Evaluation of the Internal Equilibrium Constant for 3-Oxo-Δ^5-steroid Isomerase Using the D38E and D38N Mutants: The Energetic Basis for Catalysis†

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ABSTRACT: The dissociation constant (K_D) for the complex of the intermediate dienol (**2**) and the D38N mutant of 3-oxo-Δ^5-steroid isomerase (D38N·**2**) has been determined for the isomerization of 5-androstene-3,17-dione (**1**). K_D for D38N·**2** is pH-dependent, with values of 6 nM at pH 6.9, 51 nM at pH 5.8, and 59 nM at pH 5.2. These values of K_D are used to estimate the pH-independent dissociation constant (0.7 ± 0.3 μM) for the complex of dienol and wild-type (WT) enzyme. The internal equilibrium constant (K_{int} = 0.3 ± 0.2) for the interconversion of bound substrate (WT·**1**) and bound intermediate (WT·**2**) was then calculated for WT using its K_D, the values for the external equilibrium constant for **1** ⇌ **2**, and the dissociation constant of the enzyme substrate complex (K_S). The dissociation constant (K_D) for the complex of equilenin (**4**) with WT, D38E, and D38N enzymes was also determined at pH values from 4 to 7. For the complex of **4** with D38N (D38N·**4**), K_D is pH-dependent with an apparent pK_a of about 4.5, whereas K_D for both WT·**4** and D38E·**4** is pH-independent. These values are used to give two additional estimates of the internal equilibrium constant for WT (K_{int} = 0.5 and 0.01). Analysis of these results in terms of Marcus formalism leads to the conclusion that the primary function of the enzyme is to decrease the thermodynamic barrier to formation of the intermediate by lowering ΔG° by about 10 kcal/mol. In contrast, the intrinsic free energy of activation ($\Delta G^{\ddagger}_{int}$) is only decreased by about 3 kcal/mol. These results are discussed in terms of competing theories of enzymatic enolization.

In their analysis of the reaction energetics of triosephosphate isomerase, Albery and Knowles (1976a,b) presented a general theory that relates the energetics of individual steps of an enzymatic reaction to the catalytic ability of the enzyme. They described three different methods by which an enzyme might accelerate a chemical reaction: uniform binding, differential binding, and catalysis of elementary steps. An important component of this theory of enzymatic efficiency is the ability of an enzyme to preferentially bind an intermediate relative to substrate, thus decreasing the energy difference between substrate and intermediate. For enzymes that function under reversible conditions, this differential binding would equalize the relative energies of bound substrate and bound intermediate, giving an internal equilibrium constant (K_{int}) of approximately unity. For irreversible enzymes, bound intermediate will be favored over bound substrate and K_{int} will be larger than unity (Chin, 1983; Stackhouse et al., 1985; Burbaum et al., 1989).

Recently, Gerlt and co-workers (Gerlt et al.,1991; Gerlt & Gassman, 1992) developed a different approach for the analysis of the rates of enzymatic proton transfers to and from carbon atoms adjacent to carbonyl or carboxyl groups (enolizations). They rationalized the rate constants for these reactions in terms of concerted protonation of the carbonyl group to form a neutral enol intermediate, rather than an enolate ion. This analysis has been modified and extended (Gerlt & Gassman, 1993) to include a description of the reaction energetics in terms of Marcus formalism (Cohen & Marcus, 1968; Marcus, 1969; Albery, 1980). A key prediction that emerges from this work is that the internal equilibrium constant for the formation of the intermediate enol(ate)[1] from the substrate at the enzyme active site should be substantially less than unity for most efficient catalysis. That is, the bound intermediate should be markedly less stable than the bound substrate. In contrast, considerations based upon theories of Albery and Knowles (Albery & Knowles, 1976a,b; Burbaum et al., 1989) and Benner and co-workers (Stackhouse et al., 1985) predict that stabilization of the bound intermediate should be sufficient to at least equalize the energies of bound intermediate and bound substrate. Guthrie and Kluger (1993) have also discussed this question and concluded that stabilization of the intermediate by these enzymes must be important.

Thus, the theories of Albery/Knowles and Gerlt/Gassman make clear, and distinctly different, predictions concerning the relative stabilities of the bound substrate and bound intermediate (the internal equilibrium constant, K_{int}) for enzyme-catalyzed enolizations. Unfortunately, no investigations to date have provided the requisite data to evaluate these conflicting proposals. Almost complete free energy profiles have been determined for triosephosphate isomerase from chicken muscle (Albery & Knowles, 1976a) and yeast (Nickbarg & Knowles, 1988) and for 3-oxo-Δ^5-steroid

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[1] Since the exact protonic state of intermediate in enzymatic enolizations is subject to some uncertainty, Gerlt and Gassman (1993) have used the term "enolic intermediate". We will use the terms "enol(ate)" or "enolic intermediate" when the protonic state is either unknown or irrelevant to the discussion and the terms "enol" or "enolate" when reference is to a specific ionization state.

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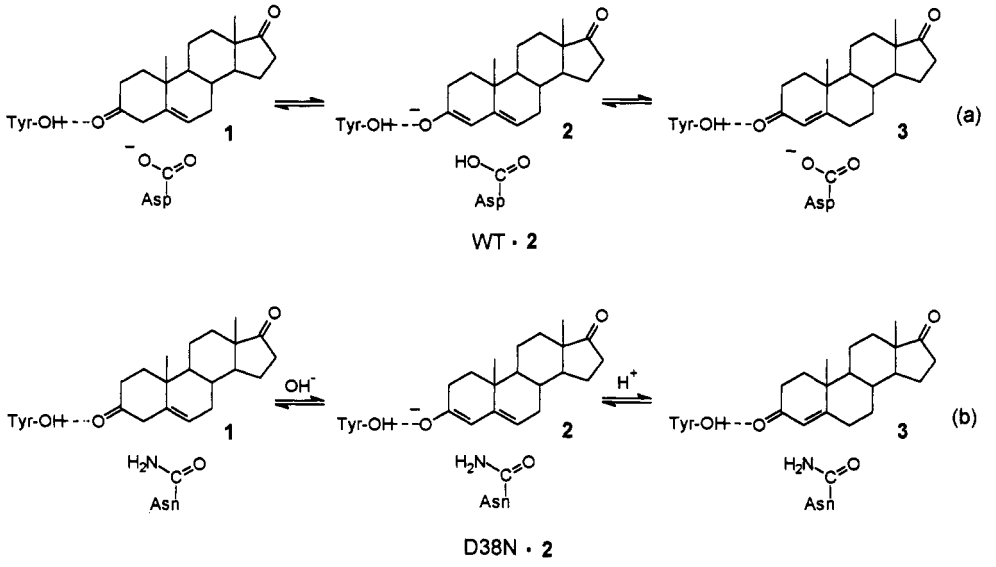
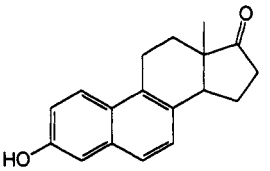
Scheme 1



isomerase (KSI)[2] from *Pseudomonas testosteroni* (Hawkinson et al., 1991b). However, in each case the energy level of the bound enolic intermediate relative to bound substrate could not be obtained. The major problem in the direct determination of the internal equilibrium constant is that the intermediate is rapidly converted to product.

We report here two methods for the estimation of the internal equilibrium constant for bound substrate and bound intermediate for wild-type KSI (WT), an enzyme that catalyzes the interconversion of β,γ-unsaturated 3-oxosteroids to their α,β-unsaturated isomers through a dienolic intermediate. Both methods are based on the use of mutants of Asp-38 (D38N and D38E). Several lines of evidence, including mutagenesis studies (Kuliopulos et al., 1989), have implicated Asp-38 as the base in this reaction, with Tyr-14 as an electrophile that stabilizes the developing charge on the carbonyl oxygen (Scheme 1a).

The first approach to the estimation of K_{int} for WT is based on the fact that D38N converts substrate (**1**) to intermediate (**2**) at the active site, but **2** is converted only very slowly to product (Xue et al., 1991). Thus, the D38N·**2** complex is relatively long-lived, and it is possible to directly determine its dissociation constant (Scheme 1b). The similarity of the structures of WT·**2** and D38N·**2** allows an estimate to be made of the dissociation constant for WT·**2**, enabling an assessment of K_{int} for WT. The second method uses the intermediate analog equilenin (**4**) as a model for the intermediate dienol (**2**). Determination of the dissociation constants for the complexes of **4** with D38N and D38E allows estimates of K_{int} for WT from the values of K_{int} for D38N and D38E.



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MATERIALS AND METHODS

Materials. 5-Androstene-3,17-dione was synthesized by G. Blotny (Pollack et al, 1989a). D38E was obtained from M. E. Zawrotny (Zawrotny et al., 1991). Water was purified by passage through an Ion Pure reverse osmosis system coupled with a tank ultrapurification system consisting of two mixed media deionization tanks, a carbon tank, a UV sterilization lamp, and a Pyroguard ultrafiltration membrane. All other reagents were reagent grade or better. SDS–PAGE gels (15%) were stained with Coomassie Blue. Recombinant WT KSI was purified from cultures of DH5α *Escherichia coli* (Eames et al., 1989).

D38N Mutant of KSI. Plasmid pKC2, containing a 1.2 kb *Pst*I restriction fragment spanning the KSI gene, was a gift from W. Benisek (Choi & Benisek, 1988). The 1.2 kb *Pst*I fragment was inserted into the *Pst*I site of M13mp8. Recombinant M13 bacteriophage was propagated on *E. coli* strain JM103, and single-stranded M13 DNA was purified (Maniatis et al., 1982). Oligonucleotide-directed mutagenesis was performed by the method of Eckstein (Taylor et al., 1985) using a protocol described in detail elsewhere (Duval et al., 1987). Mutants were identified by single-base sequence analysis and confirmed by sequencing the entire gene; no other mutations were apparent. The mutant gene was isolated from M13mp8 on a 1.2 kb *Eco*RI–*Hin*dIII fragment, inserted into the *Eco*RI and *Hin*dIII sites of pUC18 and transformed into *E. coli* strain JM83. The protein was isolated by a procedure similar to that used for WT, except that crystallization with saturated ammonium sulfate was used rather than affinity chromatography as the final purification step (Kawahara et al., 1962).

