Binding of 2-Naphthols to D38E Mutants of 3-Oxo-Δ^5-steroid Isomerase: Variation of Ligand Ionization State with the Nature of the Electrophilic Component†

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ABSTRACT: 3-Oxo-Δ^5-steroid isomerase (KSI) catalyzes the isomerization of a variety of 3-oxo-Δ^5-steroids to their conjugated Δ^4 isomers. The mechanism involves sequential enolization and ketonization, with Asp-38 acting to transfer a proton from C-4 to C-6 through a dienol(ate) intermediate. We have previously proposed that this intermediate is anionic, with stabilization provided from direct hydrogen bonding from Tyr-14 and Asp-99 to the oxygen of the steroid. In this work, we analyze the binding of substituted 2-naphthols, which are analogues of the intermediate dienol, to the D38E KSI mutant and the corresponding double mutants lacking one of the two electrophilic groups (D38E/Y14F and D38E/D99A). The binding of these naphthols to the mutant KSIs at pH 7 is described by the modified Brønsted equation: $\log K_D = \alpha(pK_a) + \text{constant}$, where K_D is the dissociation constant of the complex. The high value of α for D38E ($\alpha = 0.87 \pm 0.06$) indicates that the negative charge in these D38E−naphthol complexes is localized almost exclusively on the bound ligand. In contrast, values of α for the double mutants ($\alpha = 0.28 \pm 0.02$ for D38E/Y14F and $\alpha = 0.25 \pm 0.02$ for D38E/D99A) are consistent with very little negative charge on the oxygen of the bound naphthol. Ultraviolet spectra of 5-nitro-2-naphthol and the fluorescence spectra of equilenin bound to these mutants support this interpretation. Extrapolation of these results to the intermediate in the catalytic reaction suggests that for the reaction with D38E, the intermediate is a negatively charged dienolate with hydrogen bonding from both Tyr-14 and Asp-99. Removal of either one of these H-bond donors (Tyr-14 or Asp-99) causes destabilization of the anion and results in a dienol enzyme−intermediate complex rather than a dienolate.

A number of enzymes, such as triosephosphate isomerase (*1*), mandelate racemase (*2*), 4-oxalocrotonate tautomerase (*3*), 3-oxo-Δ^5-steroid isomerase (*4*), and citrate synthase (*5*), catalyze the abstraction of a proton α to a carbonyl group (enolization). These reactions generally involve stabilization of the incipient oxyanion by an acidic group on the enzyme, and much attention has been focused on the nature of this electrophilic catalysis (*6, 7*). Although schemes involving either proton transfer from the enzyme to the substrate or hydrogen bonding (including low-barrier hydrogen bonding) have been proposed for these reactions (*8*−*11*), in most cases there is little unambiguous evidence to enable a distinction between these possibilities to be made.

We have investigated this question for the reaction catalyzed by 3-oxo-Δ^5-steroid isomerase from *Pseudomonas testosteroni* (3-ketosteroid isomerase, KSI)[1] (*12*). KSI catalyzes the isomerization of a variety of 3-oxo-Δ^5-steroids to their Δ^4 isomers through an enolization/reketonization pathway (Scheme 1). Asp-38 functions as a base in the enolization and reprotonates the intermediate dienol(ate) at C-6 to produce the conjugated ketone, while electrophilic catalysis (hydrogen bonding) is provided by the OH group of Tyr-14 and the COOH group of Asp-99, which is un-ionized at pH 7 (*13*−*17*). Phenolic steroids, such as equilenin, act as intermediate analogues and bind as competitive inhibitors to KSI, with dissociation constants in the micromolar range (*18, 19*). Recently, we found that simple phenols bind sufficiently well to the D38N mutant of KSI so that it is possible to monitor the dissociation constants (K_D) as a function of the pK_a (*20*). A plot of K_D for a series of substituted phenols is described by a modified Brønsted equation (eq 1) that includes terms in the phenol pK_a and the substituent hydrophobicity (π). The high value of the Brønsted α (0.85 ± 0.08) suggests a structure for the D38N−phenol complex that has the negative charge almost completely on the oxygen of the phenol.

