# Reaction Energetics of a Mutant 3-Oxo-$\Delta^5$-steroid Isomerase with an Altered Active Site Base (D38E)†

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ABSTRACT: 3-Oxo-$\Delta^5$-steroid isomerase (KSI) catalyzes the isomerization of a variety of 3-oxo-$\Delta^5$-steroids to their conjugated $\Delta^4$- isomers through the formation of an intermediate dienol. Mutation of the catalytic base (Asp-38) to Glu (D38E) has been found to reduce $k_{cat}/K_m$ for the isomerization of 5-androstene-3,-17-dione (**1**) to 4-androstene-3,17-dione (**3**) by about 300-fold (Zawrotny et al., 1991). The free energy profile for the D38E enzyme was determined from a combination of steady state kinetics and stopped-flow kinetics with the independently generated dienol intermediate (**2**). A comparison of the energetics of D38E with that of the wild type enzyme (WT) shows that the only significant difference is a reduction in the rates of the chemical steps for the interconversion of **1**, **2**, and **3** on the enzyme surface by about $10^3$-fold for D38E. The relative energy levels for all bound species are nearly identical for WT and D38E, whereas the transition states for both enolization and ketonization are destabilized by 3−4 kcal/mol. The effect of the D38E mutation on the energetics of KSI is comparable to the corresponding effect of the E165D mutation on the energetics of triosephosphate isomerase (TIM).

3-Oxo-$\Delta^5$-steroid isomerase (KSI, EC 5.3.3.1) from *Pseudomonas testosteroni* catalyzes the isomerization of a variety of $\beta,\gamma$-unsaturated-3-oxosteroids to their $\alpha,\beta$-unsaturated isomers (Scheme 1). Much effort has been expended to determine both the chemical mechanism of KSI [see Pollack et al. (1989a) and Schwab and Henderson (1990) for reviews] and the energetic basis for catalysis. We have recently determined the complete free energy profile for the isomerization of 5-androstene-3,17-dione (**1**) by KSI and discussed these results in terms of competing theories of catalysis (Hawkinson & Pollack, 1994).

A substantial body of evidence (Benisek et al., 1980; Bounds & Pollack, 1987; Kuliopulos et al., 1989) has implicated Asp-38 of KSI as the residue that abstracts a proton from **1** to produce an intermediate dienolate ion, which is stabilized by the hydroxyl group of Tyr-14. This dienolate is then reprotonated by Asp-38 to produce the product ketone (**3**). We examined the sensitivity of this reaction to small changes in position and/or orientation of the carboxylate group by mutating this aspartic acid to glutamic acid. Although the functional groups of the side chains of Asp and Glu are identical, $k_{cat}$ for the mutant (D38E) is decreased by 300-fold with respect to wild type (WT) with 5-androstene-3,17-dione (**1**) as the substrate (Zawrotny et al, 1991).

In order to probe the effects of the replacement of Asp-38 by Glu on the energetics of each of the individual steps of the overall reaction, we have now determined the complete free energy profile for the reaction catalyzed by D38E. The major effect of this mutation is to raise the barriers for both enolization and ketonization by about 3−4 kcal/mol. The internal equilibrium constant for the interconversion of bound substrate and bound dienol with D38E is approximately unity, comparable to that with WT KSI. The partitioning of the intermediate dienol to reactant vs product is approximately 1:1, also unchanged from WT. The overall changes in the energetic profile of the mutant relative to WT are similar to those previously observed for the D165E mutant of triosephosphate isomerase (Straus et al., 1985).

## MATERIALS AND METHODS

*Materials.* 5-Androstene-3,17-dione (**1**) was prepared by G. Blotny of this laboratory (Pollack et al., 1989b). The 4-androstene-3,17-dione content was estimated at less than 5% by determining the absorbance at 248 nm prior to and following isomerization with KSI. 4-Androstene-3,17-dione (**3**) was purchased from Sigma and purified by recrystallization from ethyl acetate and methanol, mp 173−174 °C.

All deuterated solvents and reagents were ≥99 atom % deuterium. Bovine serum albumin (BSA) was purchased from Calbiochem and used without further purification. Other reagents were reagent grade or better.

WT and D38E were available from previous investigations (Eames et al., 1989; Zawrotny et al., 1991). Both WT and D38E were purified by modification of published procedures (Kawahara et al., 1962; Kuliopulos et al., 1987; Talalay & Benson, 1972). For the D38E purification, all glass and plasticware were new, chromic acid washed, or washed with Extran 1000 detergent to ensure that they were completely free of any residual wild type activity. WT used in this work had a specific activity of 45 000−55 000 units/mg, whereas D38E had a specific activity of 270 units/mg under standard conditions (Kawahara et al., 1962; Kuliopulos et al., 1989).

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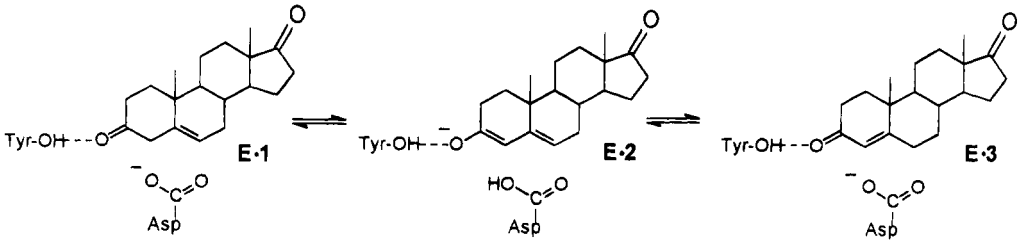
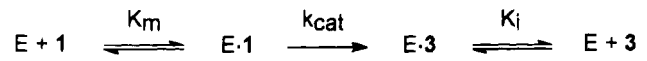
Scheme 1




Scheme 2




*Partitioning Experiments.* The enzyme-catalyzed partitioning of the dienol intermediate was determined as previously described (Eames et al., 1990; Hawkinson et al., 1991a; Hawkinson et al., 1991b) using a Hi-Tech PQ/SF-53 sequential mixing stopped-flow spectrophotometer. The reaction was usually monitored at 249.5 nm, although a few experiments were done at 243 nm.

