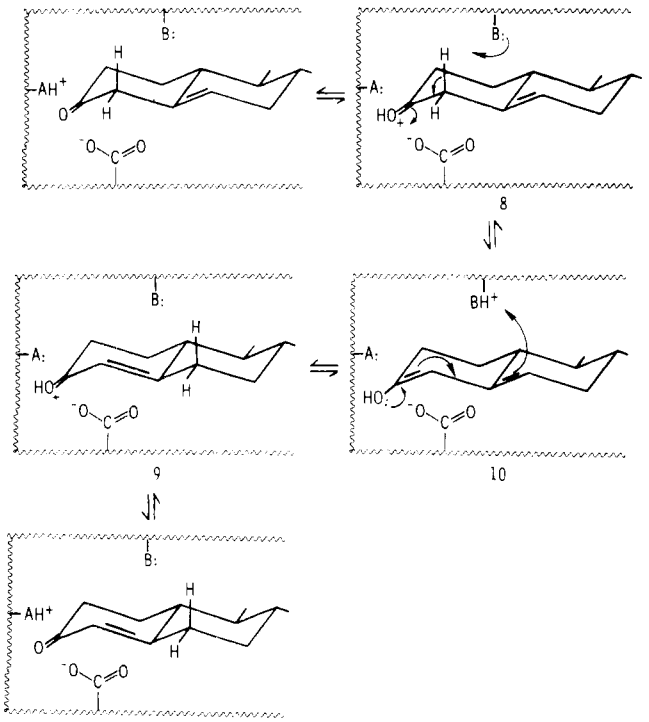
Weintraub et al. (1970) have previously determined the effect of pH on the kinetic parameters for the isomerization of 5-androstene-3,17-dione under conditions very similar to those of the present study. They report values of $pK_E = 4.7$ and $pK_{ES} = 5.6$, compared to our results of 4.3 and 4.75, respectively. In addition, Weintraub and co-workers found another pK for the E·S complex of 9.3. We attempted to examine rates in solutions of pH >9 but were unable to do so due to rapid denaturation of the enzyme (>20% loss of activity in 1 min). The cause of the descrepancy at low pH values may simply be due to the observed hollow in the pH–rate profiles, which makes accurate determination of pK's very difficult.

The $(k_{cat}/K_M)^{obsd}$ vs. pH rate profile for 5-pregnene-3,17-dione (**3**) has a lot of scatter in it, and it would be a mistake to put too much emphasis on the value of $pK_E^{app} = 4.7$ calculated from this plot. The k_{cat}^{obsd} vs. pH plot, however, seems to clearly show a pK_{ES} of ca. 5.5, or possibly larger, compared to 4.75 for both 5-androstene-3,17-dione (**2**) and 5(10)-estrene-3,17-dione (**4**). A possible explanation is the fact that the rate-limiting step in k_{cat} may be release of product for **3**, whereas the rate-limiting step for both **2** and **4** is probably

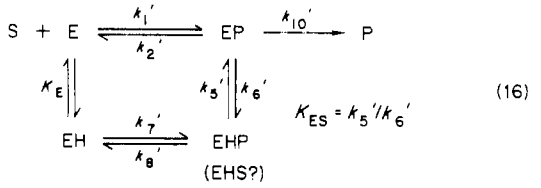
[2] Another assumption implicit in the derivation of these results is that if nonproductive binding of the substrate occurs, the ratio of nonproductive to productive binding is pH-independent (Fersht, 1984). If this assumption does not hold, pK_{ES} (or pK_{EI}) values will be perturbed, although no effect will be seen on pK_E (or pK_E^{app}). There is good evidence for nonproductive binding in isomerase–inhibitor interactions (Bevins et al., 1980) but no evidence concerning its pH dependence.

[3] The values for both k_{cat} and k_{cat}/K_M obtained by Weintraub et al. (1980) are ca. 2-fold higher than those we obtained for **4**. Their values for **2** are also 2-fold larger than either our values or those of Batzold et al. (1976). It seems probable that Weintraub et al. (1980) are reporting kinetic constants for the dimer whereas other groups are basing their values on the monomer.