$$\log K_D = 0.85(pK_a) - 0.63\pi - 6.3 \qquad (1)$$

In this work, we describe an extension of this investigation to binding constants for substituted naphthols to the D38E mutant and the two double mutants D38E/Y14F and D38E/

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[1] Abbreviations: ADA, *N*-(2-acetamido)-2-iminodiacetic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; IPTG, isopropyl β-D-thiogalactopyranoside; KSI, 3-oxo-Δ^5-steroid isomerase; SDS, sodium dodecyl sulfate; TAPS, *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; WT, wild-type 3-oxo-Δ^5-steroid isomerase.

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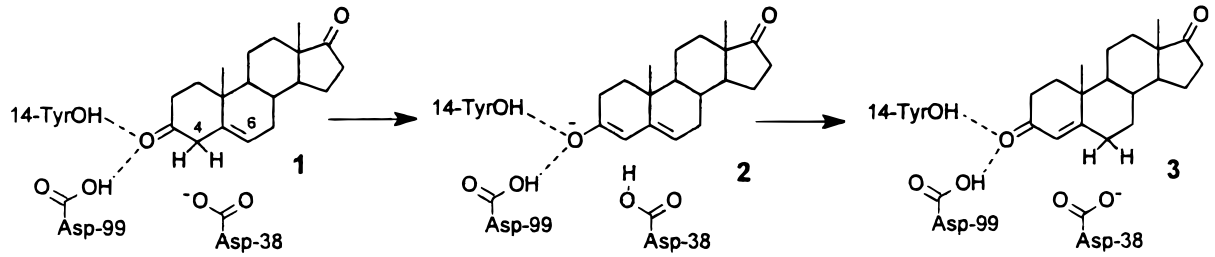
Scheme 1



D99A. The advantages to this system over the previous one are 3-fold: (1) the COOH group of Glu-38 better mimics the COOH group of Asp-38 in the wild-type KSI than does the $CONH_2$ of Asn-38 in D38N (wild-type KSI itself does not bind naphthols sufficiently well to carry out a similar study); (2) naphthols are somewhat better models for the reaction intermediate than are phenols; (3) there is no contribution to binding from hydrophobic effects for the naphthols. Studies with the two double mutants D38E/Y14F and D38E/D99A, in which one of the two electrophilic groups has been eliminated, indicate that the ionization state of the inhibitor is influenced by the number of electrophilic groups that are present in the vicinity of the phenolic oxygen.

MATERIALS AND METHODS

General. UV spectral and kinetic data were acquired at 25.0 ± 0.1 °C on a Gilford Response II or a Cary I Bio spectrophotometer. Melting points were determined in open capillaries with a Mel-Temp apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a General Electric QE-300 spectrometer, and IR spectra were recorded on a Perkin-Elmer 1420 spectrophotometer. Thin-layer chromatography (TLC) was carried on precoated (20 mm) silica gel (Merck 60 F254), while column chromatography was performed on silica gel Merck 60.

2-Naphthols were the best available commercial preparations from Aldrich or Fluka and were used without further purification. All gave a single spot on thin-layer chromatography developed with hexane/ethyl acetate (4:1 or 2:1). 2-Naphthylamine and equilenin were purchased from Aldrich and Sigma, respectively. 5-Androstene-3,17-dione (**1**) was available from previous work (*21*). Oligonucleotides were synthesized by the Biopolymer Laboratory at the University of Maryland, Baltimore. The QuickChange Site-Directed Mutagenesis Kit was purchased from Stratagene. The Wizard Plus Minipreps DNA Purification System was supplied by Promega. All other reagents were reagent grade or better. Water was purified by reverse osmosis.

6-Nitro-2-naphthol was prepared from 2-naphthylamine according to published procedures (*22*, *23*) with major modifications. We report our procedure here in its entirety.