Solutions of **1** were used within 30 min of preparation. Dienol concentrations were 10−50 $\mu$M in the observation cell. Concentrated solutions of KSI were diluted immediately before use. WT concentrations were usually 0.1−0.5 $\mu$M in the observation cell, whereas D38E concentrations were approximately 10-fold higher.

The data were fit directly to the proposed kinetic scheme with the IBM PC compatible version of the KINSIM/FITSIM programs (Barshop et al., 1983; Frieden, 1993), using the Marquardt algorithm option with equal weighting of all data points. All runs from a given experiment were fit simultaneously to give an overall best set of kinetic parameters. Separate fitting of each run followed by averaging of the results yielded similar values of the parameters.

*Steady-State Kinetics.* All steady-state kinetic measurements were made using either a Gilford Response or Response II spectrophotometer with 3.00 mL solutions (34 or 330 mM phosphate buffer, pH 7.0, with 3.3% methanol, unless otherwise indicated). Solutions of **1** were thermostated at 25.0 °C and allowed to equilibrate for at least 10 min prior to the addition of enzyme. Stock solutions of KSI were prepared by diluting concentrated KSI into solutions of 10 mg/mL BSA in 34 mM phosphate buffer; several microliters of this solution were added to initiate the reaction. The reaction was monitored at 248 nm, the absorbance maximum for **3**.

For progress curve experiments, the data were usually collected for 5−7 half-lives and never for less than three half-lives. Values for the steady-state kinetic parameters were obtained from fitting to a modified Michaelis−Menten scheme (Scheme 2), using the KINSIM/FITSIM programs. Typically, three separate time course measurements (10 and 90 $\mu$M **1** with no additional **3**, and 20 $\mu$M **1** with 50 $\mu$M **3**) were analyzed together to give the best overall fit to the kinetic parameters.

For initial rate experiments, rates were determined from the first 5% of the reaction. Concentrations of **1** were varied in the range of 15−95 $\mu$M. Kinetic parameters were determined either from the slope and intercepts of double-reciprocal plots or from nonlinear fitting to the hyperbolic form of the Michaelis-Menten equation.

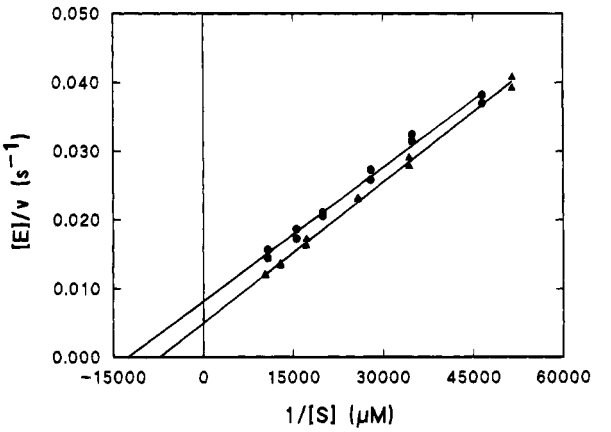



FIGURE 1: Rate of formation of **3** from the D38E catalyzed isomerization of **1** in 34 mM (▲) and 330 mM (●) phosphate buffer (pH 7.0). Final conditions: (▲) 34 mM phosphate, 3.3% methanol, pH 7.0, 25.0 ± 0.1 °C, 2.1 nM D38E, (●) 330 mM phosphate, 3.3% methanol, pH 7.0, 25.0 ± 0.1 °C, 3.7 nM D38E. Theoretical curves are calculated from the following: (▲) $k_{cat}$ = 200 s$^{-1}$, $K_m$ = 137 $\mu$M, $k_{cat}/K_m$ = 1.46 × 10$^6$ M$^{-1}$ s$^{-1}$; (●) $k_{cat}$ = 122 s$^{-1}$, $K_m$ = 79.6 $\mu$M, $k_{cat}/K_m$ = 1.54 × 10$^6$ M$^{-1}$ s$^{-1}$.

Table 1: Kinetic Constants for WT and D38E with 5-Androstene-3,17-dione as Substrate and 4-Androstene-3,17-dione as Inhibitor

| | $k_{cat}$ (s$^{-1}$) | $K_m$ ($\mu$M) | $k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$) | $K_i$ ($\mu$M) |
|---|---|---|---|---|
| WT | | | | |
| 330 mM[a,b] | 3.8 × 10$^4$ | 118 | 3.0 × 10$^8$ | 148 |
| 34 mM[c] | 6.6 × 10$^4$ | 277 | 2.4 × 10$^8$ | 162 |
| D38E | | | | |
| 330 mM[a,d] | 120 | 72 | 1.7 × 10$^6$ | nd[g] |
| 330 mM[a,e] | 160 | 63 | 2.5 × 10$^6$ | 61 |
| 34 mM[a,d,f] | 180 | 120 | 1.6 × 10$^6$ | nd[g] |

[a] Phosphate buffer, pH 7.0, 3.3% MeOH, 25.0 °C. [b] Hawkinson et al. (1991b). [c] Phosphate buffer, pH 7.0, 3.2% MeOH, 25.0 °C (Pollack et al., 1986). [d] From initial rate measurements. Runs were done in duplicate. Errors are estimated as 5% for $k_{cat}/K_m$ and 20% for $k_{cat}$ and $K_m$. [e] From progress curves fit to Scheme 2; errors are estimated as 5% for all parameters. [f] Current work and Zawrotny et al. (1991). [g] Not determined.

