# An Active Site Phenylalanine of 3-Oxo-$\Delta^5$-Steroid Isomerase Is Catalytically Important for Proton Transfer†

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ABSTRACT: 3-Oxo-$\Delta^5$-steroid isomerase (KSI) from *Pseudomonas testosteroni* catalyzes the isomerization of a variety of 3-oxo-$\Delta^5$-steroids to their conjugated $\Delta^4$-isomers through the intermediate formation of a dienolate ion. This dienolate is formed by proton transfer from C-4 of the substrate to Asp-38, which then protonates the dienolate at C-6. Catalysis is enhanced by electrophilic assistance (hydrogen bonding) to the 3-oxygen by Tyr-14. We have investigated the effect of modifying phenylalanine-101 (F101), a hydrophobic residue that is located in the binding pocket of KSI. Two mutant enzymes (F101L and F101A) of KSI were prepared, and their kinetic properties were examined with 5-androstene-3,17-dione (**1**) as the substrate. Both of the mutants show reduced values of $k_{cat}$ compared to the wild type (WT), by about 30-fold (F101L) and by 270-fold (F101A), with only a small difference in $K_m$ values. There is little change in the $K_i$'s ($\leq$4-fold) for the product 4-androstene-3,17-dione (**3**), although both enzymes bind the intermediate analog d-equilenin (**4**) about 25-fold less tightly than does the WT. Fluorescence spectra of **4** bound to each of these enzymes suggest that **4** is ionized at the active site of WT, un-ionized at the active site of F101A and a mixture of these ionization states at the active site of F101L. Free energy profiles are constructed for each of the mutant enzymes, and these are compared to the free energy profile for the WT. The results are interpreted in terms of stabilization of the intermediate dienolate and the flanking transition states by the phenyl ring of F101.

Although the importance of hydrophobic effects in the binding of small molecules to enzymes is widely accepted (Fersht, 1985), the possible contribution of hydrophobic residues to enzymatic catalysis remains generally unappreciated. However, analysis of the nature of the driving force for enzymatic rate accelerations by transition state theory (Wolfenden, 1972; Lienhard, 1973; Kraut, 1988) suggests that these residues might play an important role in catalysis. By binding more tightly to the transition state than to the substrate, residues that do not function directly in bond-forming or bond-breaking steps could enhance the catalytic ability of an enzyme.

A few reports in the literature attest to the ability of hydrophobic residues to participate in enzymatic catalysis. Benkovic and co-workers have concluded that Leu-54 is an important participant in the catalysis of hydride transfer in dihydrofolate reductase (Mayer et al., 1986), although this hydrophobic residue plays no obvious role in the mechanism. Similarly, Dupureur et al. (1992) showed that substitution of Phe-22 or Phe-106 by Ala results in decreased catalytic activity of phospholipase $A_2$. In other work, Matsui et al. (1994) examined the effect of mutations of the aromatic residue Tyr-83 on the catalytic activity of α-amylase. Substitution of Tyr-83 by the nonaromatic residues leucine and asparagine decreases $k_{cat}$ by 50−100-fold, with little change in $K_m$ (<3-fold). Replacement by Phe, however, results only in a decrease in $k_{cat}$ of <4-fold and $K_m$ < 2-fold. Hurley et al. (1993) have shown that an aromatic amino acid at position 65 is necessary for optimal electron transfer in ferrodoxin:$NADP^+$ reductase.

We report here the results of our investigation into the catalytic contribution of the active site hydrophobic residue Phe-101 to the mechanism of 3-oxo-$\Delta^5$-steroid isomerase. 3-Oxo-$\Delta^5$-steroid isomerase (EC 5.3.3.1, also called $\Delta^5$-3-ketosteroid isomerase, KSI) from *Pseudomonas testosteroni* catalyzes the conversion of a variety of 3-oxo-$\Delta^5$-steroids to their conjugated $\Delta^4$-isomers [for reviews, see Pollack et al. (1989a), Schwab and Henderson (1990), and Creighton and Murthy (1990)]. KSI is one of the most active enzymes known, with a $k_{cat}$ for 5-androstene-3,17-dione of approximately $6 \times 10^4$ $s^{-1}$ per monomer at pH 7 (Pollack et al., 1986; Kuliopulos et al., 1989). The reaction proceeds through enolization of the initial β,γ-unsaturated ketone (**1**) to form an enzyme-bound dienolate (**2**) with subsequent ketonization of **2** to give the product α,β-unsaturated ketone (**3**), as shown in Scheme 1 (Bantia et al., 1986; Eames et al., 1990).