In order to eliminate any WT activity, the enzyme (ca. 20 μM) was treated with the oxiranyl steroid (17*S*)-spiro-[androstane-17,2′-oxiran]-3α-ol (ca. 50 μM), an irreversible inhibitor of KSI (Bevins et al., 1980; Kayser et al., 1983; Bantia et al., 1985), for 15 h at 4 °C (34 mM phosphate, pH 7.0). Inactivation of wild-type activity was followed by multiple protein concentration/dilution cycles (Amicon Centriprep concentrators) until [oxirane] < 1 μM and [D38N]/[oxirane] > 300.

[2] Abbreviations: KSI, 3-oxo-Δ^5-steroid isomerase; D38N, Asp-38 to Asn-38 mutant of KSI; D38E, Asp-38 to Glu-38 mutant of KSI; WT, wild-type KSI; Tris, tris(hydroxymethyl)aminomethane; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DEAE, (diethylamino)ethyl; BSA, bovine serum albumin.

The UV Spectra of Dienol 2 and the D38N·2 Complex. These spectra were obtained using a Hi-Tech PQ/SF-53 sequential mixing stopped-flow spectrophotometer equipped with a Hi-Tech MG-3000 rapid scanning monochromator (100 ms/scan). The dienol was generated *in situ* by mixing a solution of **1** in 20% methanol (ca. 150 μM) with 1.0 N sodium hydroxide in a 1:1 ratio. This solution was allowed to age for 1.2 s, followed by rapid quenching into a buffer solution (400 mM phosphate, 4.2% methanol, pH 7.0, 1:5 mixing ratio) in the observation cell of the spectrophotometer (Eames et al., 1990; Hawkinson et al., 1991a). The final conditions were 333 mM phosphate and 5.2% methanol, pH 7.2. The UV spectrum of **2** in the absence of enzyme was obtained within 0.2 s after mixing; 10 individual spectra were averaged.

Similarly, the UV spectrum of **2** was obtained with an excess of D38N in the quench solution ($[D38N]_{final}$ = ca. 20 μM). In this case, 10 individual scans were taken at 0.1 s intervals (total time = 1.1 s) and averaged. The spectrum of D38N was also obtained in the absence of dienol. A separate experiment showed no change in the D38N spectrum over a 5 s period. Finally, the averaged D38N spectrum was subtracted from that for **2** in the presence of excess D38N to give the spectrum of **2** bound to D38N (i.e., the spectrum of D38N·**2** corrected for protein absorbance).

Binding Constant of Equilenin to Wild-Type, D38E, and D38N. Dissociation constants for the binding of equilenin (**4**) to wild-type, D38N, and D38E were determined by fluorescence titration of **4** with the appropriate enzyme using an SLM 8000 C spectrofluorimeter equipped with single photon counting. Emission intensity as a function of wavelength was expressed as a ratio to the intensity of a rhodamine B internal standard. All emission spectra were measured using vertically polarized exciting light and with the emission polarizer oriented at the magic angle (Lakowicz, 1983). Measurements were made in acetate (10 mM, pH ca. 4–5.5, 5.2% MeOH) and phosphate (10 mM, pH ca. 7, 3.3% and 5.2% MeOH) buffers. Concentrated enzyme samples were diluted to concentrations suitable for titration with the same buffers. The concentrations of stock solutions of **4**, which were prepared in MeOH, were determined by UV spectroscopy using $\epsilon_{282.5}$ = 5250 M^{-1} cm^{-1} (Davenport et al., 1986). A 5.0 μL volume of the stock solution was added to 3.0 mL of buffer, giving final concentrations of **4** of 1–3 μM for WT and D38E and 900 nM (low pH) to 88 nM (high pH) for D38N. Titrations were carried out by sequential additions of small portions (2–20 μL) of enzyme solution (to a total of between 25 and 125 μL). Before and after each addition of enzyme, the emission spectrum of **4** was scanned from 330 to 500 nm with an excitation wavelength of 298 or 290 nm (an average of 4–8 scans). The spectra of the final solutions were unchanged after 30 min. Each titration was performed in duplicate. Emission spectra for each enzyme concentration were also obtained in the absence of **4** for calculation of the difference spectra. The difference spectra were multiplied by the appropriate factor to account for dilution of **4** upon sequential addition of enzyme. The fluorescence of **4** at the emission maximum (361–363 nm) at each enzyme concentration was then used to calculate the dissociation constant for binding of **4** to isomerase by nonlinear least-squares fitting to eq 2.

Reaction of D38N with 5-Androstene-3,17-dione (1). The reaction of D38N with **1** was investigated in 34 mM buffer (acetate, pH 5.21 and 5.75; phosphate, pH 6.95) with 5.0% methanol as cosolvent, using UV spectroscopy at 258 or 260 nm to monitor product formation. The reaction was monitored through the burst phase (enolization of bound **1**) and for 6–10 min into the steady-state phase (turnover of D38N·**2**) of the reaction (Figure 1). In most cases ([D38N] < 10 μM), the reaction was initiated by addition of enzyme. However, due to the absorbance of D38N, the spectrophotometer was referenced on the enzyme solution prior to addition of **1** for reactions with high [D38N] (ca. 90 μM). For reactions with [D38N] > ca. 2 μM, a 1 cm path length cell was used (Gilford Response spectrophotometer). For the reactions with [D38N] < ca. 2 μM, a 10 cm path length cell was used (Cary 219 spectrophotometer).

Due to the relatively high concentration of D38N required to observe burst kinetics (≥9 μM) and the limited solubility of **1** in 5% methanol (concentrations higher than 100 μM can form micelles), a relatively high percentage of **1** (up to 10%) may be bound by D38N during the burst phase of the reaction. The lack of a constant concentration of **1** during the burst phase necessitated the calculation of k_2 and K_S by an iterative procedure. Nonlinear least-squares analysis of a plot of the initial velocities of the burst (v_0) against $[\mathbf{1}]_{total}$ gave initial estimates of k_3 and K_S. A plot of v_0 against $[\mathbf{1}]_{unbound}$, which was calculated using the estimated value of K_S, gave a second estimate of k_3 and K_S. This procedure was repeated until the change in the calculated value of $[\mathbf{1}]_{unbound}$ was insignificant ($\Delta[\mathbf{1}]_{unbound} < 0.01$ μM).

Reaction of D38N with the Intermediate Dienol (2). The dienol (**2**) was generated as previously described (Eames et al., 1990; Hawkinson et al., 1991a,b). Accordingly, a solution of **1** in 20% methanol was mixed in a 1:1 ratio with 1.0 N NaOH in the stopped-flow spectrophotometer to give rapid equilibrium formation of the dienolate. After a 0.5 s incubation period, the dienolate solution was quenched (1:5 ratio) in a mildly acidic buffer to yield the dienol via protonation at oxygen (final conditions: pH 5.21 and 5.75, [**2**] = ca. 21.5 μM, 333 mM acetate, 5.0% methanol; pH 6.90, [**2**] = ca. 10.5 μM, 333 mM phosphate, 5.0% methanol). Enzyme concentrations used were as follows: 15.8 and 22.7 μM at pH 5.21 and 5.75; 4.8 and 8.3 μM at pH 6.90. In the absence and presence of D38N in the quench solution, the nonenzymatic ketonization of **2**, to predominantly **1**, was monitored at 242 nm, the isosbestic wavelength of free **2** plus D38N and D38N·**2**. The amplitudes of the absorbance traces were used to calculate values of K_D as described in the Results section.

RESULTS

Preparation and Properties of D38N. The D38N mutant gene was prepared by site-directed mutagenesis and characterized by sequencing the entire gene. On SDS–PAGE gels the purified D38N enzyme shows only one band, even when the gel is overloaded, with the same mobility as wild-type (WT). The D38N mutant enzyme shows only trace activity with 5-androstene-3,17-dione (**1**) at modest enzyme concentrations ([D38N] < 0.1 μM). At higher concentrations, "burst" kinetics are observed. These characteristics, as well as the UV spectrum of the mutant protein, are consistent with those previously observed by Kuliopulos et al. (1989). As previously noted by Xue et al. (1991) with their preparations, trace amounts of WT were present in the purified D38N. This WT activity was eliminated by treating solutions of D38N with (17*S*)-spiro[androstane-17,2′-oxiran]-3α-ol, an active-site-directed alkylating agent that reacts specifically with Asp-38 of KSI (Kayser et al., 1983), until the residual activity due to D38N was constant.