Scheme I



chemical conversion of ES to EP.[4] If these assumptions are true, then the pK_{ES} values for **2** and **4** represent the true values for the E·S complex, where E·S is the enzyme complex with Δ^5-isomer (or possibly some intermediate occurring before the Δ^4-isomer, e.g., a dienol). However, the pK_{ES} for **3** is the ionization constant of the product Δ^4-ketone bound to the enzyme, since EP is now in equilibrium with E + S (eq 16) and dissociation of EP is rate-limiting.



(16)

[4] The values for k_{cat} and k_{cat}/K_M quoted by Sih and Whitlock (1968) are ca. 10-fold larger than those we find for **3**. Their values (actually determined by Ringold and Huether) appear too high, especially $k_{cat}/K_M = 2.6 \times 10^{11}$ M^{-1} min^{-1}, which is above what one would expect for even a diffusion-controlled reaction. Our value of 2.0×10^{10} M^{-1} min^{-1} is very similar to that determined for **2**, which suggests that both **2** and **3** operate at or near the diffusion limit. Sih and Whitlock's conclusion that the rate-limiting step of k_{cat} for isomerization of **3** is product release is based upon a kinetic isotope effect of $k^H_{cat}/k^D_{cat} = 1.0$ for the 4,4-dideuterio analogue and a calculated value for the rate of product release from EP (k_{10}). Although the data for this conclusion have not been published, an estimate for k_{10} can be obtained from the inhibition constant for the product Δ^4-ketone from **3** (6.4 μM) and an estimated diffusion-controlled on-rate of 2×10^{10} M^{-1} min^{-1} (Fersht, 1984). The value obtained (1.3×10^5 min^{-1}) is in good agreement with our experimentally determined k_{cat} of 1.6×10^5 min^{-1}. A similar calculation for **2** (K_I of the product = 320 μM) gives $k_{10} = 6 \times 10^6$ min^{-1} compared to a k_{cat} of 3.4×10^6 min^{-1}. For **4** (K_I of the product = 27 μM), $k_{10} = 5.4 \times 10^5$ min^{-1} whereas $k_{cat} = 5 \times 10^3$ min^{-1}. It is clear from these calculations that a chemical step is rate-limiting for **4**, but no definitive conclusions can be drawn for **2** or **3**. A k_{cat} isotope effect of 5.35 for **2** (Malhotra & Ringold, 1965), however, is strong evidence that a chemical step is at least partly rate-limiting. For **3**, the observed isotope effect on k_{cat} of 1.0 may be due to either rate-limiting product release or washout of the deuterium to solvent during the reaction, with deprotonation at C-6 slower than reprotonation at C-4. Washout can be eliminated as a cause of the low isotope effect since it has been shown that over 40% of the deuterium in the 4β-^{3}H analogue of **3** is transferred to C-6 during the isomerization (Viger et al., 1981).

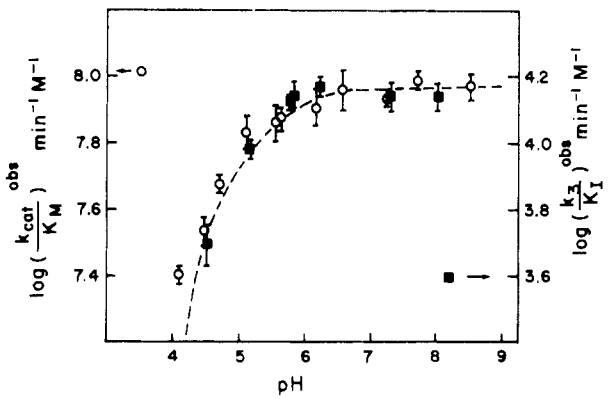


FIGURE 5: Comparison of the pH–rate profiles for log $(k_{cat}/K_M)^{obsd}$ for the isomerization of 5(10)-estrene-3,17-dione (○) and log $(k_3/K_I)^{obsd}$ for the inactivation by (3*S*)-spiro[5α-androstane-3,2′-oxiran]-17-one (■). The line is theoretical for a simple titration curve with pK = 4.75.