## RESULTS

*Determination of Steady-State Kinetic Constants.* Steady-state kinetic constants for the D38E catalyzed conversion of **1** → **3** were determined in solutions of 34 mM or 330 mM phosphate buffer at pH 7.0 with 3.3% methanol cosolvent (Figure 1 and Table 1). In addition, $k_{cat}/K_m$ for D38E was determined at pH 8.0 (34 mM phosphate) from a pseudo-first-order kinetic analysis of data at [S] ≪ $K_m$ ($k_{cat}/K_m$ = 1.6 × 10$^6$ M$^{-1}$ s$^{-1}$). At pH 7, the value of $k_{cat}/K_m$ in 330 mM buffer is comparable to the value in 34 mM buffer (1.7 × 10$^6$ and 1.6 × 10$^6$ M$^{-1}$ s$^{-1}$, respectively). However, the individual values of $k_{cat}$ and $K_m$ (120 s$^{-1}$ and 72 $\mu$M) are somewhat lower than those determined at lower buffer

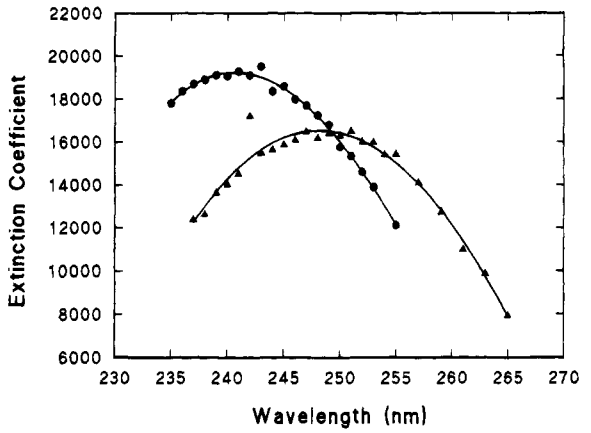



FIGURE 2: Ultraviolet absorbance spectra of **2** (●) and **3** (▲). The spectrum of **2** was calculated from the amplitude of the reaction of **2** to **1** as a function of wavelength; the spectrum of **3** was calculated from the amplitude of the reaction of **1** to **3**. Final conditions were (●) 330 mM phosphate buffer, pH 7.4, 3.3% methanol, and (▲) as above with 0.6 $\mu$M D38E.

concentration (180 $s^{-1}$ and 120 $\mu$M). A similar dependence of the kinetic constants on buffer concentration has been observed previously for catalysis by the wild type (WT) enzyme. Both $k_{cat}$ and $K_m$ decrease from 34 mM buffer (6.6 $\times$ $10^4$ $s^{-1}$ and 277 $\mu$M; Pollack et al., 1986) to 330 mM buffer, (3.8 $\times$ $10^4$ $s^{-1}$ and 118 $\mu$M; Hawkinson et al., 1991b), while $k_{cat}/K_m$ remains nearly independent of buffer concentration (3.0 $\times$ $10^8$ $M^{-1}$ $s^{-1}$ in 330 mM buffer and 2.4 $\times$ $10^8$ $M^{-1}$ $s^{-1}$ in 34 mM buffer).

The dissociation constant ($K_i$) for D38E·**3** was determined from the entire time course of the enzymatic isomerization of **1** → **3** at several initial concentrations of both **1** and **3** (330 mM phosphate; 10 $\mu$M < [**1**] < 100 $\mu$M and 1 $\mu$M < [**3**] < 55 $\mu$M). The data were analyzed by fitting to the mechanism of Scheme 2, giving $K_i$ = 61 $\mu$M. This analysis also gives values of the steady state kinetic parameters $k_{cat}$ (160 $s^{-1}$) and $K_m$ (63 $\mu$M), in agreement with the values determined from initial rates (Table 1).

*Generation of the Dienol Intermediate and Determination of the Isosbestic Point for Dienol* (**2**) and Product (**3**). 5-Androstene-3,17-dione (**1**) is unusually acidic for a ketone, with a $pK_a$ of 12.7 (Pollack et al., 1989b). Thus, treatment of **1** with base for a short period of time produces the enolate anion, which upon quenching in mildly acidic buffer is protonated at the oxygen, generating dienol **2**. We have previously shown that incubation of **1** in 0.5 M NaOH for 0.5 s followed by quenching into phosphate buffer (monobasic:dibasic phosphate 1:1) yields a solution that is 12% **1**, 83% **2**, and 5% **3** (Hawkinson et al., 1991a,b). In these solutions, **2** then ketonizes to a mixture of **1** and **3** in a ratio of ca. 97:3 (Pollack et al., 1989; Zeng & Pollack, 1991).

The ultraviolet absorption spectrum of **2** (Figure 2) was determined by monitoring the variation of the absorbance decrease as a function of wavelength for the conversion of **2** → **1**, using the procedure of Hawkinson et al. (1991a). The absorption spectrum of **3** from 237 nm to 265 nm was determined similarly by mixing **1** with KSI and monitoring the amplitude of the absorbance increase as a function of wavelength for the conversion of **1** → **3**.

The calculated absorbance spectrum of **2** ($\lambda_{max}$ = 241 nm; $\epsilon$ = 19 100 $M^{-1}$ $cm^{-1}$) obtained here is similar to the one that we reported previously ($\lambda_{max}$ = 236 nm; $\epsilon$ = 17 500 $M^{-1}$ $cm^{-1}$; Hawkinson et al., 1991a). The isosbestic point for the conversion of **2** → **3** (249.5 nm) is somewhat higher than that reported earlier by us (243 nm; Eames et al., 1990; Hawkinson et al., 1991a). Given the limitations of the method by which this value is determined, we do not believe that these differences are significant.