The complete free energy profile has been constructed for KSI, and the energies of the bound intermediate (**2**) and transition states relative to bound substrate (**1**) and product (**3**) are known (Hawkinson et al., 1991b, 1994). A variety of experiments have implicated Asp-38 and Tyr-14 as catalytically important residues (Ogez et al., 1977; Benisek et al., 1980; Bounds & Pollack, 1987; Kuliopulos et al., 1987, 1990), with Asp abstracting the proton and Tyr-14 acting to polarize the carbonyl by hydrogen bonding (Zeng et al., 1992). These two groups are the only ones that have been

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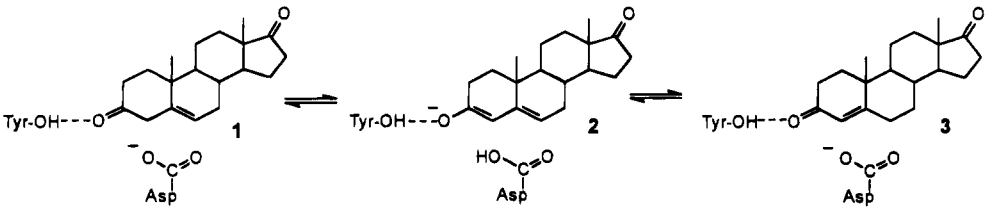
Scheme 1




proposed as functionally important for the stabilization of either the intermediate dienolate or the transition states for the reaction.

Low resolution (6 Å) X-ray measurements by Westbrook and co-workers (Westbrook & Sigler, 1984; Westbrook et al., 1984) have shown that KSI has one binding site per monomer subunit and that site is located in a deep pit. Further characterization of the active site comes from the work of Kuliopulos et al. (1987), who examined the binding of a spin-labeled substrate analog to the isomerase. Their results, along with unpublished X-ray results at 2.5 Å (E. M. Westbrook, personal communication), allow the identification of hydrophobic groups at the active site. We have mutated one of these, Phe-101, to Leu (F101L) and to Ala (F101A) and have found that, although both mutants bind substrate with similar dissociation constants to the wild type (WT), in each case, $k_{cat}$ is substantially reduced toward 5-androstene-3,17-dione (ca. 30-fold for F101L and ca. 270-fold for F101A). Thus, the presence of the phenyl ring of F101 is important for the catalysis of proton transfer by KSI.

## MATERIALS AND METHODS

*Materials.* 5-Androstene-3,17-dione was prepared as before (Pollack et al., 1989b). Recombinant KSI was available from previous work (Eames et al., 1989). Water was purified by reverse osmosis. All other materials were used as purchased.

*Synthetic Methods.* 4$\beta$[$^2$H]-5-Androstene-3,17-dione (**1d**) was prepared by modifications of published procedures (Malhotra & Ringold, 1965; Xue et al., 1990) as described below.

5-Androstene-3$\beta$,17$\beta$-diol dibenzoate was prepared according to the literature (Barton & Cox, 1948) and recrystallized from chloroform/ethyl acetate: 92%, mp 217−218 °C [lit mp 152 °C (Barton & Cox, 1948)]. Anal. calcd for $C_{33}H_{38}O_4$: C,79.41; H, 7.68. Found: C, 79.68; H, 7.87.

5$\alpha$,6$\alpha$-Oxido-androstane-3$\beta$,17$\beta$-diol dibenzoate was synthesized by the method of Malhotra and Ringold (1965). Separation of the 5$\alpha$,6$\alpha$-isomer from its 5$\beta$,6$\beta$-isomer was accomplished by flash chromatography on silica using methylene chloride as the eluting solvent. The 5$\beta$,6$\beta$-isomer eluted first and was recrystallized from ethyl acetate (30% yield, mp 204−206 °C). The 5$\alpha$,6$\alpha$-isomer eluted later (61% yield, mp 234.5−236.5 °C). Both compounds gave $^1$H NMR spectra in agreement with literature values (Xue et al., 1990).

