# Catalytic Activity of the D38A Mutant of 3-Oxo-$\Delta^5$-steroid Isomerase: Recruitment of Aspartate-99 as the Base†

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ABSTRACT: 3-Oxo-$\Delta^5$-steroid isomerase (KSI) from *Comamonas (Pseudomonas) testosteroni* catalyzes the isomerization of $\beta,\gamma$-unsaturated 3-oxosteroids to their conjugated isomers through an intermediate dienolate. Residue Asp-38 (p$K_a$ 4.57) acts as a base to abstract a proton from C-4 of the substrate to form an intermediate dienolate, which is then reprotonated on C-6. Both Tyr-14 (p$K_a$ 11.6) and Asp-99 (p$K_a \geq$ 9.5) function as hydrogen-bond donors to O-3 of the steroid, helping to stabilize the transition states. Mutation of the active-site base Asp-38 to the weakly basic Asn (D38N) has previously been shown to result in a $>10^8$-fold decrease of catalytic activity. In this work, we describe the preparation and kinetic analysis of the Ala-38 (D38A) mutant. Unexpectedly, D38A has a catalytic turnover number ($k_{cat}$) that is ca. $10^6$-fold greater than the value for D38N and only about 140-fold less than that for wild type. Kinetic studies as a function of pH show that D38A-catalyzed isomerization involves two groups, with p$K_a$ values of 4.2 and 10.4, respectively, in the free enzyme, which are assigned to Asp-99 and either Tyr-14 or Tyr-55. A mechanism for D38A is proposed in which Asp-99 is recruited as the catalytic base, with stabilization of the intermediate dienolate ion and the flanking transition states provided by hydrogen bonding from both Tyr-14 and Tyr-55. This mechanism is supported by the lack of detectable activity of the D38A/D99N, D38A/Y14F, and D38A/Y55F double mutants.

3-Oxo-$\Delta^5$-steroid isomerase ($\Delta^5$-3-ketosteroid isomerase, KSI,[1] EC 5.3.3.1) from *Comamonas testosteroni* (formerly known as *Pseudomonas testosteroni*) catalyzes the isomerization of $\beta,\gamma$-unsaturated 3-oxosteroids to their $\alpha,\beta$-conjugated isomers at a rate that approaches the diffusion limit with specific substrates (*1*). The reaction proceeds through the formation of an intermediate dienol or dienolate ion by abstraction of a proton from C-4 by Asp-38 (*2*−*4*), with the majority of the evidence favoring a dienolate rather than a dienol (*5*−*7*). Although for a long time it was thought that electrophilic assistance is provided solely by Tyr-14 (*8*, *9*), the recent determination of the solution structure revealed the existence of an additional polar group (Asp-99) at the active site (*10*). Mutation of this residue causes significant rate reductions in the D99A (ca. 3000-fold) and D99N (ca. 27-fold) mutants at pH 7.0 (*10, 11*). These results can most simply be accounted for by the formation of hydrogen bonds directly to the dienolate oxygen by both Tyr-14 and Asp-99 (Scheme 1), although a more complicated mechanism has been proposed (*12*). Support for the mechanism of Scheme 1 is provided by the 2.26 Å X-ray structure of KSI with the intermediate analogue equilenin bound in the active site (*13*). This structure shows that the phenolic oxygen of Tyr-14 and one of the carboxyl group oxygens of Asp-99 are within hydrogen-bonding distance of O-3 of the steroid. In addition, as predicted by Scheme 1, the effects of mutations of Tyr-14 to Phe (Y14F) and Asp-99 to Ala (D99A) on both $k_{cat}$ and $k_{cat}/K_M$ are additive in the Y14F/D99A mutant (*14*).

Consistent with the involvement of Asp-38 as the base, mutation of this residue to the less basic asparagine produces an enzyme (D38N) that is $10^8-10^9$-fold less active than the wild-type enzyme toward the specific substrate 5-androstene-3,17-dione (**1**) (*2, 15*).[2] For this mutant, the reaction involves formation of a tightly bound intermediate dienolate (**2**), which is then converted much more slowly to the product (**3**). We now report that replacement of Asp-38 by the nonbasic alanine (D38A) results in a much smaller decrease in activity than replacement by asparagine. Surprisingly, D38A is ca. $10^6$-fold more active than D38N and only ca. 140-fold less active than wild type (WT), suggesting the involvement of

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[1] Abbreviations: BSA, bovine serum albumin; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CD, circular dichroism; CHES, 2-(cyclohexylamino)-1-ethanesulfonic acid; D38X, mutant of 3-oxo-$\Delta^5$-steroid isomerase with Asp-38 replaced by the residue X; IPTG, isopropyl $\beta$-D-thiogalactopyranoside; $K_E$, acid dissociation constant for the free enzyme; $K_{ES}$, acidic dissociation constant for the enzyme−substrate complex; KSI, 3-oxo-$\Delta^5$-steroid isomerase (EC 5.3.3.1); PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; WT, wild type.

[2] With the D38N mutant, formation of an intermediate dienol−enzyme complex occurs with a $k_{cat}$ about $10^6$-fold less than $k_{cat}$ for wild type. This intermediate decomposes to products with a rate constant ca. 500-fold lower than its rate of formation (*2, 15*), so that the actual ratio of rate constants is $10^8-10^9$.

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Scheme 1

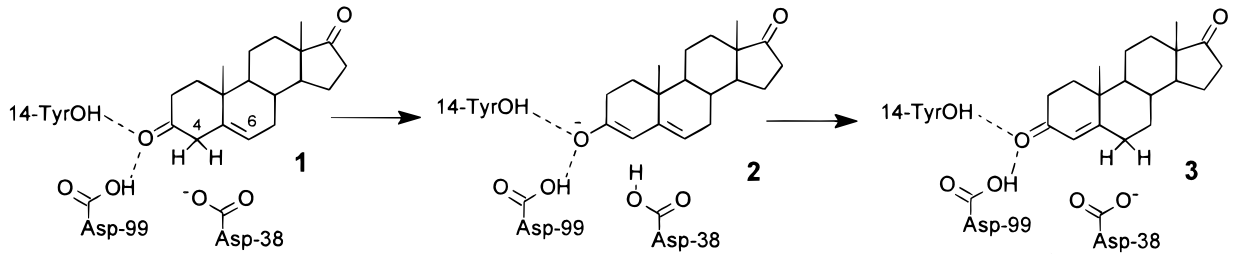



Table 1: Plasmids and Primers Used in This Study for Mutagenesis

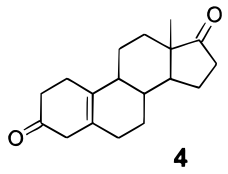
| template (KSI gene) | set of primers[a] | mutation(s) in KSI gene |
|---|---|---|
| pLDT41[b] (D99N) | 5′GGAACCCACGGGGGCTTCCACCGTGG3′<br>5′CCACGGTGGAAGCCCCCGTGGGTTCC3′ | D38A/D99N |
| $pKSI_{tac}$[c] (WT) | 5′GGAACCCACGGGGGCTTCCACCGTGG3′<br>5′CCACGGTGGAAGCCCCCGTGGGTTCC3′ | D38A |
| $pKFH_{38A}$[d] (D38A) | 5′GCCGTGGTACAACGCTTTGTGGCTGCGCG3′<br>5′CGCGCAGCCACAAAGCGTTGTACCACGGC3′ | D38A/Y14F |
| $pKFH_{38A}$[d] (D38A) | 5′CGATTTCGTGAGTTTTTCGCCAACTCGCTC3′<br>5′GAGCGAGTTGGCGAAAAACTCACGAATCG3′ | D38A/Y55F |

[a] The base change is indicated by the underlined letter. [b] From Thornburg et al. (*11*). [c] From Brooks and Benisek (*17*). [d] This study.

a new mechanism that is unavailable to D38N. These results are interpreted in terms of a new binding mode of the steroid in the catalytic site, which allows Asp-99 to be recruited as the catalytic base.