Determination of the Concentration of D38N. Concentrations of D38N were estimated initially by UV spectroscopy,

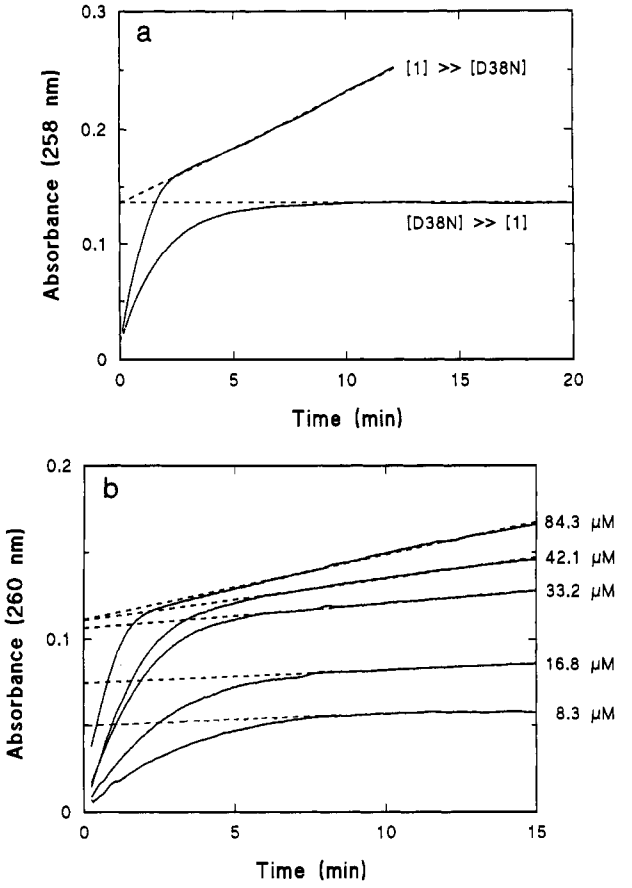


FIGURE 1: UV absorbance change as a function of time for the reaction of **1** with D38N (34 mM phosphate, pH 6.95, 5.0% MeOH): (a) [D38N] = 86 μM ≫ [**1**] = 8.3 μM; and [**1**] = 92 μM ≫ [D38N] = 8.6 μM, λ = 258 nm; (b) [D38N] = 9.0 μM; [**1**] = 8.4, 16.8, 33.2, 42.1, and 84.3 μM, λ = 260 nm. The dashed lines are extrapolations of the absorbances at long time.

using a value of 0.336 for the absorbance at 280 nm of a 1.00 mg/mL solution (Kuliopulos et al., 1989). A more precise method relies upon the observation of an initial burst of absorbance in the reaction of D38N and **1**. Mildvan and co-workers (Xue et al., 1991) have established that this initial burst is due to rapid formation of the complex between the intermediate dienol(ate) (**2**) and D38N. This complex (D38N·**2**) reacts to form product (**3**) at a much slower rate.

Figure 1a shows plots of the absorbance at 258 nm against time for the reaction of **1** with D38N at pH 6.95 under conditions of [D38N] ≫ [**1**] and [**1**] ≫ [D38N], with the limiting reagent present in a concentration of ca. 8.5 μM. Due to tight binding of **2** under these conditions (Xue et al., 1991), the magnitude of the burst is directly proportional to the concentration of the limiting reagent. In the case of [D38N] ≫ [**1**], the variation of absorbance with time shows a first-order approach to a plateau that persists for longer than 30 min. The increase in absorbance corresponding to formation of D38N·**2** is rapid (half-life ca. 70 s) and stoichiometric under these conditions. Since conversion of the intermediate D38N·**2** complex to products is slow, the magnitude of the amplitude of the absorbance change, coupled with the initial concentration of **1**, can be used to calculate the extinction coefficient of the D38N·**2** complex at 258 nm (ε 14 300 M^{-1} cm^{-1}). The concentration of D38N can then be obtained from the magnitude of the absorbance burst under conditions of [**1**] ≫ [D38N].

The UV Spectra of Dienol **2** *and the D38N·***2** *Complex.* These spectra were obtained as described in the Materials and Methods section and are shown in Figure 2. The spectrum of **2** exhibits a λ_{max} of 234–236 nm (ε 17 300 M^{-1} cm^{-1}), in agreement with the value of 236 nm (ε 17 500 M^{-1} cm^{-1}) obtained previously by a different method (Hawkinson et al., 1991a). The spectrum of the D38N·**2** complex shows a λ_{max} of 252 nm (ε 17 300 M^{-1} cm^{-1}), similar to that obtained from mixing **1** with D38N (λ_{max} ca. 258 nm, $\epsilon \approx 13\,000$ M^{-1} cm^{-1}; Xue et al., 1991). The isosbestic point for the conversion of **2** + D38N to D38N·**2** is 242 nm.

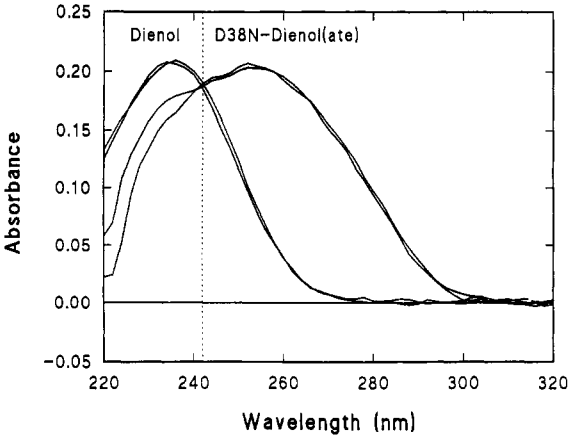


FIGURE 2: Ultraviolet spectra of dienol **2** and D38N-dienol(ate) D38N·**2**, corrected for protein absorbance (333 mM phosphate, 5.2% MeOH, pH 7.2), obtained as described in the Materials and Methods section. The concentrations of **2** and D38N·**2** were 12.1 μM. Duplicate determinations are shown for both spectra.

Binding Constant of Equilenin to Wild-Type, D38E, and D38N. The fluorescence emission spectrum of equilenin (**4**) was determined in the presence of varying concentrations of enzyme for WT, D38E, and D38N at pH values from 3.9 to 7.2. Equilenin concentrations were 0.5–3 μM (WT), 1 μM (D38E), 0.09 μM (D38N, low pH), and 0.9 μM (D38N, high pH). Representative plots for WT and D38N are shown in Figure 3; the results with D38E are similar to those with WT. In the absence of enzyme, equilenin shows a peak in the emission spectrum at 363 ± 1 nm (excitation at either 298 or 291 nm). Addition of enzyme (WT, D38E, or D38N) causes a marked decrease in the intensity of this peak.

Fluorescence intensities at 363 nm were analyzed as a function of the concentration of added enzyme to give values of the observed dissociation constant (K_D). In any given solution, the ratio of the concentration of unbound equilenin ($[4]_f$) to bound equilenin ($[4]_b$) is given by $(F - F_\infty)/(F_0 - F)$, where F is the fluorescence intensity, F_0 is the intensity in the absence of enzyme, and F_∞ is the intensity extrapolated to infinite enzyme concentration. The relationship between K_D and the observed fluorescence is given by

$$K_D = [4]_f[E]_f/[4]_b \quad (1a)$$

$$= [4]_f([E]_t - [4]_b)/[4]_b \quad (1b)$$

$$= c(F - F_\infty)([E]_t - (F_0 - F)c)/(F_0 - F)c \quad (1c)$$

where $[E]_t$ is the concentration of total enzyme in the solution, $[E]_f$ is the concentration of unbound enzyme, and $c = [4]/(F_0 - F_\infty)$. Rearranging eq 1 gives

$$[E]_t = (F_0 - F)(K_D/(F - F_\infty) + c) \quad (2)$$

The experimental data were fit to eq 2 using a nonlinear least-squares computer program based on the Marquardt algorithm. The parameters F_0, F_∞, K_D, and c were allowed

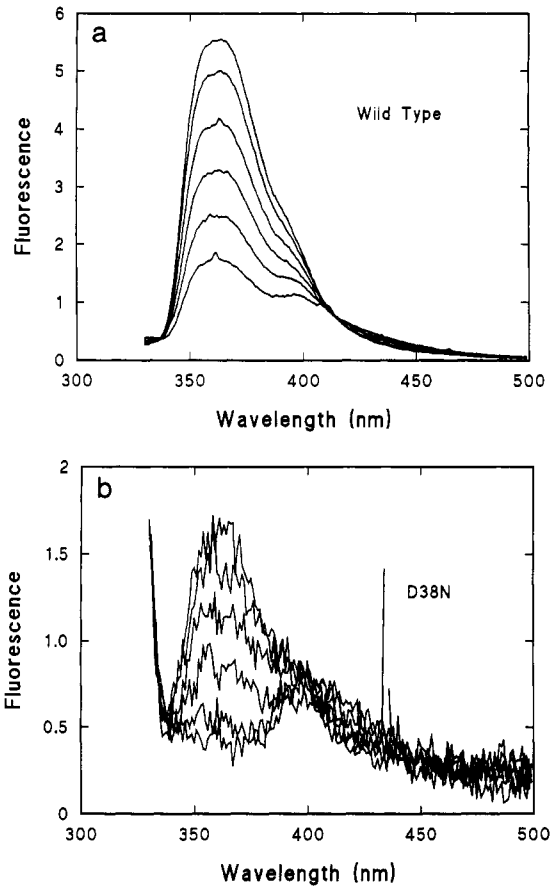


FIGURE 3: Fluorescence emission spectra of equilenin as a function of concentration of added enzyme at pH 7.1 (10 mM phosphate, 5.2% methanol) for (a) wild-type ([equilenin] = 3.36 μM) and (b) D38N ([equilenin] = 88.5 nM). Enzyme concentrations were (in order of decreasing fluorescence) 0, 0.62, 1.85, 3.06, 4.84, and 7.47 μM (wild-type) and 0, 18.8, 37.5, 56.1, 74.7, and 93.2 nM (D38N).

to vary in order to give the best fit. Calculated values of F_0 and c were generally quite close to observed values, and calculated F_∞'s were reasonable.[3] In a few cases, c had to be fixed in order for the program to converge on reasonable values of the other parameters. Representative plots of the data are given in Figure 4, and calculated values of K_D are given in Table 1.

The values for K_D for both WT·**4** (ca. 3 μM) and D38E·**4** (ca. 1 μM) are well-defined and vary little throughout the pH range 4–7. However, K_D for D38N·**4** is much more difficult to determine, especially at the higher pH values. Since K_D for D38N·**4** is substantially less than that for either WT·**4** or D38E·**4** (ca. 1–100 nM *vs* 1–3 μM), low concentrations of **4** ($\leq$0.09 μM) were necessary for some experiments with D38N, causing decreased signal/noise in the measurements (Figure 3). Furthermore, at the higher pH's the curvature in the plots for D38N is quite sharp, increasing the errors in the values of the derived parameters (Figure 4c).