6$\beta$-Chloro-androstane-3$\beta$,5$\alpha$,17$\beta$-triol 3$\beta$,17$\beta$-dibenzoate was also synthesized according to Malhotra and Ringold (1965) and recrystallized from either methanol or ethyl acetate in 92% yield: mp 213−215 °C [lit mp 223−225 °C (Malhotra & Ringold, 1965)].

6$\beta$-Chloro-4-androstene-3$\beta$,17$\beta$-diol dibenzoate was purified by flash chromatography with hexane/ethyl acetate (4:1) as the eluting solvent. Recrystallization from ethyl acetate/hexane gave an 80% yield: mp 167−169 °C [lit mp 167−169 °C (Malhotra & Ringold, 1965)].

4$\beta$-[$^2$H]-5-Androstene-3$\beta$,17$\beta$-diol was purified by flash chromatography using ethyl acetate/hexane (2:1) and was recrystallized from ethyl acetate/hexane in 63% yield: mp 179−182 °C [lit mp 175−178 °C (Malhotra & Ringold, 1965)].

4$\beta$-[$^2$H]-5-Androstene-3,17-dione (**1d**) was obtained in 65% yield and gave a $^1$H NMR identical to the literature (Xue et al., 1990), with no discernible signal due to a proton at 4$\beta$ at 500 MHz.

*Mutagenesis.* F101L and F101A mutants of KSI were prepared using the method of oligonucleotide-mediated site-directed mutagenesis described by Sayers et al. (1988), as implemented in a commercially available kit from Amersham. Positive mutations were verified by sequencing the entire gene. Mutants were directionally recloned into pUC 8 from M13mp18 using unique *Eco*RI and *Hin*dIII restriction sites for expression of the proteins.

*Protein Expression and Purification.* Recombinant plasmids were transformed into *Escherichia coli* strain NCM 533 (Shand et al., 1991), and the protein was expressed by inoculation of 10 L of 2× TY medium (16 g of Bacto tryptone, 10 g of yeast extract, 5 g of NaCl) with 500 mL of late log phase culture and incubation for 14 h at 37 °C with constant shaking. Proteins were isolated as described previously for WT and D38E (Zawrotny & Pollack, 1994).

*Kinetic and Fluorescence Methods.* Kinetic and fluorescence methods have been described (Pollack et al., 1979; Eames et al., 1989; Hawkinson, et al., 1991b; Zawrotny & Pollack, 1994). Both $k_{cat}$ and $K_m$ were determined by monitoring the change in absorbance at 248 nm (or 262 nm for high concentrations of **1**).

## RESULTS

Several mutants of KSI at position 101 were prepared in order to explore the effect of the side chain at this position on the catalytic activity. In addition to the F101A and F101L mutants, both F101Y and F101H were produced, but neither could be obtained in satisfactory purity. Each of these proteins rapidly lost activity during purification. Both F101L and F101A, however, are much less labile than F101Y and F101H. F101L was produced at about 5-fold greater levels than wild type (WT) in *E. coli* strain NCM533, whereas F101A was produced at a level 6−10-fold lower than WT. Both F101L and F101A showed only one band on an overloaded PAGE gel (Coomassie blue stain) and showed little loss in activity during storage at 4 °C for several months or for more than 1 year at −80 °C. WT used in this work had a specific activity of 56 000 units/mg, while F101L had a specific activity of 3000 units/mg, and F101A had a specific activity of 400 units/mg. Both mutants showed the charac-

Table 1: Kinetic and Inhibition Constants for the Isomerization of 5-Androstene-3,17-dione (**1**) to 4-Androstene-3,17-dione (**3**) by Wild Type 3-Oxo-$\Delta^5$-Steroid Isomerase (WT) and Its F101L and F101A Mutants