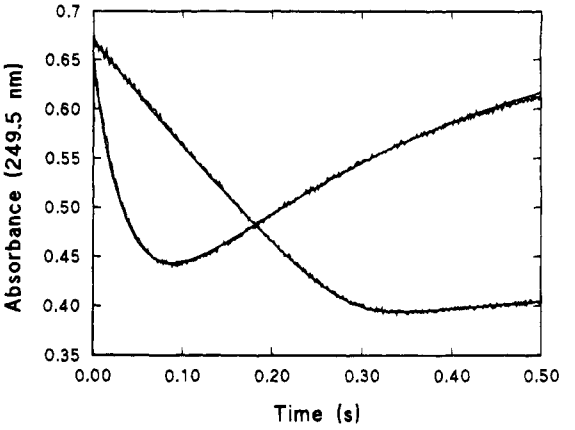
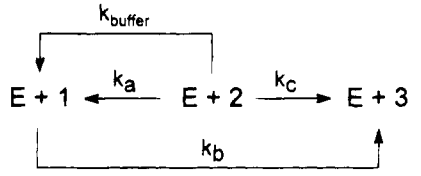



FIGURE 3: Absorbance change *vs* time at 249.5 nm for the reaction of **2** with (a) D38E and (b) WT. Solutions of **1** (ca. 500 $\mu$M in 80% $D_2O$/20% MeOD) and 1.0 N NaOD were mixed and allowed to age for 0.5 s. This solution was then quenched into five parts of a solution of 400 mM phosphate buffer in $D_2O$ containing enzyme in the observation cell of the stopped flow spectrophotometer. The final conditions were 330 mM phosphate pD 7.0, 3.3% MeOD, 25.0 °C, with (a) 0.70 $\mu$M D38E and (b) 0.063 $\mu$M WT. The theoretical curves were calculated from fits to (a) Scheme 4 with $^2K_m$ = 1.92 $\mu$M, $k_4$ = 112 $s^{-1}$, $k_b$ = 3.19 $\times$ $10^5$ $M^{-1}$ $s^{-1}$, $k_5$ = 65.2 $s^{-1}$, and (b) Scheme 3 with $k_a$ = 2.46 $\times$ $10^8$ $M^{-1}$ $s^{-1}$, $k_b$ = 4.58 $\times$ $10^7$ $M^{-1}$ $s^{-1}$, $k_c$ = 1.41 $\times$ $10^8$ $M^{-1}$ $s^{-1}$.

Scheme 3



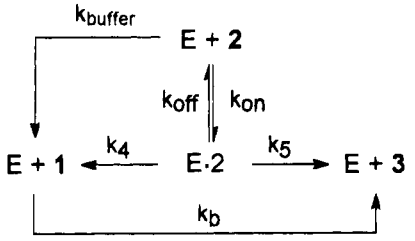

*Partitioning of the Dienol Intermediate.* Dienol **2** was generated by quenching the anion into a buffered solution of enzyme (either WT or D38E) and the reaction was monitored at the isosbestic point for **2** and **3**. In both cases, a rapid decrease in UV absorbance followed by a slower increase was observed (Figure 3). For WT (Eames et al., 1990; Hawkinson et al., 1991a,b), we have previously interpreted this result in terms of an initial decrease of absorbance due to the enzymatic conversion of **2** → **1** and subsequent increase in absorbance from the enzyme-catalyzed isomerization of **1** → **3** (Scheme 3). The absorbance variation with time for WT was treated as the sum of two pseudo-first-order reactions which were analyzed by nonlinear curve fitting to a double-exponential equation to give the apparent second order rate constants $k_a$, $k_b$, and $k_c$. In the present work, the WT data were fit directly to Scheme 3, using the KINSIM/FITSIM program.These reactions were typically monitored at the isosbestic wavelength (249.5 nm). In addition, to permit comparison with the earlier studies, separate measurements at 243 nm [the isosbestic wavelength given in Eames et al. (1990) and Hawkinson et al. (1991a,b)] were carried out and these yielded equivalent rate constants. Values for the apparent second order rate constants $k_a$, $k_b$, and $k_c$ for WT are similar to those previously determined

Table 2: Rate Constants for the Reaction of **2** with Wild Type KSI at 25 °C in 330 mM Phosphate, pH 7.4[a,b]

| % MeOH | $10^6 k_a$ ($M^{-1}$ $s^{-1}$) | $10^6 k_b$ ($M^{-1}$ $s^{-1}$) | $10^6 k_c$ ($M^{-1}$ $s^{-1}$) | $k_c/(k_a + k_c)$ |
|---|---|---|---|---|
| 2.5[c] | 160 ± 4 | 210 ± 3 | 250 ± 8 | 61 ± 5 |
| 3.3 | 225 ± 6 | 258 ± 12 | 179 ± 29 | 44 ± 3 |
| 3.3[c] | 210 ± 20 | 210 ± 40 | 230 ± 30 | 53 ± 5 |
| 10.0 | 206 ± 3 | 71 ± 1. | 87 ± 5 | 30 ± 1 |
| 10.0[c] | 270 ± 30 | 67 ± 7 | 120 ± 10 | 31 ± 3 |
| 15.0 | 129 ± 1 | 21.0 ± 0.3 | 43 ± 2 | 25.0 ± 0.2 |
| 20.0 | 60.7 ± 0.5 | 7.2 ± 0.1 | 20.3 ± 0.6 | 25 ± 2 |
| 3.3 ($D_2O$) | 214 ± 3 | 47.3 ± 0.5 | 137 ± 4 | 39 ± 4 |
| 3.3 ($D_2O$)[c] | 240 ± 40 | 46 ± 7 | 120 ± 20 | 33 ± 2 |
| 20.0 ($D_2O$) | 44.9 ± 0.2 | 1.77 ± 0.02 | 9.4 ± 0.2 | 17.3 ± 0.7 |

[a] Rate constants defined in Scheme 3 and in Hawkinson et al. (1991a,b). [b] 330 mM phosphate buffer pH 7.4, 25.0 °C. Data were collected as sets of 10 determinations and fit with the KINSIM/FITSIM programs. [c] Data from Hawkinson et al. (1991b).

Scheme 4




by Hawkinson et al. (1991b) and are given in Table 2.