## MATERIALS AND METHODS

*Materials*. Water was purified by reverse osmosis; 5-androstene-3,17-dione (**1**) was prepared as before (*16*); 5(10)-estrene-3,17-dione (**4**) and equilenin (**5**) were purchased commercially (Steraloids and Sigma, respectively). All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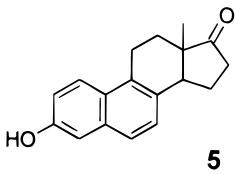
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steroids gave a single spot on thin-layer chromatography (precoated 20 mm silica gel Merck 60 F254) developed with hexane/ethyl acetate. Bovine serum albumin (BSA) was purchased from Calbiochem and used without purification. Deuterated solvents ($CH_3OD$, 99.5+ % atom D; $D_2O$, 99.8% atom D) were obtained from Aldrich. Other reagents were reagent-grade or better and are commercially available. Oligonucleotides were synthesized by the Biopolymer Laboratory at the University of Maryland, Baltimore School of Medicine. WT, D99N, and D38N/D99A KSIs were available from previous work (*11*).

*Mutagenesis and Expression Plasmids for D38A, D38A/Y14F, D38A/Y55F, and D38A/D99N*. The D38A, D38A/Y14F, D38A/Y55F, and D38A/D99N mutants of KSI were prepared by use of the QuikChange site-directed mutagenesis kit (Stratagene) and a thermocycler (Powerbloc System, Ericomp). To create each expression plasmid, a double-strand plasmid vector, either $pKSI_{tac}$ (carrying the WT KSI gene, *17*), pLDT41 (carrying the D99N KSI gene, *11*), or $pKFH_{D38A}$ (carrying the D38A gene) was used as a template with a set of two primers designed to introduce the mutation in the KSI gene (Table 1). Recombinant plasmids were transformed into Epicurian Coli XL1-Blue supercompetent cells (Stratagene), and purified from the transformants by using the Wizard Plus miniprep DNA purification system (Promega). The complete sequences of the genes were determined by the Biopolymer Laboratory at the University of Maryland, Baltimore. Experimental data were fit to equations with FigP (Biosoft), a least-squares program based on the Marquardt algorithm, with appropriate weighting.

*Protein Expression and Purification*. The D38A and D38A/D99N proteins were expressed in Epicurian Coli XL1-Blue cells by inoculation of 6 L of 2× TY-IA medium (16 g of bactotryptone, 10 g of yeast extract, and 5 g of NaCl per liter, supplemented with 1 mM IPTG and 100 $\mu$g/mL ampicillin) with 300 mL (D38A) or 45 mL (D38A/D99N) of late-log-phase culture. Incubation was performed for 43 h at 30 °C (D38A) or 24 h at 37 °C (D38A/D99N) with constant shaking. The D38A/Y14F and D38A/Y55F proteins were expressed in Epicurian Coli XL1-Blue cells by inoculation of 2 L of 2× TY-IA medium with 10 mL of late-log-phase culture. Incubation was performed for 24 h at 37 °C. Cells were harvested by centrifugation for 30 min at 5000*g* (4 °C) and dissolved in ice-cold 50 mM Tris-HCl buffer, pH 7.5. The preparation of the cell extracts and the purification of the protein were performed according to published procedures (*2*, *4*). One liter of stationary phase culture yielded 15 mg of the D38A protein, 9.6 mg of the D38A/Y14F protein, 16 mg of the D38A/Y55F protein, or 35 mg of the D38A/D99N protein. Polyacrylamide gel electrophoresis was carried out on a Bio-Rad Mini-Protean II apparatus, and the purity of the protein was determined by discontinuous SDS−PAGE on 15% gels with the buffer system of Laemmli (*18*).

*Circular dichroism* was performed at 20.0 ± 0.1 °C with a Jasco 710 spectropolarimeter. Samples were run in a 3 mL, 1 cm path-length quartz cuvette at protein concentrations of 20 $\mu$g/mL in 34 mM potassium phosphate buffer, pH 7.0. The parameters used were bandwidth, 1.0 nm; sensitivity, 50 mdeg; response time, 4 s; scan speed, 10 nm/s; and $\lambda$, 200−250 nm.

*Kinetics of the Isomerization of 5-Androstene-3,17-dione (**1**) and 5(10)-Estrene-3,17-dione (**4**).* UV spectra and kinetic data were acquired on a Gilford Response I, Response II, or Cary 1 Bio spectrophotometer equipped with thermostated sample blocks. Solutions of **1** (10−120 $\mu$M) or **4** (10−180 $\mu$M) in 3.10 mL of 34 mM potassium phosphate (3.3% methanol, pH 7.0) were incubated at 25.0 °C ± 0.1. Stock solutions of KSI were prepared by diluting concentrated KSI into solutions of 0.25% BSA in 10 mM potassium phosphate buffer, pH 7.0, and isomerization was initiated by adding the appropriate diluted KSI solution. Initial rates for 5% conversion of **1** and **4** to their conjugated isomers were determined from the change of the absorbance at 248 nm ($\epsilon$ = 16 300 $M^{-1}$ $cm^{-1}$; *19*).

*Solvent Isotope Effect.* Phosphate buffers (10 mM) were prepared in $D_2O$ from monobasic and dibasic potassium phosphate that had been recrystallized three times from $D_2O$ (99.8% atom D) and oven-dried. The pD was measured with a glass electrode, with a correction of +0.4 pH unit applied to the pH reading to account for the solvent isotope effect on the electrode response (*20*). Kinetic measurements of the isomerization of **1** were carried out as above, with the use of $CH_3OD$ as a cosolvent.

*pH−Rate Profiles.* The pH dependence of the kinetic constants for isomerization of **1** by D38A was determined at 25.0 ± 0.1 °C at constant ionic strength ($\mu$ = 0.1 M) adjusted with KCl, in 10 mM citrate (pH 3.5−5.0), 10 mM acetate (pH 4.5−6.0); 10 mM phosphate (pH 6.0−8.5), 5 mM CHES (pH 8.0−10.0), and 5 mM CAPS (pH 10.0−11.0) buffers. The stability of the D38A mutant was checked at all pH values by incubating the enzyme in the assay buffer at 25.0 ± 0.1 °C for 1 min and then initiating the reaction by addition of substrate. The observed rate was compared to the rate obtained with addition of substrate before enzyme. Specific activities were assayed under standard conditions (*21*) before and after the experiments, and in all cases at least 95% of the activity was retained.

*Native Gel Electrophoresis.* Samples (10 $\mu$L) containing 1.5−2.0 $\mu$g of either WT or mutant KSI were subjected to electrophoresis in 7.5% polyacrylamide gels at pH 7.0 and 8.2 according to a previous published procedure (*11*).