| | enzyme | | | | | |
|---|---|---|---|---|---|---|
| constant[a] | WT | F101L | F101A | WT/F101L | WT/F101A | method |
| $k_{cat}$ ($s^{-1}$) | $(4.2 \pm 1.1) \times 10^4$ | $(1.6 \pm 0.2) \times 10^3$ | $(1.8 \pm 0.1) \times 10^2$ | $26 \pm 7$ | $268 \pm 68$ | initial rate |
| | $6.1 \times 10^4$ | $1.5 \times 10^3$ | $2.2 \times 10^2$ | 41 | 277 | KINSIM |
| $K_m$ ($\mu$M) | $183 \pm 33$ | $123 \pm 7$ | $130 \pm 1$ | $1.8 \pm 0.3$ | $1.6 \pm 0.3$ | initial rate |
| | 248 | 115 | 134 | 2.2 | 1.9 | KINSIM |
| $k_{cat}/K_m$ ($M^{-1}$ $s^{-1}$) | $(2.4 \pm 0.1) \times 10^8$ | $(1.3 \pm 0.2) \times 10^7$ | $(1.4 \pm 0.1) \times 10^6$ | $18 \pm 3$ | $171 \pm 14$ | initial rate |
| | $2.2 \times 10^8$ | $1.1 \times 10^7$ | $1.5 \times 10^6$ | 20 | 150 | KINSIM |
| $K_i$ (**3**) ($\mu$M) | 220 | 800 | 600 | 0.3 | 0.4 | KINSIM |
| $K_i$ (**4**) ($\mu$M) | $2.7 \pm 0.7$[b] | 49 | 39 | 0.06 | 0.07 | first order |
| | 1.7 | 40 | 40 | 0.04 | 0.04 | KINSIM |

[a] Kinetic parameters were determined at pH 7.0 (33 mM phosphate), 3.3% methanol, and 25.0 °C. Initial rate determinations are the average of three separate runs. Errors are standard deviations. [b] 3.3% methanol and 10 mM phosphate, pH 7.15 (Hawkinson et al., 1994).

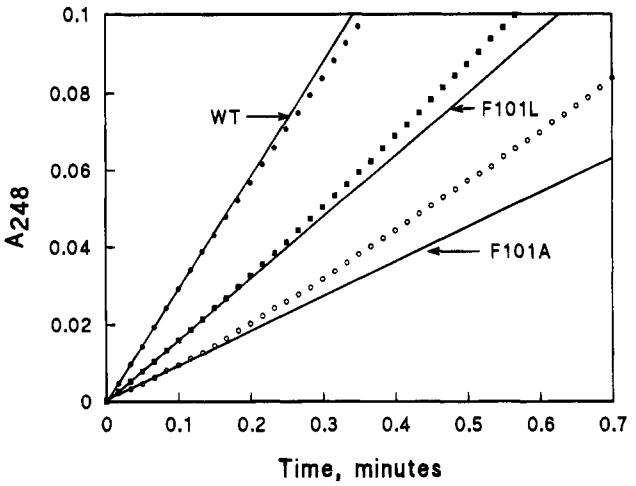
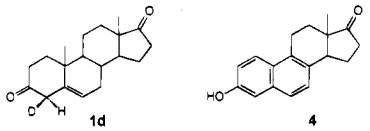



FIGURE 1: Plots of initial absorbance changes for the reaction of **1d** with WT, F101L, and F101A at pH 7.0, 25.0 °C, 3.3% methanol, and 33 mM phosphate. Initial concentration of **1** was 47 $\mu$M for **1** with all enzymes. Enzyme concentrations were $1.70 \times 10^{-9}$ M (F101L); $4.57 \times 10^{-9}$ M (F101A), and $1.35 \times 10^{-10}$ M (WT). Lines are based on absorbance changes for the first 1−2% reaction.

teristic hand-shaped UV spectrum of KSI (Kuliopulos et al., 1989).

Steady-state kinetic assays with F101A, F101L, and WT using 5-androstene-3,17-dione (**1**) as the substrate gave values for the kinetic parameters at 25 °C and pH 7.0 (Table 1). Similar results were obtained at pH 8.0. Relative to WT, $k_{cat}$ is reduced for both F101L (ca. 30-fold) and F101A (ca. 270-fold), although there is little change in $K_m$ (less than 2-fold for each mutant). The mutant enzymes were also assayed with 4$\beta$[$H^2$]-5-androstene-3,17-dione (**1d**). The




increase in absorbance at 248 nm for both F101A and F101L displays an induction period, which is more pronounced in F101A than F101L; in contrast, WT gives linear kinetics through the first 5−10% reaction (Figure 1). Kinetic parameters for F101L and F101A were determined from the initial spectral changes (ca. 1−2% reaction). These values are given in Table 2, along with calculated kinetic isotope effects.