N-(2-Naphthyl)acetamide. Acetic anhydride (3.4 mL, 40 mmol) was added to 5.0 g (35 mmol) of 2-naphthylamine in 10 mL of glacial acetic acid, and the mixture was stirred for 2 h. Acetic acid and excess acetic anhydride were removed under reduced pressure. The residue was taken up into ethyl acetate and washed with water to neutral pH. The organic phase was dried over magnesium sulfate, and after filtration, removal of the solvent under reduced pressure, and recrystallization from ethyl alcohol, 5.9 g (91%) of the product was obtained, mp 132−134 °C [lit. 132−134 °C (*24*)].

Nitration of N-(2-Naphthyl)acetamide. Nitric acid (70%, 2.6 mL) was slowly added with stirring and cooling (the temperature was kept below 40 °C) to 5.7 g (31 mmol) of *N*-(2-naphthyl)acetamide in 10 mL of acetic acid. The mixture was stirred at room temperature until the starting material disappeared by TLC (ca. 2 h) and poured onto ice. Products were extracted with ethyl acetate. The organic phase was washed with water until neutral, and dried over magnesium sulfate. Following filtration and removal of the solvent, the products were separated by column chromatography using hexane/ethyl acetate (1:1) as the solvent.

The first compound to elute was *N*-(1-nitro-2-naphthyl)-acetamide, which was recrystallized from ethyl acetate/hexane (4.28 g, 60%), mp 123−124 °C [lit. 123−124 °C (*25*)]. TLC (hexane/ethyl acetate 1:1), $R_f = 0.45$. ^{1}H NMR (acetone-d_6): δ 2.20 (s, 3H), 7.61−7.75 (m, 2H), 7.82−7.85 (m, 1H), 8.01−8.06 (m, 2H), 8.14−8.17 (m, 1H), 9.34 (br s, 1H). IR (KBr): ν 3180, 3010, 1645, 1520, 1460, 1350, 1275, 860, 820, 760 cm^{-1}.

N-(8-Nitro-2-naphthyl)acetamide eluted as a second product and was recrystallized from ethyl alcohol (600 mg, 8.4%), mp 202−203 °C [lit. 198 °C (*22*)]. TLC (hexane/ethyl acetate 1:1), $R_f = 0.29$. ^{1}H NMR (acetone-d_6): δ 2.18 (s, 3H), 7.55−7.61 (m, 1H), 8.07−8.09 (m, 2H), 8.24−8.29 (m, 2H), 8.81 (s, 1H), 9.67 (br s, 1H). IR (KBr): ν 3240, 3060, 1645, 1600, 1580, 1540, 1500, 1435, 1410, 1355, 1245, 1205, 1145, 1015, 975, 865, 825, 785, 725, 655 cm^{-1}.

N-(6-Nitro-2-naphthyl)acetamide eluted as a third substance and was recrystallized from ethyl acetate/hexane (620 mg, 8.7%), mp 229−231 °C [lit. 224 °C (*22*)]. TLC (hexane/ethyl acetate 1:1), $R_f = 0.12$. ^{1}H NMR (acetone-d_6): δ 2.18 (s, 3H), 7.79 (dd, $J = 2.1$ and 9 Hz, 1H), 8.03 (dd, $J = 2.4$ and 9 Hz, 1H), 8.15−8.23 (m, 2H), 8.58 (d, $J = 0.9$ Hz, 1H), 8.83 (d, $J = 2.1$ Hz, 1H), 9.65 (br s, 1H). IR (KBr): ν 3295, 3070, 1660, 1610, 1600, 1540, 1505, 1455, 1380, 1330, 1255, 1155, 1080, 1005, 900, 870, 800, 730 cm^{-1}.