*UV Titration of D38A.* Ultraviolet spectra of D38A (38.5 $\mu$M) were acquired as a function of pH in 1.0 cm quartz cuvettes at 25.0 ± 0.1 °C from 240 to 320 nm, with a scan speed of 100 nm/min. The buffers ($\mu$ = 0.1 M, adjusted with KCl) were 10 mM citrate (pH 4.0−5.0); 10 mM acetate (pH 5.5−6.0); 10 mM phosphate (pH 7.0−8.5); 10 mM CHES (pH 9.0−10.0), and 10 mM CAPS (pH 10.5−11.0). The p$K_a$ value was obtained by fitting the observed values of the absorbance at 295 nm ($A$) as a function of pH using eq 1, where $a_H$ is the activity of hydronium ion, $A_i$ is the absorbance of the solution at $a_H$ = 0, $A_f$ is the absorbance of the solution at $a_H$ = infinity, and $K_E$ is the acid dissociation constant.

$$A = (A_f K_E + A_i a_H)/(K_E + a_H) \quad (1)$$

*Binding constants of equilenin* (**5**) *to D38A, D38A/Y14F, D38A/Y55F, and D38A/D99N* were determined in 2.0 mL of 10 mM potassium phosphate buffer (MeOH 3.3%, pH 7.0) at 25.0 ± 0.1 °C, with 100 nM **5** and 0−100 nM protein (D38A), 2 $\mu$M **5**, and 0−8.7 $\mu$M protein (D38A/Y55F and D38A/D99N), or 1 $\mu$M **5** and 0−15 $\mu$M protein (D38A/Y14F) from the variation of fluorescence intensity as a function of protein concentration (*22*). Static fluorescence excitation and emission spectra were determined with a Fluoromax-2 spectrofluorometer. Dissociation constants ($K_D$) were obtained from fitting the data to eq 2, where $F$ is the fluorescence intensity at 364 nm, $F_0$ is the intensity of the fluorescence of **5** in the absence of protein, $F_{inf}$ is the intensity extrapolated to infinite concentration of protein, $c$ is the enzyme concentration, and [**5**] is the fixed concentration of **5**.

$$F = (F_0 - c)\{K_D/(c - F_{inf}) + [\mathbf{5}]/(F_0 - F_{inf})\} \quad (2)$$

*Fluorescence excitation spectra of equilenin* (**5**) were recorded at an emission wavelength of 400 nm. Solutions of **5** (5 $\mu$M, 3.3% MeOH) were prepared in 10 mM potassium phosphate buffer (pH 7.0) and 10 mM CAPS buffer (pH 11.0). The complexes of **5** with the mutant proteins were prepared in 10 mM potassium phosphate buffer (pH 7.0, 3.3% MeOH). For all the complexes, the concentration of equilenin was 10 $\mu$M, with enzyme concentrations of 10 $\mu$M (D38A), 139 $\mu$M (D38A/Y14F), or 24 $\mu$M (D38A/Y55F). Spectra were obtained at 25.0 ± 0.1 °C and were corrected for the fluorescence of buffer, protein, and unbound **5**.

## RESULTS

*Mutagenesis and Protein Expression.* The D38A, D38A/Y14F, D38A/Y55F, and D38A/D99N mutants of KSI were prepared using the QuikChange site-directed mutagenesis kit. The entire D38A, D38A/Y55F, and D38A/D99N genes were sequenced and shown to contain only the desired mutations; the D38A/Y14F gene also contained an additional silent mutation in the codon for Gln-12 (CA<u>A</u>). The four proteins were expressed in *Escherichia coli* (XL1-Blue), and purified to homogeneity, as determined by SDS−PAGE stained with Coomassie Blue R-250. The UV spectra of both D38A and D38A/D99N show the characteristic "hand" shape observed for native KSI, with a principal maximum at 277 nm and a minimum at 250 nm (*2*), whereas the UV spectra of D38A/Y14F and D38A/Y55F are similar to those previously observed for Y14F and Y55F, respectively (*2*). The far-UV CD spectra of D38A, D38A/Y14F, D38A/Y55F, and D38A/D99N are identical to those of WT and D99N and show a minimum at 222 nm.

*Kinetic Parameters.* Specific activities for WT (55 000 $\mu$mol $min^{-1}$ $mg^{-1}$) and D38A (600 $\mu$mol $min^{-1}$ $mg^{-1}$) were determined in 34 mM potassium phosphate buffer at pH 7.0, with 5-androstene-3,17-dione (**1**) as substrate. The D38A mutant possesses considerable activity toward **1** (ca. 1% of WT), and this activity is independent of the phosphate buffer concentration (10 or 34 mM). The double mutants D38A/Y55F, D38A/Y14F, and D38A/D99N have no detectable catalytic activity, even when 3 $\mu$M enzyme is used in the assay. From this result, an upper limit of $2.0 \times 10^{-4}$ $\mu$mol $min^{-1}$ $mg^{-1}$ can be calculated for the specific activity of these mutants, ca. ($3.0 \times 10^8$)-fold less than WT. Moreover, under assay conditions with D38A/D99N concentrations comparable to those of the substrate **1**, there is no spectral evidence for the formation of an enzyme-bound dienol(ate) intermedi-

Table 2: Solvent Isotope Effects on the Kinetic Constants for the Isomerization of 5-Androstene-3,17-dione (**1**) to 4-Androstene-3,17-dione (**3**) by WT and D38A, at 25.0 ± 0.1 °C

| KSI | solvent | pL[a] | $K_M$ ($\mu$M) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_M$ ($s^{-1}$ $M^{-1}$) | $^{H/D}(K_M)$ | $^{H/D}(k_{cat})$ | $^{H/D}(k_{cat}/K_M)$ |
|---|---|---|---|---|---|---|---|---|
| WT[b] | $H_2O$ | 7.5 | 285 | $5.3 \times 10^4$ | $1.85 \times 10^8$ | 1.38 | 1.6 | 1.15 |
| | $D_2O$ | | 206 | $3.3 \times 10^4$ | $1.6 \times 10^8$ | | | |
| D38A | $H_2O$ | 6.5 | 99 | 340 | $3.4 \times 10^6$ | 1.47 | 1.47 | 1.0 |
| | $D_2O$ | | 67 | 230 | $3.4 \times 10^6$ | | | |
| | $H_2O$ | 7.5 | 96 | 412 | $4.3 \times 10^6$ | 1.13 | 1.58 | 1.43 |
| | $D_2O$ | | 85 | 260 | $3.0 \times 10^6$ | | | |
| | $H_2O$ | 8.5 | 94 | 412 | $4.4 \times 10^6$ | 1.28 | 1.48 | 1.15 |
| | $D_2O$ | | 73 | 279 | $3.8 \times 10^6$ | | | |
| | | average | | | | 1.3 ± 0.2 | 1.5 ± 0.1 | 1.2 ± 0.2 |

[a] L = H or D. [b] Data from Xue et al. (*24*).

Table 3: Kinetic Constants for the Isomerization of 5-Androstene-3,17-dione (**1**) to 4-Androstene-3,17-dione (**3**) by WT and D38X Mutant KSIs

| enzyme | $K_M$ ($\mu$M) | limiting $k_{cat}$ ($s^{-1}$) | limiting $k_{cat}/K_M$ ($s^{-1}$ $M^{-1}$) | p$K_{E1}$ | p$K_{E2}$ | p$K_{ES1}$ | p$K_{ES2}$ |
|---|---|---|---|---|---|---|---|
| WT[a] | 240 | $5.7 \times 10^4$ | $2.3 \times 10^8$ | 4.57[b] | >9[c] | 4.75[b] | 9.3[c] |
| D38E[d] | 73 | 210 | $2.4 \times 10^6$ | 4.65 | ≥9.5 | 6.13 | 8.83 |
| D38C[d] | 21 | 15.7 | $1.2 \times 10^7$ | 8.46 | ≥9.5 | 8.27 | ≥9.5 |
| D38H[d] | 43 | 564 | $1.3 \times 10^7$ | 4.37 | 8.5 | 3.41 | 7.52 |
| D38A[e] | 122 | 400 ± 20 | $3.0 (\pm 0.2) \times 10^6$ | 4.2 ± 0.1 | 10.4 ± 0.1 | 4.5 ± 0.1 | ≥10.5 |

[a] Data from Pollack et al. (*33*). [b] p$K$ values for WT were determined with the nonsticky substrate 5(10)-estrene-3,17-dione (**4**). [c] From data of Pollack et al. (*33*) and Weintraub et al. (*53*). [d] Data from Holman and Benisek (*23*). [e] Errors are standard deviations.

ates, whereas under the same conditions the D38N mutant catalyzes the conversion of **1** to a tightly bound dienolate (*22*).