The results of our previous work with WT at 2.5−10% methanol (Hawkinson, 1991b) have been confirmed and extended to 15% and 20% methanol (Table 2). In 2.5% methanol, approximately 60% of **2** is converted to **3** and 40% is partitioned to **1** by WT. As the amount of methanol cosolvent increases, the partitioning of **2** to **3** becomes less favorable, with ca. 25% of **2** converted to **3** in 15% and 20% methanol. An analogous trend is seen in $D_2O$, with the percentage of **3** decreasing from 39% in 3.3% MeOD to 17% in 20% MeOD.

The variation of the UV absorbance for the partitioning of **2** by D38E is qualitatively similar to that with WT, with an initial decrease in absorbance followed by a slower increase. However, the shape of the curve with D38E is markedly different than that with WT and has the form typically seen with saturation kinetics (Figure 3). For the reaction with D38E, the data are not well described either by a double-exponential equation or the mechanism of Scheme 3. Thus, the data were fit to the more complete mechanism of Scheme 4 using the KINSIM/FITSIM programs to give the apparent dissociation constant for the complex of D38E·**2** ($^2K_m$) and the rate constants for conversion of D38E·**2** to **1**($k_4$) and to **3** ($k_5$). The rate constant for the conversion of **1** to **3** ($k_b$) under these conditions was not determined because the time scale required to observe the partitioning is too short to observe significant reaction.

Unlike WT, the partitioning ratio for D38E is independent of the methanol concentration. There is, however, a decrease in the partitioning from 52% **3** in aqueous solution to 37% **3** in $D_2O$ (Table 3). Furthermore, addition of methanol increases the apparent dissociation constant of the dienol ($^2K_m$) from 2.0 μM in 3.3% MeOH to nearly 35 μM in 20% MeOH. In $D_2O$ the change in $^2K_m$ is even greater, from 1.2 μM to 60 μM as the percent MeOD is increased from 3.3% to 20%.

Table 3: Rate Constants for the Reaction of **2** with D38E KSI at 25 °C in 330 mM Phosphate, pH 7.4[a]

| % MeOH | $^2K_m$ (μM) | $k_4$ ($s^{-1}$) | $k_5$ ($s^{-1}$) | $k_5/(k_4 + k_5)$ |
|---|---|---|---|---|
| 3.3 | 2.0 ± 0.6 | 320 ± 30 | 335 ± 20 | 51 ± 1 |
| 10.0 | 5 ± 1 | 300 ± 20 | 320 ± 20 | 51.7 ± 0.2 |
| 15.0 | 16 ± 3 | 300 ± 30 | 330 ± 25 | 51.7 ± 0.4 |
| 20.0 | 35 ± 9 | 230 ± 40 | 240 ± 30 | 51.5 ± 0.1 |
| 3.3 ($D_2O$) | 1.2 ± 0.3 | 110 ± 4 | 66 ± 2 | 37.9 ± 0.7 |
| 20.0 ($D_2O$) | 60 ± 13 | 80 ± 12 | 47 ± 5 | 36.7 ± 0.5 |

[a] 330 mM phosphate, pH 7.4, 25.0 °C. Rate constants defined in Scheme 4. Data collected as sets of eight determinations and fit together using the KINSIM/FITSIM programs.

## CALCULATION OF THE FREE ENERGY PROFILE

The free energy profile for the isomerization of **1** to **3** catalyzed by D38E in 3.3% methanol at pH 7 (330 mM phosphate) and 25 °C can be calculated from the partitioning ratio and the steady state kinetic parameters. Definition of the microscopic rate constants is given in Scheme 5, and the rationale for the calculation of these rate constants is given below. Although this method is based on that used previously (Hawkinson et al., 1991b), the existence of saturation kinetics with D38E requires significant modifications.

(1) For D38E, unlike WT, the chemical steps are much slower than the diffusion steps (i.e., $k_3 \ll k_2$; $k_6 \ll k_7$). Support for this assertion comes from the decrease in both $k_{cat}$ and $k_{cat}/K_m$ by > $10^2$-fold for the reaction of D38E with **1** compared to WT with **1**. In addition, $k_{cat}/K_m$ for D38E (ca. $2 \times 10^6$ $M^{-1}$ $s^{-1}$) is much lower than the diffusion limit ($10^8$−$10^9$ $M^{-1}$ $s^{-1}$). The independence of the partitioning ratio on methanol concentration also is consistent with rapid diffusion (*vide infra*). Thus, the observed rate constants $k_4$ and $k_5$ represent conversion of E·**2** to E + **1** and E + **3**, respectively.

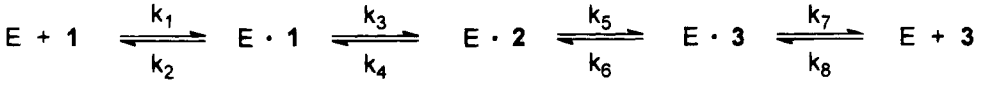
(2) With the assumption that $k_6 \ll k_7$, the expression for $k_{cat}$ is given by eq 1, which can be solved for $k_3$.

$$k_{cat} = \frac{k_3 k_5}{k_3 + k_4 + k_5} \quad (1)$$

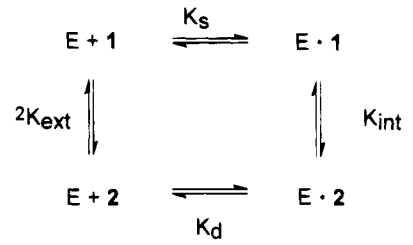
(3) The value for the internal equilibrium constant for the conversion of **1** to **2** on the enzyme surface ($K_{int}$) can be given as $K_{int} = k_3/k_4$ (ca. 1.8).