6-Nitro-2-naphthylamine. *N*-(6-Nitro-2-naphthyl)acetamide (600 mg, 2.6 mmol) and 2 mL of concentrated hydrochloric acid in 5 mL of ethyl alcohol were refluxed for 4 h. After the alcohol was removed under reduced pressure, the residue was made alkaline with 1 N sodium hydroxide and extracted with ethyl acetate. The organic phase was washed with water and dried over magnesium sulfate, and after filtration and removal of the solvent, the residue was recrystallized from ethyl alcohol (461 mg, 94%), mp 206−208 °C [lit. 207−207.5 °C (*26*)]. TLC (hexane/ethyl acetate 1:1), $R_f = 0.43$. ^{1}H NMR (acetone-d_6): δ 5.69 (s, 2H), 7.09 (s, 1H), 7.24 (dd, $J = 2.1$ and 9 Hz, 1H), 7.69 (d, $J = 9$ Hz, 1H), 7.96−8.10 (m, 2H), 8.69 (s, 1H). IR (KBr): ν 3460, 3350, 3200, 1610, 1520, 1480, 1460, 1420, 1385, 1310, 1215, 1175, 1100, 1070, 920, 895, 810, 730 cm^{-1}.

6-Nitro-2-naphthol. 6-Nitro-2-naphthylamine (432 mg, 2.3 mmol) was added to 3 mL of 60% sulfuric acid, and the mixture was stirred for 30 min. It was then cooled to 0−5 °C, and sodium nitrite (160 mg, 2.3 mmol, in 2 mL of water) was added slowly with stirring. After being stirred for 3 h, the reaction mixture was slowly added to a stirring and boiling solution of 5 mL 60% sulfuric acid and refluxed for 30 min. After cooling, the reaction mixture was poured onto ice and extracted with ethyl acetate. The organic phase was washed with water and dried over magnesium sulfate. Filtration and removal of the solvent gave the product which was purified by column chromatography using hexane/ethyl acetate (2:1) as solvent system. Recrystallization from benzene gave 262 mg (60%) of dark orange crystals, mp 158−159 °C [lit. 158−159 °C (*27*)]. TLC (hexane/ethyl acetate 2:1), $R_f = 0.34$. ^{1}H NMR (acetone-d_6): δ 7.36−7.40 (m, 2H), 7.91−7.94 (m, 1H), 8.15−8.20 (m, 2H), 8.84 (m, 1H), 9.46 (s, 1H). IR (KBr): ν 3380, 3070, 1610, 1515, 1485, 1455, 1425, 1380, 1275, 1120, 1075, 890, 850, 795, 730 cm^{-1}. UV (0.1 N NaOH) λ_{max} 433 nm.

5-Nitro-2-naphthol was prepared from 2-naphthylamine according to published procedures (*28*, *29*). The product was purified by column chromatography (2:1 hexane/ethyl acetate), mp 148−149 °C [lit. 147−149 °C (*28*)].

Spectral Determination of pK_as. Stock solutions of the naphthols in methanol were added to either buffer solutions (pH 6.0−11.0, [buffer] = 10 mM, μ = 0.05 M adjusted with KCl) or aqueous potassium hydroxide (μ = 0.05 M adjusted with KCl), and the absorbance (A) was determined at the λ_{max} of the naphtholate ion (433 nm for 6-nitro-2-naphthol, 430 nm for 5-nitro-2-naphthol, 354 nm for 6-bromo-2-naphthol, 346 nm for 2-naphthol, 360 nm for 6-methoxy-2-naphthol, and 376 nm for 2,6-dihydroxynaphthalene). The buffers used were ADA (pH 6.0−7.0), TES (pH 7.5), TAPS (pH 8.0−9.0), CHES (pH 9.5), and CAPS (pH 10.0−11.0). K_a values were obtained by fitting the data to eq 2 using nonlinear least-squares regression, where $A_0 = A$ at [H$^+$] = 0, and $A_{inf} = A$ at [H$^+$] = infinity. Spectral titration data for the diprotic acid 2,6-dihydroxynaphthalene were fit to eq 3, where c is the concentration of 2,6-dihydroxynaphthalene and ϵ_1 is the extinction coefficient of the monoprotonated species, which was treated as an adjustable parameter in the fit.

$$A = (K_a A_0 + A_{inf}[H^+])/(K_a + [H^+]) \quad (2)$$

$$A = (A_{inf} + c\epsilon_1 K_{a1}/[H^+] + A_0 K_{a1} K_{a2}/[H^+]^2)/\{1 + (K_{a1}/[H^+])(1 + K_{a2}/[H^+])\} \quad (3)$$