Binding Constant of the Intermediate Dienol to D38N. Method A. D38N was first prepared by Kuliopulos et al. (1989), who found that the catalytic activity (k_{cat}) is almost 10^6-fold less than WT at pH 7 toward 5-androstene-3,17-dione (**1**), although the K_m's are comparable for the two enzymes. Later, Xue et al. (1991) examined the reaction of D38N with **1** in more detail. They found that D38N catalyzes the conversion of **1** to **2**, which is bound tightly at the active

[3] F_∞ could not be obtained experimentally because of fluorescence due to added protein.

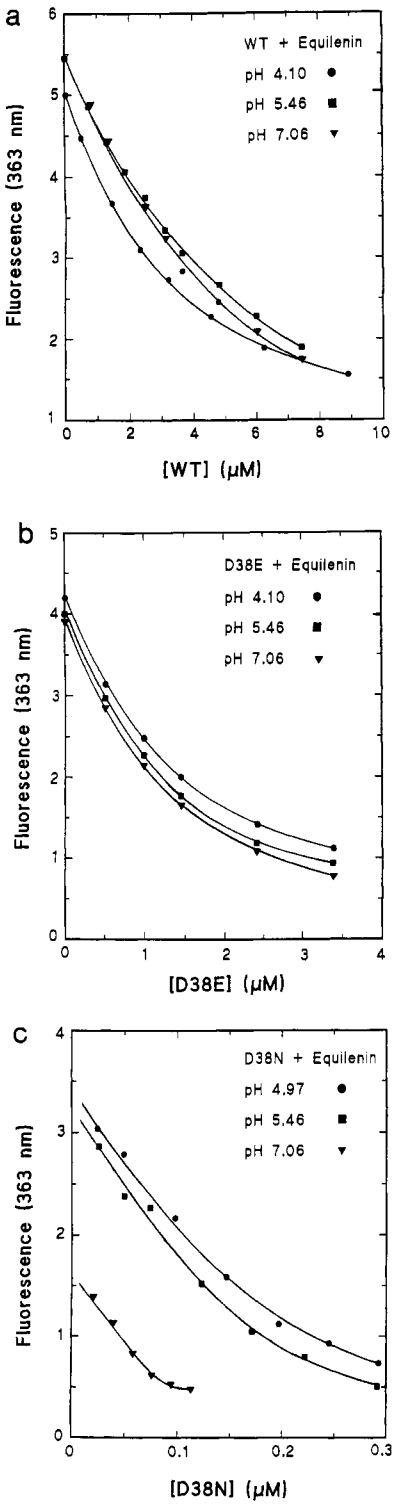


FIGURE 4: Variation of fluorescence emission of equilenin at 363 nm with concentration of added enzyme for (a) wild-type, (b) D38E, and (c) D38N. Concentrations of equilenin were (a) 0.521 μM (pH 4.10), 3.36 μM (pH 5.46 and pH 7.06); (b) 0.912 μM (pH 4.10, 5.46, and 7.06); and (c) 0.178 μM (pH 4.97 and 5.46) and 0.89 μM (pH 7.06). The excitation wavelength was either 291 or 298 nm. The lines are theoretical based upon the best fit to eq 2, as described in the text.

site, followed by slow conversion of **2** to product (**3**) (Scheme 2). Importantly, the complex of **2** and D38N can be observed spectrally (λ_{max} ca. 258 nm). Thus, mixing of excess **1** with D38N gives a burst of absorbance due to formation of the D38N·**2** complex, followed by a much slower increase, corresponding to formation of **3**.

Table 1: Dissociation Constants of Wild-Type, D38E, and D38N with Equilenin as a Function of pH[a,b]

	K_D, μM		
pH[c]	wild-type	D38E	D38N
3.87	3.3 ± 0.1		0.075 ± 0.006
			0.086 ± 0.018
4.10	3.1 ± 0.3	0.76 ± 0.13	0.101 ± 0.016
	3.8 ± 0.1	0.48 ± 0.01	0.097 ± 0.019
4.50	3.7 ± 0.5	1.3 ± 0.1	0.045 ± 0.008
			0.044 ± 0.016
5.01	3.4 ± 0.2	0.88 ± 0.14	0.014 ± 0.008
			0.008 ± 0.002
5.46	3.1 ± 0.2	0.50 ± 0.12	0.023 ± 0.013
	3.1 ± 0.3	0.91 ± 0.18	0.012 ± 0.005
7.07	2.9 ± 0.2	0.81 ± 0.08	0.001 ± 0.0005
	2.3 ± 0.1	0.74 ± 0.17	0.00012 ± 0.00005
	2.0 ± 0.3		
7.22	2.6 ± 0.4	1.24 ± 0.13	
7.15[d]	1.4 ± 0.4		
	3.3 ± 1.0		

[a] Solutions were 5.2% methanol and 10 mM buffer (acetate for pH < 6; phosphate for pH > 6). [b] Errors are internal standard deviations within a run. [c] Variations in reported pH values are ≤0.04 pH unit. [d] 3.3% methanol.

Scheme 2

$$1 + \text{D38N} \underset{}{\overset{K_S}{\rightleftharpoons}} \text{D38N}\cdot\mathbf{1} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \text{D38N}\cdot\mathbf{2} \xrightarrow{k_5} \text{D38N} + \mathbf{3}$$

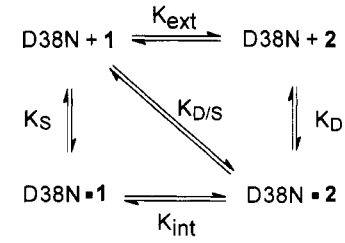
We determined both the dissociation constant K_S for D38N·**1**, which is formed rapidly, and the apparent dissociation constant $(K_{D/S})^{obs}$ for the mixture of D38N·**1** and D38N·**2**, which is formed more slowly. These two values, along with the equilibrium constant for the interconversion of **1** and **2**, allow the calculation of the dissociation constant for D38N·**2**.

D38N was added to solutions of **1** ([**1**] = 8.4, 16.8, 33.2, 42.1, and 84.3 μM, final [D38N] = 9.0 μM, pH 6.95), and the change in the UV spectrum was monitored at 260 nm, the isosbestic point for the conversion of D38N·**2** to **3**. In each case, a rapid rise in absorbance, followed by a much slower increase, was observed (Figure 1b). Initial rates at each concentration of [**1**] were determined and analyzed by iterative fitting to the Michaelis–Menten equation, giving values for K_S = 87 ± 22 μM and k_3 = 0.023 ± 0.004 s^{-1}, in reasonable agreement with the values of K_S = 80 ± 10 μM and k_3 = 0.052 ± 0.05 s^{-1} reported by Xue et al. (1991).[4] At lower pH values, the following results were obtained: pH 5.75, K_S = 39 ± 7 μM, k_3 = 0.0065 ± 0.007 s^{-1}; pH 5.21, K_S = 22 ± 3 μM, k_3 = 0.0037 ± 0.002 s^{-1}.

Extrapolation of the linear portion of the curve back to time = 0 gives the magnitude of the burst, which is proportional to the amount of enzyme-bound steroid (D38N·**1** + D38N·**2**). Solutions with [**1**] = 42.1 μM and [**1**] = 84.3 μM give extrapolations to the same absorbance value, indicating that reaction to form D38N·**2** + D38N·**1** is stoichiometric at these concentrations of **1**. Extrapolations for the lower substrate concentrations result in lower absorbance bursts, showing that complete saturation of the enzyme does not occur at these concentrations.

The apparent equilibrium established between free enzyme and substrate and the two bound species (D38N·**1** and D38N·**2**) may be described by an apparent dissociation constant $(K_{D/S})^{obs}$ given by eqs 3–6. Thus, values of $(K_{D/S})^{obs}$ were determined at the two lowest concentrations of **1** from the variation of the burst with [**1**]: $(K_{D/S})^{obs}$ = 5.0 μM at [**1**] = 8.3 μM and 4.9 μM at [**1**] = 16.8 μM. The corresponding dissociation constants $K_{D/S}$ and K_D were calculated using the relationships of eqs 7–11 and Scheme 3, along with known values of K_S and K_{ext} (2×10^{-3}; Zeng & Pollack, 1991).

Scheme 3



$$(K_{D/S})^{obs} = [\mathbf{1}][\text{D38N}]/([\text{D38N}\cdot\mathbf{2}] + [\text{D38N}\cdot\mathbf{1}]) \quad (3)$$

$$[\text{D38N}] = [\text{D38N}]_{total}(A_\infty - A)/A_\infty \quad (4)$$

where A is the magnitude of the absorbance burst at a given concentration of added **1**, and A_∞ is the magnitude of the burst at saturating [**1**].

$$[\text{D38N}\cdot\mathbf{1}] + [\text{D38N}\cdot\mathbf{2}] = [\text{D38N}]_t(A/A_\infty) \quad (5)$$

$$[\mathbf{1}] = [\mathbf{1}]_{total} - [\text{D38N}\cdot\mathbf{1}] + [\text{D38N}\cdot\mathbf{2}] \quad (6)$$

$$K_{D/S} = [\mathbf{1}][\text{D38N}]/[\text{D38N}\cdot\mathbf{2}] \quad (7)$$

$$K_S = [\mathbf{1}][\text{D38N}]/[\text{D38N}\cdot\mathbf{1}] \quad (8)$$

$$1/(K_{D/S})^{obs} = 1/K_{D/S} + 1/K_S \quad (9)$$

$$K_{ext} = [\mathbf{2}]/[\mathbf{1}] \quad (10)$$

$$K_D = K_{D/S}K_{ext} \quad (11)$$

Values of $K_{D/S}$ obtained in this manner are 4.4 ± 0.8 μM and 4.6 ± 0.8 μM, for total concentrations of **1** of 16.8 and 8.4 μM, respectively. The data at 33.2 μM substrate were not used in these calculations since the most reliable values are obtained at concentrations of D38N, **1**, and D38N·**2** that are comparable, where errors in the absorbances, concentration values, or background rates have minimal effects. A similar set of experiments at lower enzyme concentration (0.99 μM) with substrate concentrations of 1.09 and 4.36 μM gave values for $K_{D/S}$ of 0.6 ± 0.2 μM and 2.0 ± 0.5 μM, respectively. Averaging these four values gives 3 ± 2 μM for K_D at pH 6.90. Conversion of $K_{D/S}$ to the dissociation constant K_D for D38N·**2** according to eq 11 gives K_D = 6 ± 3 nM. Values of $(K_{D/S})^{obs}$, $K_{D/S}$, and K_D at pH 5.21 and 5.75 are given in Table 2.