Dissociation constants ($K_i$) were determined for the product (**3**) and the intermediate analog d-equilenin (**4**) with F101L and F101A (Table 1). $K_i$ values for both **3** and **4** were obtained by analysis of the complete time course of the reactions, using the FITSIM/KINSIM program of Frieden (Barshop et al., 1983; Frieden, 1993). Reactions with various initial concentrations of **1** (0−120 $\mu$M) and **3** (0−100 $\mu$M) were monitored until completion, and the absorbance vs time data were fit to Scheme 2 to give $K_i$. The $K_i$ of **4** was determined in a similar fashion for each enzyme. Initial concentrations of **1** (3−5 $\mu$M) and **4** (0−35 $\mu$M) were varied, with the concentration of **1** at least 20-fold less than $K_m$, so that pseudo-first-order conditions were followed. The data were fit to Scheme 3 to give the values for the kinetic constants summarized in Table 1. In addition, $K_i$ for **4** was determined under first order conditions (Pollack et al., 1979) with constant substrate concentration (5−10 $\mu$M) and variable inhibitor concentration (0−45 $\mu$M). Agreement between the two determinations of $K_i$ for **4** is excellent for both F101L and F101A.

The partitioning of the intermediate dienol on the enzyme surface was investigated by monitoring the reaction of the

Table 2: Kinetic Isotope Effects for the Isomerization of 4$\beta$[$^2$H]-5-Androstene-3,17−dione (**1d**) by F101L and F101A Mutants of 3-Oxo-$\Delta^5$-Steroid Isomerase

| | enzyme | |
|---|---|---|
| constant[a] | F101L | F101A |
| $k_{cat}$ (**1**) ($s^{-1}$) | $(1.6 \pm 0.2) \times 10^3$ | $180 \pm 10$ |
| $k_{cat}$ (**1d**) ($s^{-1}$) | $240 \pm 50$ | $53 \pm 10$ |
| $k_{cat}$ (**1**)/$k_{cat}$ (**1d**)[b] | $6.7 \pm 1.6$ | $3.4 \pm 0.7$ |
| $K_m$ (**1**) ($\mu$M) | $123 \pm 7$ | $130 \pm 1$ |
| $K_m$ (**1d**) ($\mu$M) | $100 \pm 40$ | $94 \pm 50$ |
| $K_m$ (**1**)/$K_m$ (**1d**) | $1.2 \pm 0.5$ | $1.4 \pm 0.7$ |

[a] Kinetic parameters were determined at pH 7.0 (33 mM phosphate), 3.3% methanol, and 25.0 °C. [b] Pronounced upward curvature of the initial rate plots with F101L and F101A made accurate determination of the kinetic parameters with **1d** difficult.

Scheme 2

$$E + \mathbf{1} \overset{K_m}{\rightleftharpoons} E\cdot\mathbf{1} \xrightarrow{k_{cat}} E + \mathbf{3}$$

$$E + \mathbf{3} \overset{K_i}{\rightleftharpoons} E\cdot\mathbf{3}$$

Scheme 3

$$E + \mathbf{1} \xrightarrow{k_{cat}/K_m} E + \mathbf{3}$$

$$E + \mathbf{4} \overset{K_i}{\rightleftharpoons} E\cdot\mathbf{4}$$

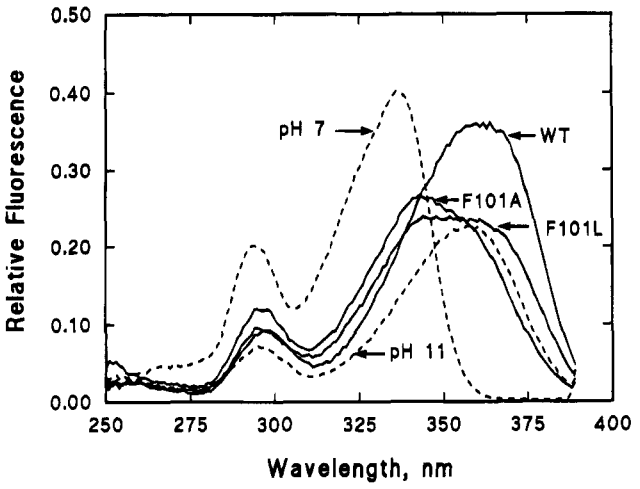



FIGURE 2: Fluorescence excitation spectra of equilenin (**4**) in water at pH 7 and 11, along with the corresponding spectra for the complex of equilenin with WT, F101L, and F101A KSI at pH 7.0 (ca. 0.2% methanol) and 25 °C. The concentration of equilenin in all cases was 11.2 $\mu$M, with enzyme concentrations of 134 $\mu$M (WT), 131 $\mu$M (F101L), and 123 $\mu$M (F101A). Doubling of the enzyme concentration gave no appreciable change in the spectra, indicating that equilenin is virtually completely bound at this low concentration of methanol.