As with WT and the other Asp-38 mutants (D38X), such as D38E, D38C, and D38H (*23*), D38A is able to catalyze the isomerization of both **1** and **4**. Kinetic parameters for the isomerization of **1** and of **4** were determined in 34 mM phosphate buffer (pH 7.0) at 25.0 ± 0.1 °C. Linear kinetics were observed through the first 5−10% of the reactions, and the first 2−3% of the reactions were used to determine initial rates. The values of $k_{cat}$ and $k_{cat}/K_M$ for isomerization of **1** are 460 ± 50 $s^{-1}$ and 2.8 (± 0.2) × $10^6$ $M^{-1}$ $s^{-1}$, respectively, and the values of $k_{cat}$ and $k_{cat}/K_M$ for isomerization of **4** are 0.77 (± 0.08) $s^{-1}$ and 1.19 (± 0.01) × $10^4$ $M^{-1}$ $s^{-1}$, respectively. Rates of isomerization of **1** by D38A were obtained in both $H_2O$ and $D_2O$ at pL (L = H or D) 6.5, 7.5, and 8.5 in 10 mM phosphate buffer at 25.0 ± 0.1 °C (Table 2). The solvent isotope effect on the kinetic parameters of D38A is independent of the pL between 6.5 and 8.5, with $^{H/D}(k_{cat}/K_M) = 1.3 \pm 0.2$, $^{H/D}(k_{cat}) = 1.5 \pm 0.1$, and $^{H/D}(K_M) = 1.2 \pm 0.2$. These values are similar to those previously observed with WT at pH 7.5 (*24*).

The pH dependence of $k_{cat}$ and $k_{cat}/K_M$ for the isomerization of **1** by D38A was determined at 25.0 ± 0.1 °C and constant ionic strength ($\mu$ = 0.1 M) from weighted least-squares analysis of plots of $1/v$ vs 1/[**1**] at each pH. Over the pH range investigated (~3.5 to ~11.0), the enzyme is stable for the time required to make the rate measurements (<1 min). Since $k_{cat}/K_M$ for D38A is at least an order of magnitude lower than $k_{cat}/K_M$ for WT, which reacts at nearly the diffusion-controlled rate with **1**, it can be assumed that **1** is not a sticky substrate for D38A. Thus, plots of $(k_{cat}/K_M)^{obs}$ vs pH and $(k_{cat})^{obs}$ vs pH should provide p$K_a$ values for the free enzyme (p$K_E$) and the enzyme−substrate complex (p$K_{ES}$), respectively (*25, 26*). The observed kinetic parameters were fit to eqs 3 and 4 (*27*) to give the kinetic constants and the p$K_a$ values, which are given in Table 3, along with values

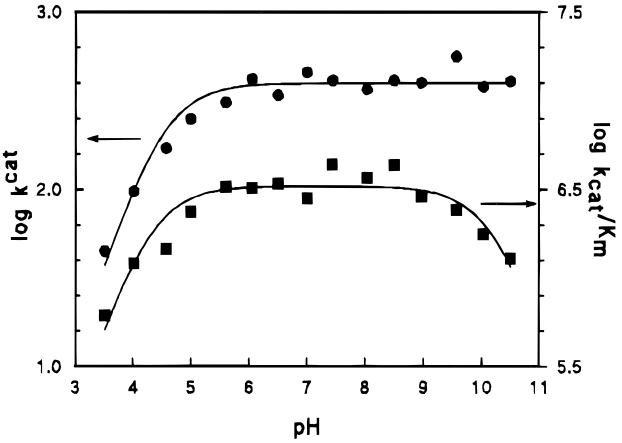



FIGURE 1: Plots of log $(k_{cat})^{obs}$ (●) and log $(k_{cat}/K_M)^{obs}$ (■) for the isomerization of 5-androstene-3,17-dione (**1**) catalyzed by D38A as a function of pH at 25 °C. The curves are theoretical, based on eqs 3 and 4 and the parameters given in Table 3.

determined previously for WT and other D38X mutants.

$$(k_{cat}/K_M)^{obs} = (k_{cat}/K_M)/\{1 + ([H^+]/K_{E1}) + (K_{E2}/[H^+])\} \quad (3)$$

$$(k_{cat})^{obs} = k_{cat}/(1 + [H^+]/K_{ES1}) \quad (4)$$

The limiting values for D38A of $k_{cat}$ (400 ± 20 $s^{-1}$) and $k_{cat}/K_M$ [3.0 (± 0.2) × $10^6$ $M^{-1}$ $s^{-1}$] represent a decrease relative to WT by ca. 140-fold and 75-fold, respectively. The log $(k_{cat}/K_M)$ vs pH profile (Figure 1) shows a limiting slope of +1 on the acidic side with an apparent p$K_{E1}$ value of 4.2 ± 0.1, and a limiting slope of −1 on the basic side with an apparent p$K_{E2}$ value of 10.4 ± 0.1. The log $(k_{cat})$ vs pH curve (Figure 1) exhibits dependence upon a single p$K_a$ (p$K_{ES1}$ 4.5 ± 0.1) at low pH.

*Native Gel Electrophoresis*. The relative charges of WT and mutants lacking Asp-38 and/or Asp-99 were determined by native gel electrophoresis at pH 7.0 (Figure 2) and 8.2.

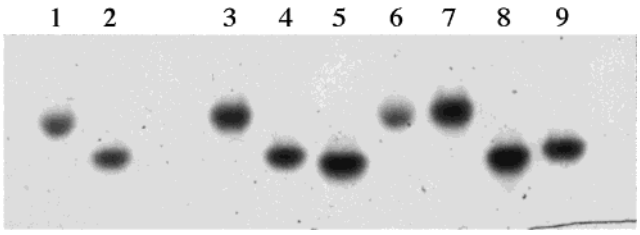



FIGURE 2: Native gel electrophoresis of WT and mutant KSI proteins at pH 7.0. The direction of migration is toward the anode at the bottom of the gel. WT is in lanes 2, 5, and 9; D38A is in lanes 4 and 8; D38N/D99A is in lanes 1 and 7; and D38A/D99N is in lanes 3 and 6.

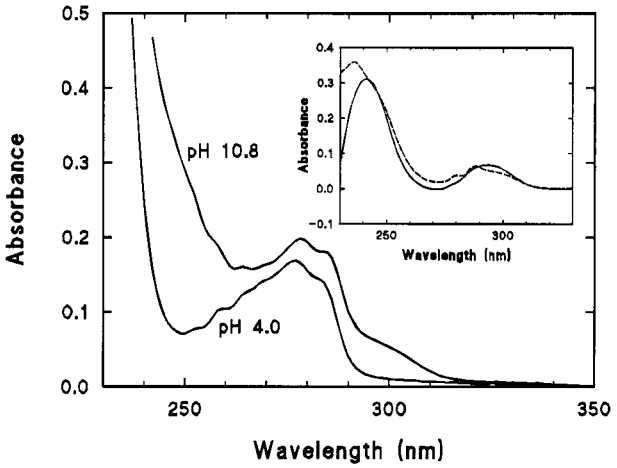



FIGURE 3: Absorbance spectra of D38A (38 $\mu$M) at pH 4.0 (10 mM citrate) and 10.8 (10 mM CAPS). Inset: Dashed line, difference spectra of D38A at pH 10.8 and pH 4.0); solid line, difference spectra of tyrosine at pH 10.8 and pH 4.0. Both concentrations are 38 $\mu$M.