Method B. The value of K_D can also be determined by an examination of the ketonization of the dienol in the presence of D38N. Dienol **2** was generated in a sequential mixing stopped-flow apparatus, as previously described (Eames et al., 1990: Hawkinson et al., 1991a,b), by quenching a solution of the dienolate anion ($\mathbf{2}^-$) in a mildly acidic buffer (final conditions: 333 mM buffer, 5.0% methanol; acetate, pH 5.75 and 5.21; phosphate, pH 6.90). The absorbance was monitored at 242 nm, the isosbestic point for **2** + D38N ⇌ D38N·**2**.

In the absence of enzyme (Figure 5A) **2** ketonizes to give predominantly **1**, with minor amounts (ca. 3%) of **3** (Zeng & Pollack, 1991). The amplitude of the trace is proportional to the concentration of **2** in the optical cell immediately after the

[4] The larger value of k_2 obtained by Xue et al. (1991) might be due to the slight WT impurity in their preparations.

Table 2: Rate and Equilibrium Constants for the Reaction of D38N with **1** as a Function of pH[a]

pH	K_S, μM	k_3, s^{-1}	$(K_{D/S})^{obs}$, μM	$K_{D/S}$, μM	K_D, nM
5.21	22 ± 3	(3.7 ± 0.2) × 10^{-3}	13 ± 1	29 ± 7	59 ± 15[b] 210 ± 50[c]
5.75	39 ± 7	(6.5 ± 0.7) × 10^{-3}	15 ± 3	26 ± 7	51 ± 15[b] 80 ± 20[c]
6.95	87 ± 22	(2.3 ± 0.4) × 10^{-2}	3 ± 2	3 ± 2	6 ± 3[b] 9 ± 1[c]

[a] All solutions contained 5.0% methanol. pH values were ±0.03. [b] Method A (see text). [c] Method B (see text).

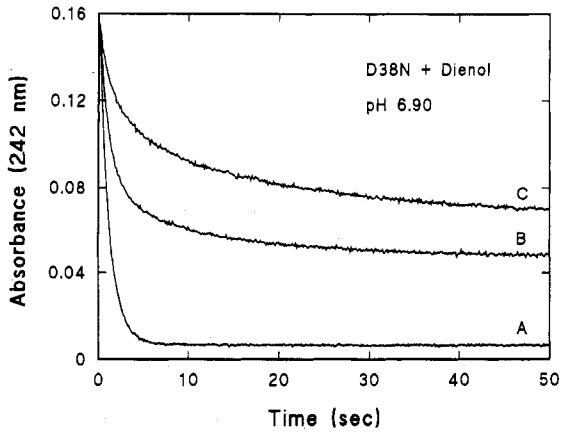


FIGURE 5: UV absorbance change at 242 nm for the ketonization of **2** to **1** (333 mM phosphate, pH 6.90, 5.0% MeOH). [**2**] = 10.5 μM; [D38N] = 0 (A), 4.8 μM (B), and 8.3 μM (C).

quench, which allows calculation of the initial concentrations of **1** and **2** (Pollack et al., 1989).

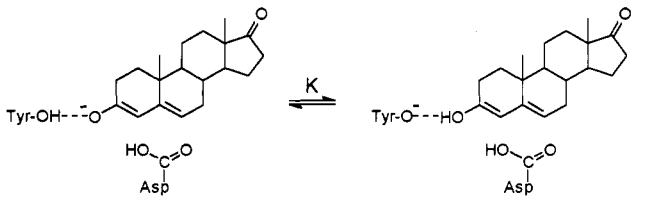
Inclusion of D38N in the quench solution results in a decrease in the amplitude of the absorbance drop, which depends upon the concentration of D38N. The simplest interpretation of this data is that D38N rapidly binds some fraction of the **2** in solution as D38N·**2**. Residual *unbound* **2** is converted nonenzymatically to **1** and a minor amount of **3**. The difference in the amplitudes of the absorbance drops in the absence and presence of D38N can be used to calculate how much **2** has been sequestered by the enzyme at the end of the reaction. Along with the appropriate value of K_S from method A, the final concentrations of **1** and D38N can also be calculated. Values of $K_{D/S}$ and K_D are then calculated by eqs 7 and 11, respectively.

The value for K_D of 9 ± 1 nM at pH 6.90 is in good agreement with the value of 6 ± 3 obtained by method A at pH 6.95. Values of K_D at lower pH's (80 ± 20 nM at pH 5.75 and 210 nM ± 50 nM at pH 5.21) are also similar to the values obtained with method A. Because of the sensitivity of the parameters derived from method B to the exact values of the concentrations used in the calculations, we have somewhat more confidence in the values from method A.

DISCUSSION

Mechanism of Action of Steroid Isomerase. 3-Oxo-Δ^5-steroid isomerase (KSI, EC 5.3.3.1) is a member of a class of enzymes that function by abstraction of a proton from a carbon adjacent to a carbonyl group [for reviews, see Pollack et al. (1989b) and Schwab and Henderson (1990)]. KSI catalyzes the isomerization of a variety of 3-oxo-Δ^5-steroids to their conjugated Δ^4-isomers through an intermediate enol(ate), with Asp-38 acting as the base and Tyr-14 stabilizing

Scheme 4



the developing charge on the carbonyl oxygen of the substrate. Although there is general agreement about the function of Asp-38, the exact nature of the stabilization of the intermediate and the adjacent transition states by Tyr-14 has been subject to some debate. On the basis of the additivity of the effects of mutations at Asp-38 and Tyr-14, Kuliopulos et al. (1989) concluded that these two residues function in the same step during the reaction. This "concerted" action of Tyr-14 and Asp-38 could be due either to a proton in-flight from the OH group of Tyr-14 to O-3 of the steroid during abstraction of the proton at C-4 by Asp-38 or, alternatively, to hydrogen bonding stabilization of the incipient anion.

Spectroscopic studies with the intermediate analogs 3-amino-1,3,5(10)-estratrien-17β-ol and equilenin (Zeng et al., 1992) suggest that the bound intermediate (**2**) is ionized as the dienolate ion at the active site, rather than being present as the dienol (Scheme 1a). These results are consistent with stabilization of the intermediate and transition state(s) by hydrogen bonding, rather than by proton transfer. Gerlt and Gassman (1993) also argue for hydrogen bonding as the mechanism of stabilization in their description of an "enolic" intermediate. They describe the intermediate as "neither an enolate ion nor a neutral enol, but a species in which the proton from the general acidic catalyst is *partially* transferred to the carbonyl oxygen of the substrate keto tautomer of the carbon acid...". Xue et al. (1991) showed that the intermediate dienol(ate) of **1** bound to D38N exhibits UV spectral characteristics of both a dienol and a dienolate, suggesting that the intermediate may be an equilibrium mixture of the dienol and the dienolate ion (Scheme 4).

However, as emphasized by Guthrie and Kluger (1993), the exact position of the proton is unimportant from an energetic viewpoint. Since the pK_a's of the dienol and Tyr-14 are likely to be similar at the active site,[5] the equilibrium constant for the reaction in Scheme 4 is near unity and the energetics of the conversion of **1** → **2** on the enzyme surface depend only slightly on whether the intermediate is the dienol or the dienolate ion. What is important for the enzyme is that the *system* be stabilized, presumably through hydrogen bonding. Thus, the energetics of the reaction can be analyzed independently of any assumption about the position of the proton in the hydrogen bond.

Binding Constant of the Dienol to D38N. Mildvan and co-workers (Kuliopulos et al., 1989; Xue et al., 1991) have previously examined the active-site mutant isomerase in which the catalytic base (Asp-38) has been changed to Asn-38 (D38N). While this mutation decreases k_{cat} toward 5-androstene-3,17-dione by a factor of $10^{5.6}$-fold relative to the wild-type (WT) enzyme, it produces little change in K_m (3-fold decrease) and no change in the dissociation constant for the competitive inhibitor 19-nortestosterone (Kuliopulos et al., 1989). D38N catalyzes enolization of the bound substrate at a substantial rate, but turnover of the enzyme–intermediate

[5] The pK_a's of these groups have been measured in solution and found to be 10.0 for the dienol (Zeng & Pollack, 1991) and 11.6 for Tyr-14 (Li et al., 1993).

complex to product is quite slow, leading to the observation of a pronounced initial burst phase in the reaction (Xue et al., 1991). Thus, D38N acts to sequester the dienolic intermediate.

Xue et al. (1991) reported that D38N reacts with **1** to form the enzyme–dienol intermediate (D38N·**2**) stoichiometrically. From a calculated upper limit for the rate constant for dissociation of D38N·**2**, they estimated the dissociation constant for D38N·**2** to give D38N plus **2** at pH 7 to be $\leq 10^{-11}$ M^{-1}, substantially less than the value we determine here (ca. 6×10^{-9} M^{-1}). This discrepancy appears to be due to the method by which Xue et al. calculated the upper limit for the rate of dissociation of the D38N·**2** complex. They measured the first order rate constant for conversion of D38N·**2** to D38N + **3** at a concentration of D38N·**2** of 23 μM, with a 7 μM excess of free enzyme ($k = 9.4 \times 10^{-5}$ s^{-1}). Since only a small fraction of the **2** released forms **3** (ca. 2–4%; Pollack et al., 1987; Zeng & Pollack, 1991), Xue et al. calculated that the upper limit for the rate of dissociation of D38N·**2** is 2.3×10^{-3} s^{-1}. With the assumption of a diffusion controlled rate of association of D38N with **2** to form D38N·**2**, the dissociation constant of D38N·**2** was calculated to be $\leq 10^{-11}$.