enzymes with an independently generated dienol. We (Hawkinson et al., 1991a,b) have previously shown that the dienol can be generated by the reaction of **1** with sodium hydroxide and rapid quenching of the solution into acidic or neutral buffer. Treatment of this dienol with enzyme allows the partitioning of the bound complex (E·**2**) to reactants and products to be observed [47:53 for WT at 3.3% MeOH, pH 7.0 (Hawkinson et al., 1991a,b)]. Using similar methods, we examined the partitioning of the dienol with F101L, F101A, and WT at pH 7.4. With both F101L and F101A, the intermediate enzyme−dienol complex reverts to reactants somewhat more rapidly than to products (61:39 for F101L, 76:24 for F101A and 43:57 for WT at pH 7.4, 3.3% MeOH).

The nature of the interactions of the enzymes with the intermediate dienolate (**2**) was probed by determining the fluorescence spectra of **4** bound to each of the enzymes. Figure 2 shows excitation spectra for **4** in aqueous buffer at pH 7 and 11, as well as the spectra in the presence of sufficient enzyme to completely bind **4** for WT, F101L, and F101A. As previously observed by Eames et al. (1989), the spectrum of the WT·**4** complex is similar to that of ionized equilenin (pH 11). In contrast, F101A·**4** gives a spectrum that bears a closer resemblance to un-ionized equilenin (pH 7), although a shoulder at about 360 nm may indicate the presence of some ionized equilenin. The F101L·**4** complex has a spectrum that suggests a mixture of both ionized and un-ionized **4**.

## DISCUSSION

Before discussing the effects of mutations of F101, it is necessary to eliminate some trivial explanations for the differences in the activities of the enzymes. (1) Both $k_{cat}$ and $K_m$ were also determined at pH 8 for each enzyme, and the values are virtually identical with those at pH 7, excluding the possibility that the results are an artifact of the pH used for the measurements. (2) The activity that is observed for the mutants is not due to contaminating WT activity, since the $K_m$'s of F101L and F101A are different from WT, and $K_m$ is a property of the catalyst that is independent of concentration. In addition, the different behavior of the three enzymes toward **1d** shows that the activities do not come from the same source. (3) The possibility that the lowered $k_{cat}$ values for the mutants are due to large amounts of contaminating inactive enzyme is rendered unlikely by the UV spectra of the mutants, which show the characteristic "hand" shape for native KSI (Kuliopulos et al., 1989).

In order to determine whether mutation of F101 to L or A causes a change in the rate determining step of the reaction, the isomerization was monitored with 4$\beta$-[$^2$H]-5-androstene-3,17-dione (**1d**). For both F101L and F101A, the initial absorbance is nonlinear, showing an induction period followed by an increase in rate; in contrast, WT shows a slight downward curvature, which is expected as substrate reacts (Figure 1). The most likely explanation for this phenomenon is enzymatic catalysis of deuterium exchange of the substrate with solvent for the mutants. We have shown that substantial exchange of substrate protons occurs with the D38E mutant of KSI (M. E. Zawrotny and R. M. Pollack, unpublished results), and it is likely that the same happens with the F101L and F101A mutants. Since the intermediate enzyme−dienol complex partitions preferentially to reactants with the two F101 mutants, there should be substantial accumulation of undeuterated substrate as the reaction progresses. Therefore, rate constants for the mutants with **1d** were obtained from the initial rates. Substantial kinetic isotope effects are observed on $k_{cat}$ for both F101L and F101A (Table 2), as has been observed previously with WT [$k_{cat}$ (**1**)/$k_{cat}$ (**1d**) = 6.1 (Xue et al., 1990)]. These kinetic isotope effects provide strong evidence that chemical steps are rate-determining for $k_{cat}$ for all three enzymes, rather than a physical step, such as dissociation of the product. Further evidence for this assertion comes from a comparison of the dissociation constants ($K_i$) of the product (**3**) for each enzyme (Table 1). Since the dissociation constant varies by less than 4-fold, it is unlikely that the rate of product release is sensitive to the nature of the residue at position 101. Thus, the decreased $k_{cat}$ for each of the mutants is due to a decrease in the rate(s) of one or both of the proton transfer steps at the enzyme active site.