pH Dependence of the Inactivation by (3S)-Spiro[5α-androstane-3,2′-oxiran]-17-one (7β). Several 3β-oxiranes of the androstane and pregnane series have been shown to be irreversible active-site-directed inhibitors of the isomerase (Pollack et al., 1979; Penning, 1985; R. M. Pollack, C. L. Bevins, and R. H. Kayser, unpublished results). Other oxiranes that irreversibly inhibit are 17β-oxiranes (Bevins et al., 1980). We have recently shown (Bevins et al., 1984) that inhibition by (3*S*)-spiro[5α-androstane-3,2′-oxiran]-17β-ol (**1**β) results in the formation of two products, each with an ester bond, one between C-3 of the steroid and an enzymic carboxylate and the other between C-2′ and an enzymic carboxylate. Although we believe both products result from modification of the same carboxylate residue of the enzyme, this has not yet been demonstrated.

We have also shown (Bevins et al., 1984) that the carboxylate(s) involved is (are) located on the α-side of the bound steroid and thus are unlikely to be acting as the base in the catalytic reaction. We postulated, instead, that the carboxylate(s) may be acting via electrostatic catalysis to increase the enzymatic rate of isomerization of 3-oxo-Δ^5-steroids (Scheme I). If this postulate is true, then one would expect a correlation between the pH–rate profiles of inactivation by 3β-oxiranes and the catalytic reaction. (3*S*)-Spiro[5α-androstane-3,2′-oxiran]-17-one (**7**β) was chosen instead of **1**β since **7**β reacts more readily than **1**β and thus is more amenable to study at the extremes of pH. The pH–rate profile for inactivation by **7**β was examined from pH 4.5 to pH 8.0. Lower pH values could not be used due to significant enzyme denaturation over the time necessary for reaction. The pK values obtained (pK_E = 4.76, pK_{EI} = 4.85) are similar to those for 5(10)-estrene-3,17-dione (**4**) isomerization (pK_E = 4.57, pK_{ES} = 4.74).[5] Figure 5 shows the similarity of the plots of log $(k_{cat}/K_I)^{obsd}$ vs. pH for the inactivation by **7**β. These results are consistent with the involvement of the same residue in both reactions, suggesting that the enzyme carboxylate residue(s) involved in the inactivation by **7**β may be important for catalysis of the isomerization reaction, supporting our model of electrostatic catalysis (Scheme I). It should be noted, however, that only one pK_a is seen in the catalytic reaction. If this pK_a

[5] The pK values for isomerization of **4** are obtained from data at pH ≥4.09, whereas the pK's from inactivation by **7**β only include points down to pH 4.48 due to competing denaturation over the time needed to monitor inactivation. If the pK's from the isomerization of **4** are recalculated by using only data at pH ≥4.48, then the values obtained are pK_E = 4.68 ± 0.07 and pK_{ES} = 4.90 ± 0.10, virtually identical with those from **7**β inhibition.

is due to the carboxylate(s) involved in inhibition by **7β**, then the pK_a of the base necessary for proton transfer does not show up in the pH–rate profile. This result requires that the pK_a of this base be less than 4. Although one initially might expect a group acting to abstract a proton to be more basic, it must be kept in mind that this group must then act as an acid to protonate the dienol intermediate. To be most effective, the base B should have a pK_a slightly greater than that of the protonated Δ^5-ketone (8 ⇌ 10) and slightly less than that of the protonated Δ^4-ketone (9 ⇌ 10). Thus, a relatively weak base might, in fact, be a good candidate for B.[6]

Supplementary Material Available

Four tables giving the values of the observed kinetic constants as a function of pH for compounds **2**, **3**, **4**, and **7β** (4 pages). Ordering information is given on any current masthead page.

Registry No. **2**, 571-36-8; **3**, 1236-09-5; **4**, 3962-66-1; **7β**, 90991-90-5; EC 5.3.3.1, 9031-36-1.

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[6] Another possibility, of course, is that the similarity in the pK_a's of inhibition and catalysis is an artifact and that the two reactions are not related. This possibility must be considered in light of the finding of Benisek and Ogez (1982) that one of the histidine residues of the isomerase has a pK_a of 4.68 in the native enzyme and 4.90 in the complex with estradiol. However, these authors concluded, partly on the basis of a lack of effect of bound estradiol on the high- and low-pH limiting chemical shifts of the histidine C-2 protons, that the NMR results do not provide evidence for a catalytic histidine residue at the active site.