At both pH values, D38A migrates similarly to WT, whereas the D38A/D99N double mutant migrates with D38N/D99A.

*UV Titration of D38A.* UV spectra of D38A were recorded between 240 and 320 nm from pH 4.0 to 11.0, a pH range in which the protein is stable. An increased pH has two major effects on the spectrum of D38A, which are similar to those observed during the UV-monitored pH titration of WT and D38H (*23*). The absorbance at 240 nm increases strongly, and a red shift of 2 nm is observed for peaks between 240 and 320 nm (Figure 3). These changes in the spectra are reversible; after acidification of a solution of pH 10.0 to either pH 6.0 or 7.0, the spectrum does not differ from the spectrum at pH 7.0. The change of absorbance at 295 nm, indicative of tyrosine ionization ($\epsilon$ = 2540 $M^{-1}$ $cm^{-1}$; *28*), shows sigmoidal behavior as a function of pH (Figure 4). A p$K_a$ value of 10.0 ± 0.4 was obtained after the data were fitted to eq 1.

*Equilenin Binding.* The dissociation constants ($K_D$) of the complexes of equilenin (**5**) with each of the four mutants, determined by fluorescence titration with the appropriate enzyme, at pH 7.0 (3.3% MeOH), are 6 ± 1 nM for D38A, 4 ± 1 $\mu$M for D38A/Y14F, 204 ± 3 nM for D38A/Y55F, and 390 ± 80 nM for D38A/D99N. Figure 5 shows the excitation spectra for **5** in buffers at pH 7.0 and 11.0, as well as the spectra of **5** complexed to D38A, D38A/Y14F, and D38A/Y55F at pH 7.0. Under the conditions used for this experiment, >97% of **5** is bound to the D38A mutants, as calculated from the $K_D$ values.

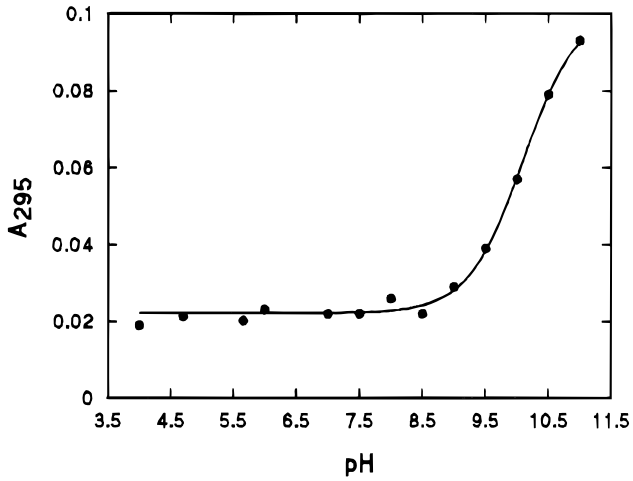



FIGURE 4: Absorbance of D38A (38.5 $\mu$M) at 295 nm as a function of pH. The data were fit to eq 1 and give a p$K_E$ value of 10.0 ± 0.4.

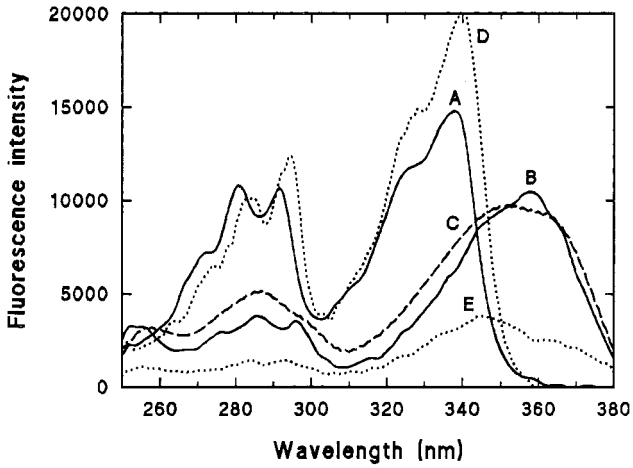



FIGURE 5: Fluorescence excitation spectra of equilenin (5 $\mu$M) at pH 7.0 (solid line A) and pH 11.0 (solid line B), along with the corresponding spectra for the complexes of equilenin (10 $\mu$M) with D38A (10 $\mu$M, dashed line C), D38A/Y14F (139 $\mu$M, dotted line D), and D38A/Y55F (24 $\mu$M, dotted line E), in 10 mM phosphate buffer, pH 7.0 (ca. 3.3% methanol).

## DISCUSSION

*Properties of the D38A Mutants.* To compare the kinetic behavior of a series of mutants, it is necessary to consider whether structural modifications have occurred within the series. The similarity of the UV spectra of the KSI mutants with the UV spectra of either WT, Y14F, or Y55F and the similarity of the far-UV CD spectra of WT, D99N, D38A, D38A/Y14F, D38A/Y55F, and D38A/D99N indicate that these mutations do not significantly modify the secondary structure of the proteins. In addition, the ability of the intermediate analogue equilenin (**5**) to bind to D38A, D38A/Y14F, D38A/Y55F, and D38A/D99N suggests that the binding sites of all the mutants are intact.

Activity due to contamination by small amounts of WT KSI can be excluded because (1) there are differences in $K_M$ between D38A and WT, and $K_M$ is a function only of the identity of the catalyst; (2) the activity of D38A differs markedly from that of WT at high pH; and (3) D38A has a different behavior than WT toward several 3$\beta$- and 17$\beta$-oxiranyl steroids. These 3$\beta$- and 17$\beta$-oxiranyl steroids are active-site-directed alkylating agents that react specifically

with Asp-38 in WT (*29*−*31*), whereas kinetic experiments and mass spectrometry analysis show that the inhibition of D38A is a more complicated process with at least a two-step time-dependent inhibition, which results in a noncovalent enzyme−inhibitor complex (F. Hénot, D. Fabris, and R. M. Pollack, unpublished results).

*Identification of the Catalytic Residues in the D38A Mutant.* The generally accepted mechanism for KSI (Scheme 1) involves Asp-38 acting to transfer a proton from C-4 to C-6. The most critical piece of data implicating Asp-38 as the base was provided by Mildvan's group (*2, 15*), who demonstrated that the D38N mutant is about $10^8$-fold less active than the wild-type enzyme for the specific substrate 5-androstene-3,17-dione (**1**). Other D38X mutants with residues at position 38 that could substitute for Asp-38 as a base have been shown to be substantially more active than D38N. Thus, D38E, D38C, and D38H all have activities ($k_{cat}/K_M$) ranging from 1% to 5.6% that of WT (*23*), as shown in Table 3. The pH−rate profiles for WT (*32, 33*) and several mutants (D38E, D38C, D38H, D99A, and D99N) (*11, 23*) can be interpreted as a dependence of the activity on the deprotonated form of the residue at position 38 (Table 3). For both WT and D38E, p$K_a$ values between 4 and 5 are observed, as expected for Asp and Glu carboxylates. For the Cys and His mutants (D38C, D38H), the observed values are 8.46 and 4.37, respectively, consistent with ionization of an SH (D38C) or a buried imidazole (D38H). The slope of the acid limb of these plots of +1 indicates the intervention of only one basic residue for all mutants.