Construction, Expression, and Purification of Mutant KSIs. Site-directed mutagenesis was performed using the QuickChange Site-Directed Mutagenesis Kit. The recombinant plasmid containing the D38E KSI gene (pUC18-KSI$_{D38E}$) was available from previous work (*30*) and used as the template for the double mutants D38E/Y14F and D38E/D99A. The mutation converting Tyr-14 to Phe was obtained with the primers 5′-GCCGTGGTACAACGCTTTGTGGCTGCGC-3′ and 5′-GCGCAGCCACAAAGCGTTGTACCACGGC-3′ (an additional silent mutation at position 36 in the KSI gene eliminated an *Eco*47III restriction site). The Asp-99 to Ala mutant (D38E/D99A) was made by using the primers 5′-GGTTGCGCCGATCGCTCACTTTCGC-3′ and 5′-GCGAAAGTGAGCGATCGGCGCAACC-3′ (an additional silent mutation at position 291 created a *Pvu*I restriction site). Following transformation of the recombinant plasmids into Epicurian Coli XL-1 Blue supercompetent cells, clones carrying the desired mutations were identified by restriction digestion with *Eco*47III (D38E/Y14F) or *Pvu*I (D38E/D99A). The entire KSI gene in positive clones was then sequenced by the Biopolymer Laboratory at the University of Maryland, Baltimore.

D38E/D99A was expressed in Epicurian Coli XL-1 Blue cells (Strategene), whereas the D38E/Y14F expression plasmid was transformed into the TG1 strain of *Escherichia coli*. For production of all three D38E mutants, cultures were grown in 2× YT medium containing 100 μg/mL ampicillin for 16−18 h at 37 °C, and the expression of mutant KSIs was induced by the addition of 0.75 mM IPTG at the start of growth. Purification of the proteins to electrophoretic homogeneity was carried out as previously described (*31*). Concentrations of the mutant enzymes were determined by UV spectroscopy; a value of 0.336 was used for the absorbance at 280 nm of a 1.00 mg/mL solution of D38E and D38E/D99A (0.226 for D38E/Y14F) (*14*).

Steady-State Kinetics. Steady-state kinetic parameters for the conversion of 5-androstene-3,17-dione (**1**) to 4-androstene-3,17-dione (**3**) were determined in 34 mM potassium phosphate buffer, pH 7.0 [3.3% (v/v) methanol], according to published procedures (*32*). The concentrations of **1** used were 10−95 μM, and the enzyme concentrations were 0.5 nM for D38E, 1.6 μM for D38E/D99A, and 6.1 μM for D38E/Y14F. Initial rates for D38E and D38E/D99A were fit to eq 4, whereas kinetic data for D38E/Y14F were fit to eq 5 (since the concentration of D38E/Y14F is not negligible compared to [**1**]).

$$[E]/v = (1/k_{cat}) + (K_m/k_{cat})(1/[\mathbf{1}]) \quad (4)$$

$$v = (k_{cat}/2)\{(K_m + [E] + [\mathbf{1}]) - \{([\mathbf{1}] + K_m - [E])^2 + 4K_m[E]\}^{1/2}\} \quad (5)$$

Inhibition constants for the reversible inhibition of the D38E mutants by 2-naphthol were determined using the standard assay mixture containing 10−95 μM **1** and different, fixed concentrations of inhibitor (0−350 μM). In the presence of 2-naphthol, k_{cat} values remained unchanged, and the apparent K_m values were fit to eq 6.

$$K_{m(app)} = (K_m/K_i)[I] + K_m \quad (6)$$

Fluorescence Spectroscopy. Emission spectra were recorded at 25.0 ± 0.1 °C using either an SLM 8000C or a FluoroMax-2 spectrofluorometer. Dissociation constants for the binding of naphthols to the D38E mutants were determined by fluorescence quenching titration (*20*). Measurements were made in 34 mM potassium phosphate buffer, pH 7.0 [1.7% (v/v) methanol]. The binding stoichiometry of 5-nitro-2-naphthol to the D38E mutants was calculated according to the method of Penning et al. (*33*). Due to its poor solubility, the binding constants between equilenin and D38E/Y14F or D38E/D99A were determined by titration of equilenin with increasing concentrations of the enzymes (*19*).