(4) Eq 2 can be derived from the thermodynamic relationship between the equilibrium constants for the interconversion of **1** and **2** both on and off the enzyme surface and the dissociation constants for E·**1** and E·**2** (Scheme 6). This equation, along with the expression for the apparent dissociation constant for E·**2** (eq 3), allows the calculation of

Scheme 5



Scheme 6




$k_{on}$ and $k_{off}$ for **2** [$(3.4 \pm 1.0) \times 10^8$ $M^{-1}$ $s^{-1}$ and $23 \pm 12$ $s^{-1}$, respectively] and the dissociation constant for **2** ($K_d$ $=0.067 \pm 0.017$ $\mu$M).

$$\frac{k_{off}}{k_{on}} = K_d = \frac{^2K_{ext}}{K_{int}(K_s)} \quad (2)$$

$$^2K_m = K_d + \frac{k_4 + k_5}{k_{on}} \quad (3)$$

(5) The apparent second-order rate constant for the D38E catalyzed conversion of **1** to **3** ($k_{cat}/K_m$) is given as

$$\frac{k_{cat}}{K_m} = \frac{k_1k_3k_5}{k_2k_4 + k_2k_5 + k_3k_5} \quad (4)$$

In principle, eq 4, in conjunction with the fact that $K_s = K_m = k_2/k_1$, should enable the direct calculation of both $k_1$and $k_2$. However, in practice this calculation is extremely sensitive to the values of $k_{cat}$ (used to calculate $k_3$) and $K_m$, neither of which are known with sufficient accuracy. Therefore, the rate constants for the association of D38E with both **1** and **3** ($k_1$ and $k_8$) are assumed to be equal to those for WT ($8.6 \times 10^8$ $M^{-1}$ $s^{-1}$; Hawkinson et al., 1991b). The values of $k_1$ and $K_m$ then determine $k_2$, which is also equal to $k_7$ since $K_s = K_i$ and $k_1 = k_8$.

(6) The only remaining rate constant ($k_6$) can be calculated from the expression for $k_{cat}/K_m$ in the reverse direction [$(k_{cat}/K_m)_{rev}$; eq 5] and the requirement that $(k_{cat}/K_m)_{rev} = (k_{cat}/K_m)/^3K_{ext}$, where $^3K_{ext}$ is the equilibrium constant for the isomerization of **1** to **3** in aqueous solution ($^3K_{ext} = 2400$; Pollack et al., 1989b).

$$\left(\frac{k_{cat}}{K_m}\right)_{rev} = \frac{k_4k_6k_8}{k_4k_6 + k_4k_7 + k_5k_7} \quad (5)$$

A summary of the values of these rate constants and the Gibbs free energies associated with them, along with the values for WT, is given in Table 4 and shown in Figure 4.

## DISCUSSION

Replacement of D with E (or vice versa) can have significant effects on the catalytic ability of enzymes that use a carboxylate group to abstract a proton from a carbon acid. Straus et al. (1985) replaced the active site base Glu-165 by an aspartic acid (E165D) in triosephosphate isomerase (TIM) and found that the rate of interconversion of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate ($k_{cat}$) is slowed by 1000-fold relative to WT. With citrate synthase, Alter et al. (1990) showed that replacement of the putative base (Asp-375) by glutamic acid (D375E) in the pig enzyme reduces $k_{cat}$ by 420-fold. Similarly, for KSI, $k_{cat}$ for D38E is ca. 300-fold lower than for WT with 5-androstene-3,17-dione.

Table 4: Calculated Rate Constants for the Wild Type and D38E KSI Catalyzed Isomerization of 5-Androstene-3,17-dione (**1**) to 4-Androstene-3,17-dione (**3**) at pH 7.4 and 25 °C[a-c]

| D38E[b] | | wild type[c] | | |
|---|---|---|---|---|
| rate constant | $\Delta G^\ddagger$ | rate constant | $\Delta G^\ddagger$ | $\Delta\Delta G^\ddagger$ |
| $k_1 = 8.6 \times 10^8$ $M^{-1}$ $s^{-1}$ | | $k_1 = 8.6 \times 10^8$ $M^{-1}$ $s^{-1}$ | | |
| $k_2 = 6.6 \times 10^4$ $s^{-1}$ | 10.9 | $k_2 = 8.6 \times 10^4$ $s^{-1}$ | 10.7 | 0.2 |
| $k_3 = 420$ $s^{-1}$ | 13.9 | $k_3 = 1.7 \times 10^5$ $s^{-1}$ | 10.3 | 3.6 |
| $k_4 = 320$ $s^{-1}$ | 14.0 | $k_4 = 3 \times 10^5$ $s^{-1}$ | <10.0 | >4.0 |
| $k_5 = 330$ $s^{-1}$ | 14.0 | $k_5 = 1 \times 10^5$ $s^{-1}$ | <10.6 | >3.4 |
| $k_6 = 0.11$ $s^{-1}$ | 18.7 | $k_6 = 40$ $s^{-1}$ | 15.3 | 3.5 |
| $k_7 = 6.6 \times 10^4$ $s^{-1}$ | 10.9 | $k_7 = 1.3 \times 10^5$ $s^{-1}$ | 10.5 | 0.4 |
| $k_8 = 8.6 \times 10^8$ $M^{-1}$ $s^{-1}$ | | $k_8 = 8.6 \times 10^8$ $M^{-1}$ $s^{-1}$ | | |

[a] Rate constants as defined in Scheme 5 and in Hawkinson et al. (1991b). [b] 330 mM phosphate buffer, pH 7, 3.3% methanol. The details of the calculations are given in the text. [c] Values taken from Hawkinson et al. (1991b). Rate constants for $k_4$ and $k_5$ are calculated with the assumption that $K_{int} = 0.6$.