However, these authors did not consider the possibility of *reassociation* of **2** with free enzyme. Free **2** formed by dissociation of D38N·**2** will regenerate D38N·**2** with a rate constant equal to the association rate constant ($\geq 10^8$ M^{-1} s^{-1}) multiplied by the concentration of free enzyme (ca. 7×10^{-6} M^{-1}) or ≥ 700 s^{-1}. Under the conditions used (ca. 50 mM buffer, pH 7), the rate constant for the buffer catalyzed reaction of free **2** to give **1** is ≤ 2 s^{-1} (Hawkinson et al., 1991a). The corresponding rate constant to give **3** is about 30-fold less (≤ 0.1 s^{-1}; Zeng & Pollack, 1991). Thus, the buffer catalyzed rate constant for production of **3** from free **2** is ca. 10^4-fold less than that for reassociation of **2** with the enzyme. Consequently, only about 1 molecule in 10^4 of those that dissociate from D38N·**2** forms **3** by this pathway. Since the observed rate constant for conversion of D38N·**2** to **3** is about 10^{-4} s^{-1}, the maximum value for the dissociation rate constant for D38N·**2** is on the order of 1 s^{-1}, ca. 400-fold larger than the value of 2.3×10^{-3} s^{-1} calculated by Xue et al. (1991). This value, combined with the rate constant for association, gives an upper limit for K_D of about 10 nM, in agreement with the K_D determined in the present work (ca. 6 nM).

Internal Equilibrium Constant and Free Energy Profile for D38N. The value of the dissociation constant K_D can be used to calculate the internal equilibrium constant for D38N from the relationship $K_{int} = (K_S/K_D)K_{ext}$ (Scheme 3) and to construct a free energy profile for the isomerization of **1** catalyzed by D38N (Figure 6). Values for K_S, k_3, and k_5 are available from the work of Xue et al. (1991), and we have redetermined K_S and k_3 here. The remaining rate constant (k_4) can be calculated from the internal equilibrium constant ($K_{int} = k_3/k_4$). At pH 6.9, $K_{int} = 30 \pm 15$, whereas at lower pH values K_{int} is somewhat less (2.8 ± 0.6 at pH 5.8 and 1.9 ± 1.1 at pH 5.2). The favorable internal equilibrium constant at pH 6.9, coupled with the unfavorable partitioning ($k_4/k_5 = 8$), explains the slow breakdown of the intermediate to products relative to its formation. The intermediate gets caught in an energetic "pit" that retards its reaction to product, as well as its reversion to substrate.

Binding of Equilenin to Wild-Type, D38N, and D38E. Chromophoric steroids, such as equilenin (**4**), were employed as tools to investigate the nature of the intermediate in the KSI reaction as early as 1963 (Wang et al., 1963). The characteristic absorption and fluorescence spectra of equilenin and other aromatic A-ring steroids have been interpreted in

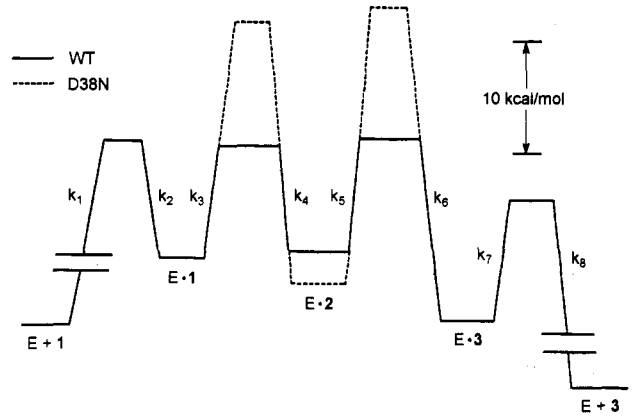


FIGURE 6: Free energy profile for the isomerization of 5-androstene-3,17-dione (**1**) to 4-androstene-3,17-dione (**3**) catalyzed by wild-type (WT) and D38N. Data for WT are taken from Hawkinson et al. (1991a) with the equilibrium constant (K_{int}) determined here. For D38N, rates of formation of the D38N·**1** complex from D38N and **1** and the binding of the product (**4**) are assumed to be the same as for WT.

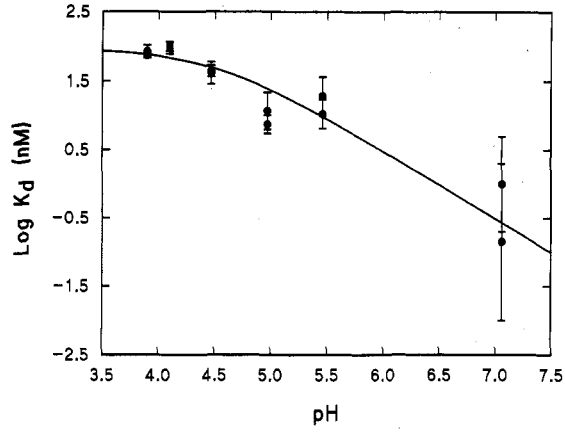


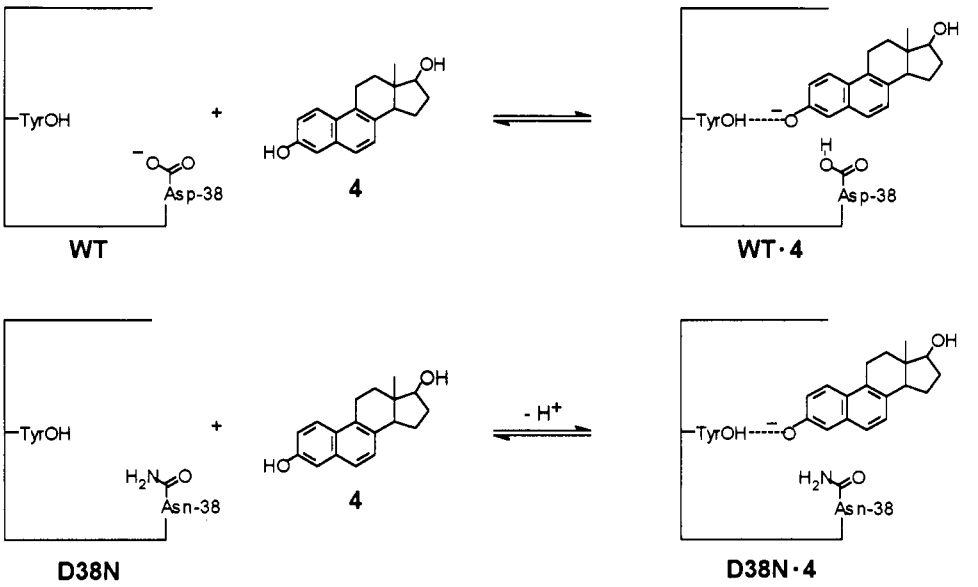
FIGURE 7: Plot of log K_D *vs* pH for the D38N·**4** complex. The line is theoretical, based upon the following relationship with a pK_a of 4.5 for the complex, and a limiting value of 90 nM for K_D at low pH (K_D^{lim}):

$$K_D = ([H^+] + K_D^{lim})/(K_a + pH)$$

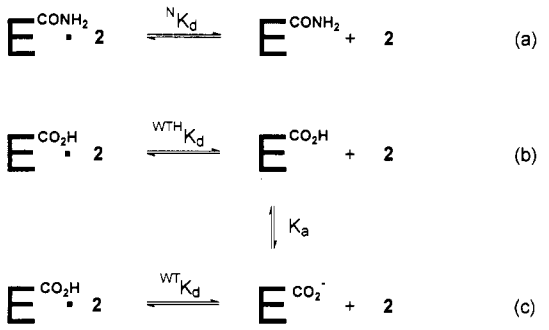
terms of ionization of the phenolic group at the active site (Bevins et al., 1986; Kuliopulos et al., 1989; Eames et al., 1989; Zeng et al., 1992). The structural similarity of equilenin and the intermediate dienol (**2**) suggests that this dienol may also be ionized at the active site of KSI. As expected for an analog of **2**, equilenin binds well to both WT and to D38E (K_D ca. 1 μM), about 100-fold better than the substrate binds ($K_S \approx 100$ μM; Hawkinson et al., 1991b).

Although the dissociation constants for both WT·**4** and D38E·**4** are pH-independent, the dissociation constant for D38N·**4** decreases with increasing pH (Table 1). At pH 7, the binding of equilenin to D38N is 10^3-fold tighter than it is to either WT or D38E . At lower pH's, equilenin binding to D38N is weaker, but still substantially stronger than to either WT or D38E (by ca. 10-fold at pH 4). The pH dependence of the binding of equilenin to D38N suggests that a proton is released by either the enzyme or substrate upon binding. Consistent with this hypothesis, the variation of the observed dissociation constant with pH can be accounted for by ionization of a group on the enzyme with a pK_a of approximately 4.5 (Figure 7). For both the WT and D38E enzymes, the independence of the observed dissociation

Scheme 5



Scheme 6

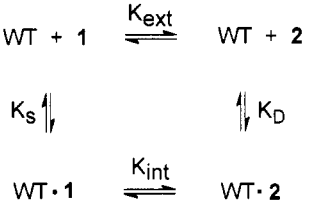


constants with pH argues for no change in net protonic state upon binding.

These results may be accounted for in terms of the model of Scheme 5. Although binding of **4** to WT leads to fluorescence spectral changes in **4** that are indicative of ionization of the phenolic hydroxyl group, the apparent dissociation constant is pH-independent, consistent with the proton being transferred to an enzymatic residue, rather than being lost to the solution. The pH dependence of the apparent dissociation constant for the equilenin·D38N complex, however, suggests that a proton is lost to solvent. The simplest explanation for this difference is that Asp-38 is the base in the WT, and the mutation of Asp → Asn in D38N lowers the basicity sufficiently to make abstraction of the proton by this residue unlikely.