Extrapolation of these results would suggest that replacement of Asp-38 by Ala should lead to an inactive (or at least greatly maimed) enzyme. Thus, it is surprising to find that D38A has a relatively high activity, with a $k_{cat}$ value ca. $10^6$-fold greater than D38N and only about 140-fold less than WT. Furthermore, the pH−rate profiles for both $k_{cat}/K_M$ and $k_{cat}$ of D38A (Figure 1) still exhibit the acid limb with a p$K_a$ of 4.2, seemingly inconsistent with the generally accepted assignment of Asp-38 as catalytic base in WT. These results suggest that there is a new mechanism for D38A, in which another residue acts as the base.

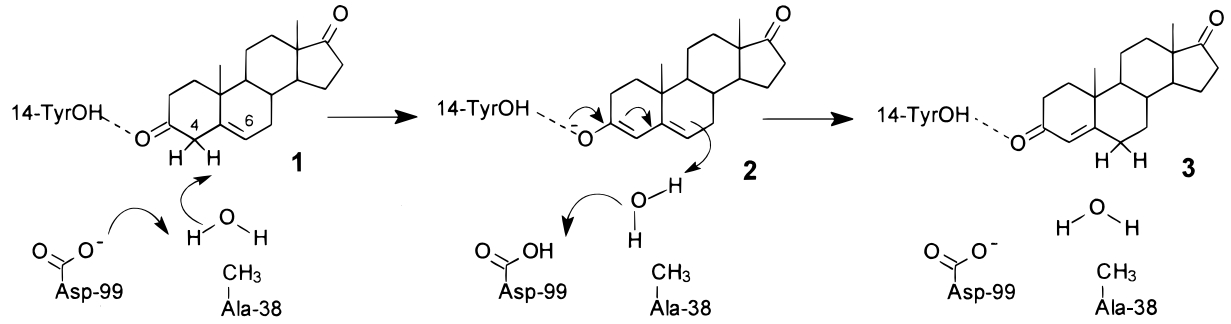
Precedent for a different mechanism comes from several previous examples of catalytic rescue in mutant proteins by another residue of the active site. In ribonuclease T1, protonated His-40 participates in electrostatic stabilization of the transition state but becomes the general base when the catalytic base Glu-58 of WT is replaced by alanine (*34*). In the E177A mutant of ricin A, the noncatalytic residue Glu-208 substitutes for the carboxylate of Glu-177 of WT to stabilize the transition-state oxycarbonium ion (*35*). Finally, in the H256A mutant of fructose-2,6-biphosphatase, His-390 takes over the function of the wild-type nucleophile His-256 and may activate a water molecule for hydrolysis of the substrate (*36*).

The recent elucidation of the structure of WT KSI, coupled with kinetic studies, has revealed that only four potential functional groups are present at the active site: Tyr-14, Asp-38, Tyr-55, and Asp-99 (*2, 10, 13, 37*). Three of these (Tyr-14, Asp-38, and Asp-99) have been implicated in the mechanism, but mutation of Tyr-55 to phenylalanine has only a modest (4-fold) effect on the catalytic ability of the enzyme (*2*). In the absence of Asp-38, the most reasonable candidate for the catalytic residue having a p$K_a$ value of 4.2 in D38A is Asp-99, although this residue has a p$K_a$ value of ≥9.5 in the wild-type enzyme (*11*). To examine the possibility that the p$K_a$ of Asp-99 is perturbed to 4.2 in D38A, the relative charges of D38A and WT were determined by native gel electrophoresis. At pH values of 7.0 (Figure 2) and 8.2 (data not shown), D38A comigrates with WT, indicating no difference in the net charge. Since Asp-38 is ionized under these conditions in WT (*11*), these results are consistent with the ionization of a residue in D38A that is not ionized in WT. To determine whether Asp-99 is this ionizing residue, the migration of D38A/D99N was compared to that of WT. D38A/D99N does not comigrate with WT but instead migrates with D38N/D99A, a mutant with one less negative charge than WT. Hence, the p$K_a$ of ca. 4.2 in the pH−rate profile of D38A is likely due to the negative charge of ionized Asp-99. The large decrease in p$K_a$ of Asp-99 in D38A relative to WT may be rationalized by the effect of Coulombic interactions that can affect the ionization of active-site residues (*38*−*41*). Loss of the strong electrostatic interaction due to the negative charge of Asp-38 located about 4 Å from Asp-99, and a probable increase of the polarity of the active site (replacement of Asp by Ala generates a cavity, which may be filled by water molecules), could significantly lower the p$K_a$ of Asp-99. In this context, replacement of Asp-38 of KSI by Asn (D38N) or by His (D38H) results in a weaker electrostatic interaction, reducing the p$K_a$ of Asp-99 from ≥9.5 to ∼8.0 (*11, 23*). A similar effect is seen in other enzymes, such as xylanase of *Bacillus circulans*, in which substitution of Glu-78 in WT by Gln decreases the p$K_a$ of Glu-172 from 6.7 to 4.2 (*42*). The high p$K_a$ of Glu-172 in the WT is likely caused by the electrostatic repulsion between the two buried and distant (6.5 Å from $C^\delta$ to $C^\delta$) carboxylic groups, which is eliminated upon mutation. A weak electrostatic interaction of Glu-35 of hen egg white lysozyme with Asp-52, located about 7 Å away, may account for the increase in the p$K_a$ of Glu-35 to ∼ 6.2, and this p$K_a$ is decreased by more than one pH unit when Asp-52 is mutated to Asn (*43, 44*). Last, His-373 has a p$K_a$ ∼15 in the reduced flavocytochrome *b*2 because of electrostatic interactions with Asp-282 and the anionic reduced cofactor (*45, 46*).

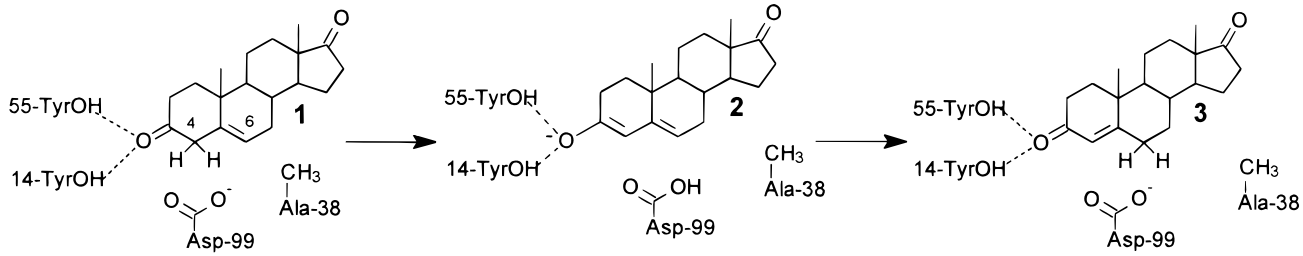
The possible involvement of Asp-99 in catalysis was investigated by introducing a second mutation (Asn-99) into D38A that eliminates the carboxyl group of Asp-99 (D38A/D99N). Asn preserves the side chain volume of Asp but does not ionize at neutral pH. If Asp-99 is the catalytic base in D38A, then the D38A/D99N mutant should show little activity. However, if Asp-99 is not ionized in D38A and acts as a hydrogen-bond donor, as in the wild type, a much more modest decrease in $k_{cat}$ should be observed. Thus, $k_{cat}$ for D99N is decreased by a factor of about 25-fold relative to WT (*11*), reflecting the weaker ability of Asn to donate a hydrogen-bond compared to a carboxylic group. The lack of detectable activity for the D38A/D99N double mutant, corresponding to a decrease in $k_{cat}$ of ≥$10^6$-fold relative to D38A, is consistent with Asp-99 being the catalytic base in the D38A mutant, rather than a hydrogen-bond donor.