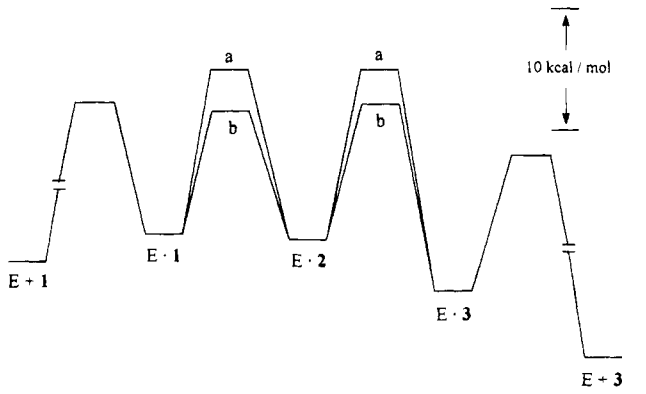



FIGURE 4: Free energy profile for the isomerization of 5-androstene-3,17-dione (**1**) to 4-androstene-3,17-dione (**3**) by (a) D38E and (b) WT steroid isomerase. The energy levels for the release of **1** and **3** for D38E are assumed to be equal to those for WT, based on the assumption that the two enzymes bind **1** and **3** with the same rate constant. The relative levels of unbound species are dependent on concentration and are not specified.

In order to understand the origin of these rate reductions, it is necessary to analyze the energetics of the reactions of both the WT and mutant enzymes. Raines et al. (1986) determined the free energy profile of E165D TIM and compared it with the profile for WT (Albery & Knowles, 1976a). They found that deletion of the methylene group at position 165 has little effect on the relative energies of bound substrate, intermediate and product, but decreases the stability of the transition states for proton transfer by about 3 kcal/mol.

KSI catalyzes an enolization/ketonization reaction similar to that of TIM, except that the proton transfer is 1,3, rather than 1,2 as with TIM. Analysis of the free energy profile for D38E KSI gives the effect of *insertion* of a methylene group into the catalytic carboxylate, the complementary experiment to that with E165D TIM.

*Possible Sources of Error and Validity of the Method.* Before discussing the nature of the energetics of D38E, it is necessary to show that the observed catalysis is due to the mutant and is not the result of a small amount of WT impurity. Two major lines of evidence show that the activity is not due to contaminating WT. (1) The $K_m$ values for D38E and WT differ by a factor of two in both 34 and 330 mM

phosphate buffer. Since $K_m$ is a function only of the identity of the catalyst and not of its concentration, the activity with D38E cannot be due to contaminating WT. (2) The kinetic form of the reaction of the dienol (**2**) with enzyme is quite different for WT and D38E. For WT, the data fit a simple double-exponential equation quite well, whereas for D38E a more complex expression is needed.

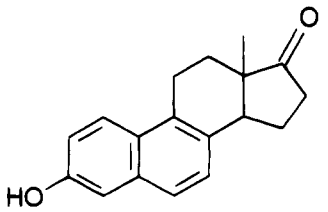
The basis for the determination of the free energy profile for D38E is the observation of the partitioning of the intermediate dienol by the enzyme. In order for this method to be reliable, the enzyme bound dienol intermediate formed from treatment of the dienol with D38E must be the same as the intermediate along the reaction pathway. Although there has been some concern that the enzyme may not be in the correct protonic state for reaction with the dienol (Xue et al., 1990), we have addressed this question previously for WT and have concluded that the same bound dienol is formed from enzyme plus dienol as from enzyme plus substrate (Hawkinson et al., 1991a,b).

The microscopic rate constants are limited by the errors inherent in the individual experiments, which result in errors on the order of 20% in the calculated rate constants in Table 4. Errors of this order of magnitude are insignificant on a free energy scale (ca. 0.1 kcal/mol), so that the overall features of the free energy profile are unaffected.

*Determination of the Internal Equilibrium Constant.* Gerlt and Gassman (1993) have proposed a general theory to explain the rapid rates of enzymatic enolizations. An important component of this theory is the prediction that the internal equilibrium constant for the interconversion of substrate and enol ($K_{int}$ = [E·**2**]/[E·**1**]) should be much less than unity. We have previously estimated $K_{int}$ for WT to be ca. 0.3 and argued that this result conflicts with the Gerlt/Gassman proposal (Hawkinson et al., 1994). Other estimates for $K_{int}$ for WT are $\leq$0.6 (Hawkinson et al., 1991b) and $\geq$0.2 (Brooks & Benisek 1994).

The procedure used for the determination of the free energy profile of D38E is similar to that used previously for WT (Hawkinson et al., 1991b). However, in contrast to the reaction of **2** with WT, the reaction of **2** with D38E shows saturation kinetics, enabling the determination of $K_{int}$ (= 1.8) from the kinetic parameters. This work represents the first direct determination of an internal equilibrium constant for an enzymatic enolization.

This result enables an additional estimate of $K_{int}$ for WT to be obtained. We (Hawkinson et al., 1994) have determined the dissociation constant ($K_d$) for the intermediate analog d-equilenin (**4**) for both WT (2.4 $\mu$M) and D38E (0.78




**4**

$\mu$M). If the dissociation constants of **2** parallel those for **4**, then $K_d$ for WT·**2** should be about 3-fold larger than $K_d$ for D38E·**2**. Coupled with a difference in the dissociation constants in 330 mM phosphate for WT·**1** (100 $\mu$M; Hawkinson et al., 1991b) and D38E·**1** (63 $\mu$M), $K_{int}$ for WT can be calculated to be about 1.9-fold lower than $K_{int}$ for D38E or ca. 1.0, in reasonable agreement with the previous estimates of 0.2 to 0.6.

*Energetics of D38E and WT.* The free energy profiles for both D38E and WT are shown in Figure 4. For WT, all four of the energy barriers are kinetically significant (Hawkinson et al., 1991b). The transition states for the $k_1$ and $k_5$ steps differ by only 0.1 kcal/mol, while the transition state for the $k_3$ step is about 0.3−0.4 kcal/mol lower. Although the transition state for the $k_7$ step is much lower in energy than the other three, $k_7$ is still partially rate determining since the magnitudes of $k_7$ and the other rate constants are similar.