Table 1: Kinetic Constants for the Isomerization of 5-Androstene-3,17-dione (**1**) to 4-Androstene-3,17-dione (**3**) Catalyzed by D38E, D38E/Y14F, and D38E/D99A[a]

enzyme	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}\,s^{-1}$)	K_D (μM)[b]
D38E[c]	180	120	1.6×10^6	0.78 ± 0.13[d]
D38E/Y14F	0.009 ± 0.001	84 ± 11	107 ± 16	17 ± 3
D38E/D99A	0.042 ± 0.001	18 ± 1	$(2.3 \pm 0.1) \times 10^3$	5.9 ± 1.6

[a] All rate measurements were made at 25.0 ± 0.1 °C in 34 mM potassium phosphate, pH 7.0 [3.3% (v/v) methanol]. [b] Dissociation constants for the complexes with equilenin. Dissociation constants were determined at 25.0 ± 0.1 °C in 34 mM potassium phosphate, pH 7.0 (1.7% methanol). [c] From Zawrotny and Pollack (*21*). [d] Data from Hawkinson et al. (*19*); 10 mM phosphate, pH 7.07 (5.2% methanol).

Samples were excited at 310 nm, and the emission maximum was 364 nm.

Excitation spectra of equilenin (3 μM) were recorded under neutral (34 mM potassium phosphate, pH 7.0, 1.7% methanol) and basic conditions (0.1 M NaOH, 1.7% methanol) and in the presence of the D38E mutants (10 μM D38E; 30 μM D38E/Y14F or D38E/D99A). The spectra of bound equilenin were derived by subtracting the fluorescence spectra of the enzyme and of the residual free equilenin, the concentration of which was calculated from the dissociation constants of the complexes. The emission wavelength was at 400 nm.

UV Spectral Study of the Complexes between D38E Mutants and 5-Nitro-2-naphthol. The UV spectrum of 5-nitro-2-naphthol (50 μM) was recorded under neutral (34 mM potassium phosphate, pH 7.0, 1.7% methanol) and basic conditions (0.1 M NaOH, 1.7% methanol) and in the presence of the D38E mutants (50 μM D38E; 100 μM D38E/Y14F or D38E/D99A in 34 mM potassium phosphate, pH 7.0, 1.7% methanol). The spectra of bound 5-nitro-2-naphthol were derived as described above for the spectra of bound equilenin.

RESULTS

Characterization and Kinetic Properties of the D38E Mutants. The genes for the double mutant KSIs D38E/Y14F and D38E/D99A were prepared by site-directed mutagenesis. The proteins were expressed in *E. coli* and purified to homogeneity, as judged by the presence of a single band on overloaded SDS−polyacrylamide gels. One liter of stationary phase culture gave ~5 mg of D38E/Y14F or ~30 mg of D38E/D99A. The UV spectra of D38E/D99A and D38E/Y14F at pH 7.0 resemble very closely those of WT and Y14F, respectively (*14*). Both double mutants have an absorption maximum at 277 nm and a minimum at 250 nm.

Specific activities for these preparations were 270 units/mg for D38E (*21*), 0.15 unit/mg for D38E/D99A, and 0.016 unit/mg for D38E/Y14F under standard conditions (*31*). The kinetic constants for the isomerization of **1** to **3** catalyzed by the D38E mutants were determined at pH 7.0 and are listed in Table 1. Relative to D38E (*21*), k_{cat} is lower for D38E/D99A (ca. 4000-fold) and D38E/Y14F (ca. 20 000-fold). The second-order rate constant k_{cat}/K_m is also substantially reduced for both double mutants (ca. 700-fold for D38E/D99A and ca. 15 000-fold for D38E/Y14F). These rate decreases are similar to those for the D99A and Y14F mutants relative to WT, suggesting additivity of the effects of the single mutations on both k_{cat} and k_{cat}/K_m for the D38E/D99A and D38E/Y14F double mutants. Thus, the D99A (*31*) and Y14F (*14*) mutations cause a ca. 3000- and 45 000-fold decrease in k_{cat}, respectively. Similarly, k_{cat}/K_m is decreased ca. 2200-fold for D99A (*31*) and ca. 16 000-fold for Y14F (*14*) relative to WT.