An additional catalytic residue is implicated by the dependence of $k_{cat}/K_M$ on a group with a p$K_E$ of 10.4. This p$K_a$ may be assigned to an active-site tyrosine residue (Tyr-14 or -55) based upon the difference spectrum of D38A at pH 10.8 and 4.0, which resembles the difference spectrum

Scheme 2




Scheme 3




of the tyrosine anion and tyrosine itself (Figure 3, inset). Analysis of the absorbance of D38A at 295 nm as a function of pH from 4.0 to 11.0 (Figure 4) gives a p$K_a$ of 10.0 ± 0.4, consistent with the titration of a tyrosine group. From this titration, an extinction coefficient of ca. 2600 $M^{-1}$ $cm^{-1}$ was calculated for the anion, in good agreement with the literature value for tyrosine of 2540 $M^{-1}$ $cm^{-1}$ (*28*), suggesting that one tyrosine titrates in this region. The lack of any observable ionization of tyrosine at lower pH values rules out assignment of the low p$K$ in both the pH−rate profile and the native gel electophoresis to a tyrosine residue.

*Mechanism of the D38A Mutant of KSI.* Although the above discussion implicates Asp-99 as the base, in the 2.26 Å X-ray KSI structure of D38N KSI complexed with the bound intermediate analogue equilenin (*13*), Asp-99 is too far from C-6 to act directly to abstract a proton from C-4 and subsequently return it to C-6. However, Asp-99 could act as a general base to deprotonate a water molecule, which in turn could enolize **1**, with reketonization of **2** by protonation of C-6 through the water molecule (Scheme 2). To examine this possibility, solvent isotope effects on both $k_{cat}/K_M$ and $k_{cat}$ were determined at pH 6.5, 7.5, and 8.5. The observed solvent isotope effect on $k_{cat}$ (1.5 ± 0.1) at all three pHs is similar to that for WT (Table 2) and substantially lower than the effect expected for a proton transfer to/from a water molecule in a rate-limiting step.

Alternatively, binding of substrate to D38A could occur in a manner that is different from that with WT and D38N. Mutation of Asp-38 or Asn-38 to Ala produces a larger cavity, which might allow the steroid to go more deeply into the catalytic site, enabling deprotonated Asp-99 to act *directly* as a base. In this model, stabilization of the intermediate and the flanking transition states is provided by hydrogen bonding from Tyr-14 and/or Tyr-55 (Scheme 3), consistent with the second p$K_a$ of ca. 10.4 in the pH−rate profile. This p$K_a$ value is ca. 1.6 pH unit lower than the p$K_a$ value of Tyr-14 determined previously for the Y55F/Y88F mutant (*47*). As expected for a neutral acid (tyrosine), the p$K_a$ is shifted to a higher value in the enzyme−substrate complex, due to increased hydrophobicity of the catalytic site upon steroid binding.

Support for this hypothesis is provided by the lack of detectable activity in either of the two double mutants D38A/Y14F and D38A/Y55F, which both show decreased activity of $10^6$-fold or more relative to D38A. This result is reminiscent of the decreased activity of the Y14F mutant relative to WT of ca. $10^5$-fold (*2*), which supports the hydrogen-bonding role of this residue in WT. Thus, both Tyr-14 and Tyr-55 are important for catalysis in the D38A mutant, in contrast to WT, in which Tyr-55 gives only a minor contribution to catalytic activity (*2*). These results are consistent with a binding mode in D38A that involves hydrogen bonding from both Tyr-14 and Tyr-55.

The mode of binding and ionization state of the intermediate can be investigated by the change in fluorescence when the intermediate analogue equilenin (**5**) binds to the active site (*48*, *49*). The intrinsic fluorescence of WT ($\lambda_{max}$ 305 nm) arises almost exclusively from its three tyrosines (Tyr-14, Tyr-55, and Tyr-88), with Tyr-14 having the highest quantum yield (*50*). The fluorescence emission spectra of D38A, D38A/Y14F, D38A/Y55F, and D38A/D99N also show maxima at 305 ± 1 nm. Addition of equilenin significantly decreases the intensity of this peak in a concentration-dependent manner for the D38A mutants, similar to what has been observed for WT (*48*, *49*) and D38N (*7*, *22*), indicative of binding of **5** to the catalytic site of these mutants. The D38A mutant binds equilenin strongly ($K_D$ ca. 6 nM), and the similarity of the excitation spectrum of **5** bound to D38A at pH 7.0 with the spectrum of uncomplexed **5** at pH 11.0 (Figure 5) is consistent with ionization of the equilenin hydroxyl in this complex.

A second mutation of either Tyr-14 or Tyr-55 to phenylalanine results in a less stable complex with equilenin (by ca. 700-fold for D38A/Y14F and by 34-fold for D38A/Y55F), but the effect is substantially less than that for the loss of activity by these same mutations (≥$10^6$-fold in each case). In the case of D38A/Y14F, the complex with **5** gives an excitation spectrum that bears a close resemblance to *un-*

*ionized* **5**, whereas for the D38A/Y55F·**5** complex, the spectrum indicates a mixture of both ionized and un-ionized **5** (Figure 5). Interpretation of these data is complicated by the fact that **5** can bind in two modes to KSI, one of which resembles the intermediate and one that involves backward binding with the D ring near the catalytic residues (*51*). If the fluorescence spectrum of the complex resembles ionized **5**, it is likely that the ligand is bound in an analogous manner to the intermediate, with hydrogen bonding to the anionic oxygen. However, if the fluorescence spectrum resembles un-ionized **5**, no conclusions can be drawn about the mode of binding. Thus, the 700-fold decrease in the binding of **5** to D38A/Y14F relative to D38A, coupled with a spectrum of the complex that resembles un-ionized **5**, is consistent with an important role for Tyr-14 in the binding of the anion of **5** and presumably the dienolate intermediate. In contrast, the minimal loss of binding affinity of D38A/Y55F for **5** compared to D38A, along with a spectrum that shows some dienolate character, would appear to be incompatible with a direct hydrogen-bonding role for Tyr-55. A likely explanation for this apparent inconsistency is that **5** can bind to D38A/Y55F (and possibly to D38A) as it does to WT, with the two hydrogen bonds being provided by a protonated Asp-99 and Tyr-14. Thus, the mode of binding of equilenin to D38A and its mutants may be different from the catalytically important complex with the dienolate ion.

A comparison of the proposed mechanism for D38A (Scheme 3) with the mechanism of WT (Scheme 1) shows that the same motif is utilized for both enzymes. In each case, the carboxylate of an aspartic acid transfers a proton from C-4 to C-6, and the intermediate dienolate ion is stabilized by direct hydrogen bonds from two residues (Asp-99/Tyr-14 for WT and Tyr-14/Tyr-55 for D38A). Given the same general mechanism for the two enzymes, it is of interest to consider the reasons for the disparity in activity. Perhaps the carboxylate of Asp-99 in D38A is not in the ideal position for proton abstraction/donation. In this vein, the D38E mutant of KSI, in which the COOH group of residue 38 has been moved ca. 1 Å relative to WT, shows a 100-fold decrease in catalytic activity (*52*). Alternatively, Tyr-14 and Tyr-55 in D38A may not be optimally positioned for stabilization of the intermediate dienolate ion by hydrogen bonding.