In contrast to WT, the free energy profile for D38E shows only two kinetically significant barriers to reaction. The barriers for the two chemical processes ($k_3$ and $k_5$) are approximately 3 kcal/mol higher than those for the two diffusion processes ($k_1$ and $k_7$). Somewhat surprisingly, the partitioning of the intermediate dienol is nearly unaffected by the D to E mutation.

Thus, the only major difference in the free energy profiles is the rates of the chemical steps. The insertion of an extra methylene group in D38E does not destabilize either the enzyme−substrate complex or the enzyme−dienol complex. However, this methylene group causes a significant change (ca. 300-fold) in the rate constants for the chemical steps. In the terminology of Albery and Knowles (1976b), the difference in the reactivity of the two isomerases is due to "catalysis of elementary steps".

*Effect of Methanol on the Partitioning of the Dienol.* The different response of the partitioning ratios for WT and D38E to changes in methanol concentration is consistent with the free energy profiles. The partitioning ratio of **2** to **1** and **3** by WT depends markedly on the methanol concentration (Table 2), decreasing from 61% **3** in 2.5% methanol to 25% **3** in 15% and 20% methanol. Similarly, the fraction of **3** that is formed in $D_2O$ decreases from 36% in 3.3% methanol to 17% in 20% methanol. In contrast, the partitioning of **2** catalyzed by D38E shows a constant amount of **3**, independent of the methanol concentration in both water (51%) and $D_2O$ (37%).

These results can be rationalized in terms of the microscopic rate constants. The ratio of [**3**]/[**1**] that is formed is given by eq 6. Since the proton transfer reactions occur at

$$\frac{[\mathbf{3}]}{[\mathbf{1}]} = \frac{k_5(k_2 + k_3)}{k_2 k_4} \qquad (6)$$

the active site, which is protected from solvent (Eames et al., 1989), the rate constants $k_4$ and $k_5$ are expected to be nearly independent of the concentration of methanol. In contrast, the rate constant for the dissociation of E·**2** to E + **1** ($k_2$) is likely to have a strong dependence on methanol concentration. For D38E, $k_2 \gg k_3$ at all concentrations of methanol and the ratio [**3**]/[**1**] becomes equal to $k_5/k_4$. For WT, however, $k_2$ and $k_3$ are of the same order of magnitude at low methanol concentrations. Thus, as the concentration of methanol increases, $k_2$ increases and the fraction of **3** decreases. The independence of the partitioning ratio in 15% and 20% methanol suggests that at these concentrations of methanol $k_2 \gg k_3$, and the observed partitioning of WT·**2** approaches $k_5/k_4$.

Since the reaction of D38E plus **2** shows saturation kinetics, the solvent kinetic isotope effect on $k_4$ ($k_{H_2O}/k_{D_2O}$ = 3.0) and $k_5$ ($k_{H_2O}/k_{D_2O}$ = 5.1) can be directly calculated. The isotope effects on both steps are consistent with a rate determining proton transfer. Because saturation of WT by **2** does not occur, values for $k_{H_2O}/k_{D_2O}$ cannot be obtained for WT, but the difference in partitioning, even in 20% methanol, indicates that the solvent isotope effects on $k_4$ and $k_5$ are not identical.

*Comparison of the Energetics of D38E KSI and E165D TIM.* Knowles and co-workers have determined the free energy profiles for wild type (Albery & Knowles, 1976a) and E165D (Raines et al., 1986) TIM from chicken muscle and from yeast (Nickbarg & Knowles, 1988). Wild type TIM, like KSI, is a nearly optimal catalyst with the apparent second order rate constant ($k_{cat}/K_m$) close to the diffusion limit. These enzymes both catalyze enolization/ketonization reactions (1,2 proton transfer for TIM; 1,3 proton transfer for KSI). In each case the mutation decreases the rates of the proton transfer steps $10^2-10^3$-fold without changing the internal equilibria.

The similarity of the effects of mutations in these two enzymes is unexpected since the E165D mutation in TIM pulls the catalytic carboxylate away from the substrate, whereas the D38E mutation in KSI likely moves it closer to the substrate. In each case the decrease in the rate of proton transfers is $10^2-10^3$-fold, and the partitioning of the intermediate is only slightly perturbed by the mutations. One might have expected that the rates of protonation of the enol intermediates at the two possible positions would be affected differently by movement of the carboxylate.

Joseph-McCarthy et al. (1994) have determined the crystal structure of E165D TIM bound to the intermediate analog phosphoglycolohydroxamate and compared it to the structure of WT TIM bound to the same inhibitor (Davenport et al., 1991). Although the overall structures of the mutant and the wild type are virtually identical, Asp-165 of the mutant is moved somewhat, relative to Glu-165 of the wild type. Thus, the distance from the carboxylate oxygens to the inhibitor by is increased about 1 Å. In addition, the *anti* orbitals of D165E appear to be better oriented for proton transfer than the *syn* orbitals, although the opposite is true in the WT. Gandour (1981) has suggested that *syn* orbitals of carboxylates are more basic than *anti* orbitals. Although no definitive structure is available for KSI, there may be a similar change in structure between WT KSI and D38E. The combination of a modified stereoelectronic orientation of the orbitals and movement of the oxygen may also account for the greater catalytic ability of WT KSI relative to D38E. More detailed analysis awaits the determination of the structure of KSI.

## CONCLUSIONS

(1) Insertion of an additional methylene group into the side chain of Asp-38 of KSI to produce D38E has little effect on the internal equilibrium constants, but decreases the rate constants for all of the proton transfers by $10^2-10^3$-fold.

(2) This effect is similar to that produced by the E165D mutation in triosephosphate isomerase, in which a side chain methylene group is *deleted*. In both cases the partitioning of the enzyme is virtually unaffected.

(3) The internal equilibrium constant for D38E for the interconversion of enzyme-bound substrate and intermediate dienol is 1.8. This represents the first direct determination of the internal equilibrium constant for an enzymatic enolization.

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