An analysis of the kinetic constants allows a determination of the effects of these mutations on the stability of bound substrate, product, and transition state(s). The logarithm of the apparent second-order rate constant $k_{cat}/K_m$ is proportional to the energy difference between the enzyme-bound transition state and the free enzyme plus substrate. Thus, the decrease in $k_{cat}/K_m$ is due to weakened interactions between the enzyme and the transition state. Similarly, the effect of a mutation on the binding of the product to the enzyme is given by the difference in $K_i$ for the wild type and mutant enzyme. The effect of substrate binding is somewhat more complicated, as it appears in the complex constant $K_m$. Nevertheless, $K_m$ can be taken as approximately equal to the dissociation constant of the substrate in many cases. In particular, if chemical steps are rate-determining, $K_m$ reduces to $K_s$, as is the case for F101A and F101L (vide infra). For WT, the situation is somewhat more complicated, but $K_m$ is a fair estimate of $K_s$ (Hawkinson et al., 1991b).

Since F101 is one of several phenylalanine residues that form a portion of the active site, it might be expected that the phenyl group of this amino acid would contribute

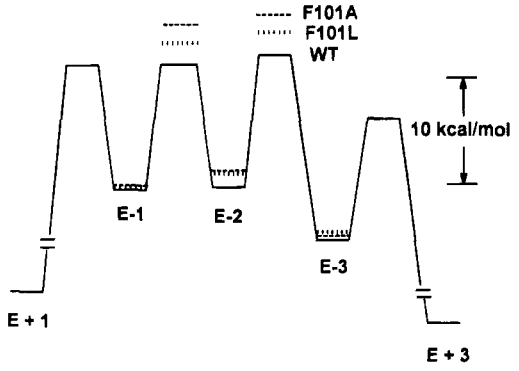



FIGURE 3: Free energy profiles for WT (solid line), F101L (dotted line), and F101A (dashed line). Methods used to generate these profiles are discussed in the text.

nonspecifically to binding of the steroid ring system. That is, the hydrophobic interaction would be of similar energy for all species along the catalytic pathway: reactants, transition state(s), intermediate, and product. However, this is clearly not the case. If it were, mutation of F101 to a less hydrophobic group would increase the dissociation constants of *all* species by a similar amount. There would be an increase in $K_m$ for **1** and $K_i$ for **3**, but little change in $k_{cat}$.

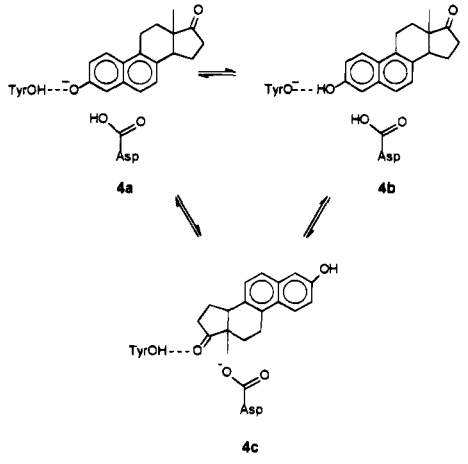
Surprisingly, the $K_m$ values for both mutants (120−130 $\mu$M) are similar to $K_s$ for WT (Hawkinson et al., 1991b). Since $k_{cat}/K_m$ for the mutants is substantially less than the rate constant for diffusion, $K_m$ is approximately equal to $K_s$. Thus, there is no significant difference in the interaction of the substrate with the side chain of amino acid residue 101 in the wild type and the mutants. The lack of a substantial effect on $K_m$ suggests that there is little interaction between the phenyl ring of F101 and the bound substrate.