## REFERENCES


1. Hawkinson, D. C., Eames, T. C. M., and Pollack, R. M. (1991) *Biochemistry 30*, 10849−10858.
2. Kuliopulos, A., Mildvan, A. S., Shortle, D., and Talalay, P. (1989) *Biochemistry 28*, 149−159.
3. Benisek, W. F., Ogez, J. R., and Smith, S. B. (1980) *Ann. N.Y. Acad. Sci. 346*, 115−130.
4. Zawrotny, M. E., Hawkinson, D. C., Blotny, G., and Pollack, R. M. (1996) *Biochemistry 35*, 6438−6442.
5. Zeng, B., and Pollack, R. M. (1991) *J. Am. Chem. Soc. 113*, 3838−3842.
6. Zeng, B., Bounds, P. L., Steiner, R. F., and Pollack, R. M. (1992) *Biochemistry 31*, 1521−1528.
7. Petrounia, I., and Pollack, R. M. (1998) *Biochemistry 37*, 700−705.
8. Zhao, Q., Li, Y.-K., Mildvan, A. S., and Talalay, P. (1995) *Biochemistry 34*, 426−434.
9. Zhao, Q., Abeygunawardana, C., Talalay, P., and Mildvan, A. S. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 8220−8224.
10. Wu, Z. R., Ebrahimian, S., Zawrotny, M. E., Thornburg, L. D., Perez-Alvarado, G. C., Brothers, P., Pollack, R. M., and Summers, M. F. (1997) *Science 276*, 415−418.
11. Thornburg, L. D., Hénot, F., Bash, D. P., Hawkinson, D. C., Bartel, S. D., and Pollack, R. M. (1998) *Biochemistry 37*, 10499−10506.
12. Zhao, Q., Abeygunawardana, C., Gittis, A. G., and Mildvan, A. S., (1997) *Biochemistry 36*, 14616−14626.
13. Cho, H.-S., Ha, N.-C., Choi, G., Kim, H.-J., Lee, D., Oh, K.-S., Kim, K. S., Lee, W., Choi, K. Y., and Oh, B.-H. (1999) *J. Biol. Chem. 274*, 32863−32868.
14. Pollack, R. M., Thornburg, L. D., Wu, Z. R., and Summers, M. F. (1999) *Arch. Biochem. Biophys. 370*, 9−15.
15. Xue, L. A., Kuliopulos, A., Mildvan, A. S., and Talalay, P. (1991) *Biochemistry 30*, 4991−4997.
16. Pollack, R. M., Zeng, B., Mack, J. P. G., and Eldin, S. (1989) *J. Am. Chem. Soc. 111*, 6419−6423.
17. Brooks, B., and Benisek, W. F. (1992) *Biochem. Biophys. Res. Commun. 184*, 1386−1392.
18. Laemmli, U. K. (1970) *Nature 227*, 680−685.
19. Kawahara, F. S., Wang, S.-F., and Talalay, P. (1962) *J. Biol. Chem. 237*, 1500−1506.
20. Schowen, K. B., and Schowen, R. L. (1982) *Methods Enzymol. 87c*, 551−606.
21. Kuliopulos, A., Talalay, P., and Mildvan, A. S. (1990) *Biochemistry 29*, 10271−10280.
22. Hawkinson, D. C., Pollack, R. M., and Ambulos, N. P., Jr. (1994) *Biochemistry 33*, 12172−12183.
23. Holman, C. H., and Benisek, W. F. (1995) *Biochemistry 34*, 14245−14253.
24. Xue, L., Talalay, P., and Mildvan, A. S. (1990) *Biochemistry 29*, 7491−7500.
25. Fersht, A. R. (1984) *Enzyme Structure and Mechanism*, 2nd ed., W. H. Freeman, New York.
26. Knowles, J. R. (1976) *Crit. Rev. Biochem. 4*, 165−173.
27. Cleland, W. W. (1977) *Adv. Enzymol. Relat. Areas Mol. Biol. 45*, 273−388.
28. Nilsson, A., and Lindskog, S. (1967) *Eur. J. Biochem. 2*, 309−317.
29. Pollack, R. M., Kayser, R. H., and Bevins, C. L. (1979) *Biochem. Biophys. Res. Commun. 91*, 783−790.
30. Bevins, C. L., Bantia, S., Pollack, R. M., Bounds, P. L., and Kayser, R. H. (1984) *J. Am. Chem. Soc. 106*, 4957−4962.
31. Bounds, P. L., and Pollack, R. M. (1987) *Biochemistry 26*, 2263−2269.
32. Weintraub, H., Alfsen, A., and Baulieu, E.-E. (1970) *Eur. J. Biochem. 12*, 217−221.
33. Pollack, R. M., Bantia, S., Bounds, P. L., and Koffman, B. D. (1986) *Biochemistry 25*, 1905−1911.
34. Steyaert, J., Hallenga, K., Wyns, L., and Stanssens, P. (1990) *Biochemistry 29*, 9064−9072.
35. Kim, Y., Misna, D., Monzingo, A. F., Ready, M. P., Frankel, A., and Robertus, J. D. (1992) *Biochemistry 31*, 3294−3296.
36. Mizuguchi, H., Cook, P. F., Tai, C.-H., Hasemann, C. A., and Uyeda, K. (1999) *J. Biol. Chem. 274*, 2166−2175.
37. Cho, H.-S., Choi, G., Choi, K. Y., and Oh, B.-H. (1998) *Biochemistry 37*, 8325−8330.
38. Sternberg, M. J. E., Hayes, F. R. F., Russel, A. J., Thomas, P. G., and Fersht, A. R. (1987) *Nature 330*, 86−88.
39. Jackson, S. E., and Fersht, A. R. (1993) *Biochemistry 32*, 13909−13916.
40. Loewenthal, R., Sancho, J., Reinikainen, T., and Fersht, A. R. (1993) *J. Mol. Biol. 232*, 574−583.
41. Nakamura, H. (1996) *Q. Rev. Biophys. 29*, 1−90.
42. McIntosh, L. P., Hand, G., Johnson, P. E., Joshi, M. D., Korner, M., Plesniak, L. A., Ziser, L., Wakarchuk, W. W., and Withers, S. G. (1996) *Biochemistry 35*, 9958−9966.
43. Inoue, M., Yamada, H., Yasukochi, T., Kuroki, R., Miki, T., Horiuchi, T., and Imoto, T. (1992) *Biochemistry 31*, 5545−5553.
44. Bartik, K., Redfield, C., and Dobson, C. M. (1994) *Biophys. J. 66*, 1180−1184.
45. Lederer, F. (1992) *Protein Sci. 1*, 540−548.
46. Gondry, M., and Lederer, F. (1996) *Biochemistry 35*, 8587−8594.

47. Li, Y. K., Kuliopulos, A., Mildvan, A. S., and Talalay, P. (1993) *Biochemistry 32*, 1816−1824.
48. Wang, S. F., Kawahara, F. S., and Talalay, P. (1963) *J. Biol. Chem. 238*, 576−585.
49. Eames, T. C., Pollack, R. M., and Steiner, R. F. (1989) *Biochemistry 28*, 6269−6275.
50. Wu, P., Li, Y.-K, Talalay, P., and Brand, L. (1994) *Biochemistry 33*, 7415−7422.
51. Bevins, C. L., Pollack, R. M., Kayser, R. L.; and Bounds, P. L. (1986) *Biochemistry 25*, 5159−5164.
52. Zawrotny, M. E. and Pollack, R. M., (1994) *Biochemistry 33*, 13896−13902.
53. Weintraub, H., Alfsen, A., Baulieu, E.-E. (1970) *Eur. J. Biochem. 12*, 217−221.

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