Binding Constant of the Dienol (2) to Wild-Type KSI. Although it is impossible to directly determine the dissociation constant for WT·**2**, its value can be estimated from that for D38N·**2** (Scheme 6). Since the $CONH_2$ group of Asn-38 is isosteric and isoelectronic to the COOH group of Asp-38, it is reasonable to assume that the dissociation constant for the complex of **2** and D38N ($^{N}K_D$, Scheme 6a) is similar to that for the complex of **2** and protonated WT ($^{WTH}K_D$, Scheme 6b). With this assumption, the dissociation constant for WT·**2** at pH 7 ($^{WT}K_D$, Scheme 6c) can be calculated from the relationship $^{WT}K_D = {}^{WTH}K_D(K_a + [H^+]/[H^+])$, where $K_a = 1.8 \times 10^{-5}$ (Pollack et al., 1986). Calculations using the results in Table 2 give an average value for the dissociation constant for WT·**2** of 0.7 ± 0.4 μM.

Scheme 7



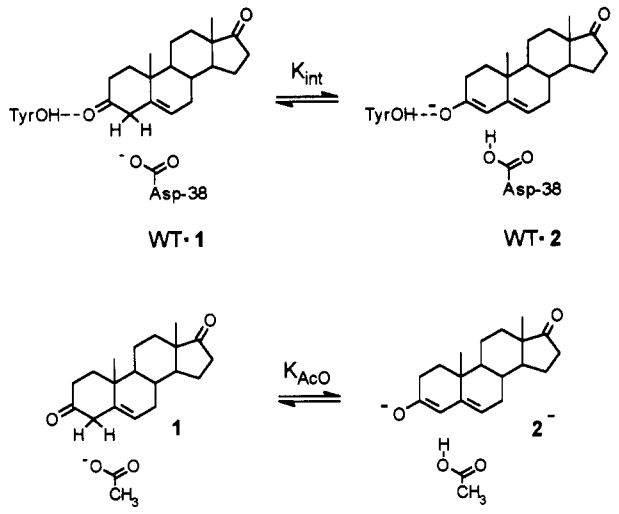
These results suggest that **2** binds more tightly to D38N than to WT at pH 7 because formation of D38N·**2** is accompanied by a proton transfer from **2** to hydroxide ion, whereas formation of WT·**2** requires transfer of a proton from **2** to a carboxylate (Asp-38). Thus, part of the intrinsic binding energy in WT is used to enable the formation of protonated Asp-38 in neutral solution.

Internal Equilibrium Constant and Free Energy Profile for Wild-Type KSI. We recently reported a determination of the free energy profile for the KSI-catalyzed isomerization of 5-androstene-3,17-dione (Hawkinson et al., 1991b). The major point of uncertainty in this free energy profile was the relative stability of the enzyme–dienolate complex WT·**2** and the enzyme–substrate complex WT·**1**. In that work, it was only possible to calculate a maximum value of the internal equilibrium constant ($K_{int} \leq 0.6$) for interconversion of the enzyme–substrate and enzyme–intermediate complexes (Scheme 7). The calculation of the dissociation constant for WT (ca. 0.7 μM) from the current investigation, coupled with values for K_S (100 μM) and K_{ext} (2×10^{-3}), allows a determination of the internal equilibrium constant ($K_{int} = 0.3 \pm 0.2$) and completes the free energy profile (Figure 6).

An additional estimate for K_{int} can be obtained from our determination of the internal equilibrium constant for D38E·**1** ⇌ D38E·**2** ($K_{int} = 1.9$; Zawrotny and Pollack, unpublished). Although this value is somewhat higher than that for WT, D38E also binds equilenin more strongly than does WT (by about 4-fold). If the ratio of the dissociation constants for WT·**2** and D38E·**2** is the same as for WT·**4** and D38E·**4**, then the K_{int} for WT would be 1.0, in satisfactory agreement with our estimated value from this work.

Before discussing the implications of K_{int}, it is important to consider the reliability of this estimate. We previously calculated a maximum value for K_{int} of 0.6 (Hawkinson et al.,

Scheme 8



1991b), and a minimum value can be obtained from the data of Brooks and Benisek (1994). They concluded that the maximum value for the rate constant for regeneration of WT·**1** from WT·**2** (k_4) is 9×10^5 s^{-1}. Coupled with the known value for the rate constant for WT·**1** to WT·**2** ($k_3 = 1.7 \times 10^5$ s^{-1}; Hawkinson et al., 1991b), this result gives a minimum value for K_{int} (k_3/k_4) of 0.2. The K_{int} obtained here agrees well with these estimated limits.

Although the above comparisons give credence to our estimate of K_{int} for WT, some caution is suggested by the results from the application of this method to the calculation of the dissociation constant for WT·**4**. When the same rationale that was used to determine the dissociation constant for WT·**2** from that for D38N·**2** is applied to D38N·**4**, a dissociation constant for WT·**4** of approximately 100 nM is obtained, in contrast to the experimentally determined value of ca. 3 μM. An error of this magnitude in the estimation of K_{int} for WT would give a corrected value for K_{int} of about 0.01. At present, we do not have an explanation for this difference of about 30-fold between the calculated and observed values for WT·**4**. Nevertheless, the agreement of K_{int} with the limits calculated by Hawkinson et al. (1991b) and by Brooks and Benisek (1994), and with K_{int} for D38E, gives us confidence that an internal equilibrium constant of about 0.3 for WT·**2** is correct. Even if the true value of the internal equilibrium constant is somewhat lower, the following analysis is qualitatively unaffected.

Relevance to the Gerlt/Gassman and Albery/Knowles Analyses. Gerlt and Gassman (1993) propose that K_{int} for enzymes that catalyze enolizations, such as KSI, should be substantially less than unity, whereas considerations based on the work of Albery and Knowles (1976b) and Stackhouse et al. (1985) predict a value close to or greater than unity. It is clear from the value of K_{int} (0.3) for KSI that the energies of the bound intermediate and bound substrate in this case are similar, inconsistent with the expectation from Gerlt/Gassman.

It is instructive to consider the corresponding value for the equilibrium constant for the model reaction acetate + **1** $\rightleftarrows$ **2**$^-$ + acetic acid (Scheme 8). The equilibrium constant for abstraction of a proton from **1** by acetate ion ($K_{AC} = 10^{-8}$) can be calculated from the pK_a's of acetic acid (4.7) and **1** (12.7; Pollack et al. 1989a), corresponding to a ΔG° at room temperature of approximately 11 kcal/mol. Similarly, ΔG° for WT·**1** $\rightleftarrows$ WT·**2** calculated from K_{int} is 0.7 kcal/mol. Binding to the enzyme stabilizes the intermediate by about 10 kcal/mol, or greater than 90% of the energy difference between reactants and products for the model reacion of acetate with **1**. Clearly, the enzyme has greatly reduced the kinetic barrier to enolization by differential binding of the intermediate relative to the substrate, as suggested by Albery and Knowles (1976b).

Analysis in Terms of Marcus Formalism. Marcus formalism (Cohen & Marcus, 1968; Marcus, 1969; Albery, 1980; Gerlt & Gassman, 1993) enables a separation of the relative contributions of the thermodynamic and kinetic contributions to catalysis, analogous to differential binding and catalysis of elementary steps in the terminology of Albery and Knowles (1976b). According to Marcus formalism, the free energy of activation of a reaction ($\Delta G^\ddagger$) may be divided into two parts: (1) the free energy of the reaction (ΔG°) and (2) the intrinsic free energy of activation ($\Delta G^\ddagger_{int}$), which is the free energy of activation for a reaction in which the reactants and products are of equal stability. These terms are related by eq 12, and the position of the transition state along the reaction coordinate ($x^\ddagger$) is given by eq 13. Gerlt and Gassman (1993) proposed that the transition states for enzymatic enolizations occur "late" on the reaction coordinate and resemble the enolic intermediates. Thus, although there may be contributions from a lowering of the free energy of the reaction by binding of the intermediate to the enzyme, the major driving force according to this theory is a substantial reduction in $\Delta G^\ddagger_{int}$ from the corresponding nonenzymatic reaction.

$$\Delta G^\ddagger = \Delta G^\ddagger_{int}(1 + \Delta G^\circ/4\Delta G^\ddagger_{int})^2 \quad (12)$$

$$x^\ddagger = 0.5 + \Delta G^\circ/8\Delta G^\ddagger_{int} \quad (13)$$

An estimate for ΔG° (0.7 kcal/mol) for the enzymatic reaction was obtained above and $\Delta G^\ddagger$ may be calculated from the rate constant for conversion of E·**1** to E·**2** ($k = 1.7 \times 10^5$ s^{-1}, $\Delta G^\ddagger = 10.3$ kcal/mol; Hawkinson et al., 1993) and the internal equilibrium constant ($K_{int} \approx 0.3$). With a knowledge of $\Delta G^\ddagger$ and ΔG°, $\Delta G^\ddagger_{int}$ can be estimated as 10 kcal/mol.