In contrast, there is a substantial decrease on $k_{cat}$ (and $k_{cat}/K_m$) for the mutants relative to the WT. This decrease may be explained by a more favorable interaction of the phenyl group of F101 with the transition state, relative to the interactions of the smaller aliphatic side chains of leucine or alanine. Thus, rather than the expected uniform binding effect on all species, there is preferential stabilization of the transition state(s) by the phenyl ring of F101.

Although the above results reflect the effects of the mutations on the relative stabilities of the bound transition state(s) versus the bound substrate, they provide no information about the stability of the bound intermediate with the different enzymes. An estimate of this effect was obtained by determining the $K_i$ values for the phenolic steroid d-equilenin (**4**), a stable analog of the intermediate **2**. Table 1 shows that **4** binds more tightly to WT than to either of the mutants by approximately 25-fold. The decreased ability of **4** to bind to F101L and to F101A suggests that the intermediate dienolate may also bind better to WT than to the mutants (Hawkinson et al., 1994).

The effect of mutations at position 101 may be summarized in a reaction coordinate diagram for each of the three enzymes. Using methods described previously (Hawkinson et al., 1991b, 1994), the free energies of each of the species along the reaction coordinate may be estimated (Figure 3). Briefly, the relative energies of the bound substrate and product are determined from the measured dissociation constants of **1**, since $K_s \approx K_m$. The energies of the bound intermediates are estimated from the relative dissociation

Scheme 4




constants of **4** to WT and the two mutants, assuming that the effect of enzyme mutation on the binding of the dienol **2** is the same as the effect on the analog **4**. The rate constants for association of **1** and **3** with the enzymes are assumed to be diffusion controlled, and the heights of the transition states for proton transfer are given by $k_{cat}$ and the observed partitioning ratio. From this figure it is apparent that the major effect of mutation of F101 to a less hydrophobic residue is to destabilize both the bound intermediate and the flanking transition states by approximately 2−3 kcal/mol.

The fluorescence spectra of **4** bound to the active site of each of the enzymes were used to probe the nature of the ionization state of the bound intermediate. The excitation spectra of **4** bound to WT, F101L, and F101A are shown in Figure 2, along with the spectra of **4** at pH 7 and 11. The similarity of the excitation spectrum of the WT·**4** complex ($\lambda_{max}$ ca. 360 nm) to that of ionized equilenin ($\lambda_{max}$ ca. 358 nm) has been interpreted (Zeng et al., 1992, and references therein) in terms of ionization of equilenin at the active site. The $\lambda_{max}$ of the F101A·**4** complex is shifted to ca. 343 nm, suggesting that the ionization state of **4** at the active site may resemble un-ionized equilenin ($\lambda_{max}$ ca. 336 nm). The F101L·**4** complex shows a spectrum that appears to have two peaks, indicating that both structures may be present.

The spectrum of ionized equilenin probably results from structure **4a** in Scheme 4, analogous to the intermediate dienolate ion that is postulated to be involved in the isomerization of **1**. The spectrum assigned to neutral equilenin, however, may be due to either of two possibilities. Structure **4b** might result from a perturbation in the relative acidities of equilenin and Tyr-14, with the inhibitor bound to the active site in the normal fashion. Alternatively, equilenin might be bound "backward", with the D ring in the A ring binding pocket (**4c**). We have previously shown that steroids in general, and an equilenin derivative in particular, bind in both modes to KSI (Bevins et al., 1986).

The greater contribution of the spectrum of **4a** for WT is consistent with stabilization of this form, relative to **4b** and **4c**, by the phenyl ring of F101 of WT. The occurrence of a greater proportion of neutral equilenin with the mutants could be due to an increase in the amount of **4b**, either because of an increase in the acidity of Tyr-14 or a decrease in the acidity of the hydroxyl proton of bound equilenin. Alternatively, the proportion of **4c** could increase due to a decreased basicity of Asp-38 or a decreased acidity of the

C-4 proton of **1**. Since **4** is an analog of the intermediate **2**, the effects of the mutations at position 101 on the position of the proton of bound **2** may be similar.

Although the exact mechanism by which F101 enhances the catalytic ability of KSI is not clear, it is apparent that the aromatic ring of F101 preferentially stabilizes the transition state(s) and the intermediate relative to the reactant and the product. In this manner, it contributes substantially (ca. 100-fold) to the catalytic ability of the enzyme.

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