How do these values compare to the corresponding nonenzymatic reaction of acetate-catalyzed isomerization of **1** to **2**? Although $\Delta G^\ddagger_{int}$ for this reaction has not yet been measured, it may be estimated in the following manner. The Brønsted β value for oxygen bases has not been determined, but $\beta \approx 0.68$ with tertiary amine bases (Perrera et al., 1980). In addition, for a similar reaction, the general base catalyzed isomerization of 3-cyclohexenone, $\beta = 0.6$ for tertiary amines and 0.5 for oxygen bases (Whalen et al., 1976). Using $\beta = 0.6$ and the experimentally determined rate constant for acetate-catalyzed isomerization of **1** to **2** (6×10^{-4} M^{-1} s^{-1}; Zeng & Pollack, 1991), extrapolation of the rate constant to p$K = 12.7$ (Δp$K = 0$) gives the intrinsic rate constant ($k_0 \approx 40$ M^{-1} s^{-1}). For comparison of this second order rate constant to the first order enzymatic rate constant, we convert the second order rate constant into a first order one by assuming

[6] In order to compare KSI and acetate as catalysts, it is necessary to consider the reaction from an encounter complex of acetate and **1**, analogous to the enzyme–substrate complex of KSI. This requires the estimation of an equilibrium constant for this encounter complex. Hine (1971) has calculated that the equilibrium constant for formation of an encounter complex with the nucleus of the basic atom positioned either above or below the acidic carbon should be about 0.017 M^{-1}. Thus, the first order rate constant for decomposition of the encounter complex is equal to (40 M^{-1} s^{-1})/0.017 = 2400 s^{-1}. We recently (Hawkinson et al., 1991b) assumed an association constant of 0.1 M in our discussion of the free energy profile of KSI. This value would be more appropriate for an encounter complex with a random orientation of the base relative to the acidic carbon. The analysis of energetics is only slightly affected by the exact number used.

formation of an encounter complex with acetate having a dissociation constant of about 60 M.[6] The "model" intrinsic rate constant then becomes 2400 s^{-1}, and the intrinsic barrier about 13 kcal/mol. Guthrie (1991) has recently calculated a similar intrinsic barrier (10.7 kcal/mol) for proton transfers from the α-carbons of carbonyl compounds to hydroxide ion. The qualitative nature of the arguments that follow does not depend appreciably on the exact value of the intrinsic barrier assumed.

A comparison of the energetics of the enzymatic and model reactions shows that ΔG° is reduced from 11 kcal/mol for the model reaction to less than 1 kcal/mol for KSI, while $\Delta G^{\ddagger}_{int}$ is reduced from 13 to 10 kcal/mol. Thus, the major function for the enzyme is to equalize the free energies of reactant and intermediate by decreasing ΔG° ($\Delta\Delta G^\circ = 10$ kcal/mol), with a lesser change in the intrinsic activation barrier ($\Delta\Delta G^{\ddagger}_{int} = 3$ kcal/mol). This result is in accord with the conclusion of Guthrie and Kluger (1993) that stabilization of the intermediate is important for enzymatic enolizations.

Structure of the Transition State. The finding that K_{int} is near unity for WT suggests that the transition state for proton transfer in the enolization of **1** at the enzyme site is nearly symmetrical with regard to proton transfer. Marcus theory (eq 13), as well as the Hammond postulate (Hammond, 1955), predicts that the transition state should be about halfway along the reaction coordinate for $K_{int} = 1.0$ and late along the reaction coordinate for $K_{int} \ll 1.0$. Independent evidence concerning the position of the transition state may be obtained from the observed kinetic isotope effects on k_{cat} for the reaction of 4β-deuterated 5-androstene-3,17-dione (**1**-4βD) *vs* its 4β-protonated analog (**1**-4βH). The substrate deuterium isotope effect on k_{cat} ($k_{4\beta H}/k_{4\beta D}$) is 6.1 (Xue et al., 1990). The rate-limiting step for k_{cat} is a mixture of deprotonation of **1**, protonation of **2**, and loss of **3** from the enzyme–product complex (Hawkinson et al., 1991b). Since only the first two of these will be subject to a kinetic isotope effect, the true isotope effect for the proton transfer steps is likely to be even higher than 6.1. An isotope effect of this magnitude suggests that the proton is approximately half-transferred in the transition state (Kresge, 1976). However, Holman and Benisek (1994) concluded from their studies of a KSI mutant with alanine-3-sulfinate at residue 38 that the Brønsted β is about 0.75, suggesting a relatively late transition state.

Conclusion. As emphasized by Guthrie and Kluger (1993), the thermodynamic instability of the dienolic intermediate must be overcome for efficient enzymatic catalysis. The energetics of KSI are consistent with the proposal of Albery and Knowles that this is accomplished by differential binding of the intermediate, increasing the internal equilibrium constant toward unity. The lowering of the energies of the adjacent transition states produced by this thermodynamic stabilization of the intermediate accounts for the major fraction of the enzymatic rate enhancement (ca. 10 kcal/mol). In contrast, the intrinsic free energy of activation ($\Delta G^{\ddagger}_{int}$) is only decreased by ca. 3 kcal/mol.

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REFERENCES

Albery, W. J. (1980) *Annu. Rev. Phys. Chem. 31*, 227–263.
Albery, W. J., & Knowles, J. R. (1976a) *Biochemistry 15*, 5627.
Albery, W. J., & Knowles, J. R. (1976b) *Biochemistry 15*, 5631.
Bantia, S., Bevins, C. L., & Pollack, R. M. (1985) *Biochemistry 24*, 2606.
Bevins, C. L., Kayser, R. H., Pollack, R. M., Ekiko, D. B., & Sadoff, S. (1980) *Biochem. Biophys. Res. Commun. 95*, 1131.
Bevins, C. L., Pollack, R. M., Kayser, R. H., & Bounds, P. L. (1986) *Biochemistry 25*, 5159.
Brooks, B., & Benisek, W. F., (1994) *Biochemistry 33*, 2682.
Burbaum, J. J., Raines, R. T., Albery, W. J., & Knowles, J. R. (1989) *Biochemistry 28*, 9293.
Chin, J. (1983) *J. Am. Chem. Soc. 105*, 6502.
Choi, K. Y., & Benisek, W. F. (1988) *Gene 69*, 121.
Cohen, A. O., & Marcus, R. A. (1968) *J. Phys. Chem. 72*, 4249.
Davenport, L., Knutson, J. R., & Brand, L. (1986) *Biochemistry 25*, 1186.
Duval, E. J., Ambulos, N. P., Jr., & Lovett, P. S. (1987) *J. Bacteriol. 169*, 4235.
Eames, T. C. M., Pollack, R. M., & Steiner, R. F. (1989) *Biochemistry 28*, 6269.
Eames, T. C. M., Hawkinson, D. C., & Pollack, R. M. (1990) *J. Am. Chem. Soc. 112*, 1996.
Frieden, C. (1993) *Trends Biochem. Sci. 18*, 58.
Gerlt, J. A., & Gassman, P. G. (1992) *J. Am. Chem. Soc. 114*, 5928.
Gerlt, J. A., & Gassman, P. G. (1993) *J. Am. Chem. Soc. 115*, 11552.
Gerlt, J. A., Kozarich, J. W., Kenyon, G. L., & Gassman, P. G. (1991) *J. Am. Chem. Soc. 113*, 9667.
Guthrie, J. P. (1991) *J. Am. Chem. Soc. 113*, 7249.
Guthrie, J. P., & Kluger, R. (1993) *J. Am. Chem. Soc. 115*, 11569.
Hammond, G. S. (1955) *J. Am. Chem. Soc. 77*, 334.
Hawkinson, D. C., Eames, T. C. M., & Pollack, R. M. (1991a) *Biochemistry 30*, 6956.
Hawkinson, D. C., Eames, T. C. M., & Pollack, R. M. (1991b) *Biochemistry 30*, 10849.
Hine, J. (1971) *J. Am. Chem. Soc. 93*, 3701.
Holman, C. M., & Benisek, W. F. (1994) *Biochemistry 33*, 2682.
Kawahara, F. S., Wang, S.-F., & Talalay, P. (1962) *J. Biol. Chem. 237*, 1500.
Kayser, R. H., Bounds, P. L., Bevins C. L., & Pollack, R. M. (1983) *J. Biol. Chem. 258*, 909.
Kresge, A. J. (1976) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrup, D. B., Eds.) pp 37–63, University Park Press, Baltimore.
Kuliopulos, A., Mildvan, A. S., Shortle, D., & Talalay, P. (1989) *Biochemistry 28*, 149.
Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, p 131, Plenum, New York.
Li, Y.-K., Kuliopulos, A., Mildvan, A. S., & Talalay, P. (1993) *Biochemistry 32*, 1816.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY.
Marcus, R. A. (1969) *J. Am. Chem. Soc. 91*, 7224.
Nickbarg, E. B., & Knowles, J. R. (1988) *Biochemistry* 27, 5939.
Perrera, S. K., Dunn, W. A., & Fedor, L. R. (1980) *J. Org. Chem., 45*, 2816.
Pollack, R. M., Bantia, S., Bounds, P. L., & Koffman, B. M. (1986) *Biochemistry 25*, 1905.
Pollack, R. M., Mack, J. P. G., & Eldin, S. (1987) *J. Am. Chem. Soc. 109*, 5048.
Pollack, R. M., Zeng, B., Mack, J. P. G., & Eldin, S. (1989a) *J. Am. Chem. Soc. 111*, 6419.
Pollack, R. M., Bounds, P. L., & Bevins, C. L. (1989b) in *The Chemistry of Enones* (Patai, S., Ed.) Chapter 13, John Wiley and Sons, New York.

Schwab, J. M., & Henderson, B. S. (1990) *Chem. Rev. 90*, 1203.
Stackhouse, J., Nambier, K. P., Burbaum, J. J., Stauffer, D. M., & Benner, S. A. (1985) *J. Am. Chem. Soc. 107*, 2757.
Taylor, J. W., Ott, J., & Eckstein, F. (1985) *Nucleic Acids Res. 13*, 8765.
Wang, S.-F., Kawahara, F. S., & Talalay, P. (1963) *J. Biol. Chem. 238*, 576.
Whalen, D. L., Weimaster, J. F., Ross, A. M., & Radhe, R, (1976) *J. Am. Chem. Soc. 98*, 7319.
Xue, L., Talalay, P., & Mildvan, A. S. (1990) *Biochemistry 29*, 7491.
Xue, L., Kuliopulos, A., Mildvan, A. S., & Talalay, P. (1991) *Biochemistry 30*, 4991.
Zawrotny, M. E., Ambulos, N. P., Lovett, P. S., & Pollack, R. M. (1991) *J. Am. Chem. Soc., 113*, 5890.
Zeng, B., & Pollack, R. M. (1991) *J. Am. Chem. Soc. 113*, 3838.
Zeng, B., Bounds, P. L., Steiner, R. F., & Pollack, R. M. (1992) *Biochemistry 31*, 1521.