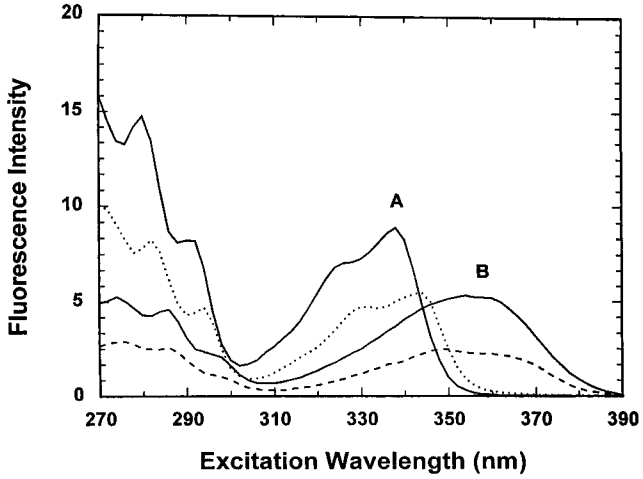


FIGURE 1: Fluorescence excitation spectra of equilenin. Solid line A: 3 μM equilenin in 34 mM potassium phosphate buffer, pH 7.0, 1.7% (v/v) methanol. Solid line B: 3 μM equilenin in 0.1 M NaOH, 1.7% methanol. Dashed line: Calculated spectrum of 2.7 μM equilenin bound to D38E. The spectrum was calculated based on a K_D of 0.78 μM (*19*) by subtracting the spectra of D38E and free equilenin (0.3 μM) from the spectrum of a solution of 10 μM D38E and 3 μM equilenin in 34 mM potassium phosphate, pH 7.0, 5.2% methanol. Dotted line: Calculated spectrum of 2.5 μM equilenin bound to D38E/D99A. The spectrum was calculated on the basis of K_D = 5.9 μM by subtracting the spectra of D38E/D99A and free equilenin (0.5 μM) from the spectrum of a solution of 30 μM D38E/D99A and 3 μM equilenin in 34 mM potassium phosphate, pH 7.0, 1.7% methanol. The emission wavelength was 400 nm.

Binding of Equilenin to the D38E Mutants. Dissociation constants for the binding of equilenin to the double mutants were determined at pH 7.0 (1.7% methanol) and are listed in Table 1. D38E/D99A exhibits a ca. 20-fold and D38E/Y14F a ca. 60-fold lower affinity for equilenin relative to D38E [the dissociation constant for the binding of equilenin to D38E was determined in 5.2% methanol; it is expected to be ca. 3-fold lower in 1.7% methanol (*34*)]. Similar results for the binding of phenolic steroids to KSI single mutants lacking Tyr-14 or Asp-99 have been reported (*14, 15*).

We also examined the excitation spectra of equilenin, both free in solution and bound to the D38E mutants. The spectrum of the D38E−equilenin complex (corrected for the absorbance of the protein) is similar to that of ionized equilenin and identical to that of equilenin bound to WT (*35*). In contrast, the spectra of equilenin bound to D38E/D99A (Figure 1) and D38E/Y14F (not shown) show a marked resemblance to the spectrum of un-ionized equilenin. Analogous observations have been made for the binding of phenolic steroids to the Y14F, D99A, and D38H/D99A KSI mutants (*14, 36*).

Dissociation Constants of the Complexes of 2-Naphthols and the D38E Mutants. 2-Naphthol is a modest competitive inhibitor of the D38E mutants at pH 7.0 (3.3% methanol); kinetic analysis gives K_i values of 158 ± 25 μM for D38E, 377 ± 65 μM for D38E/Y14F, and 174 ± 29 μM for D38E/

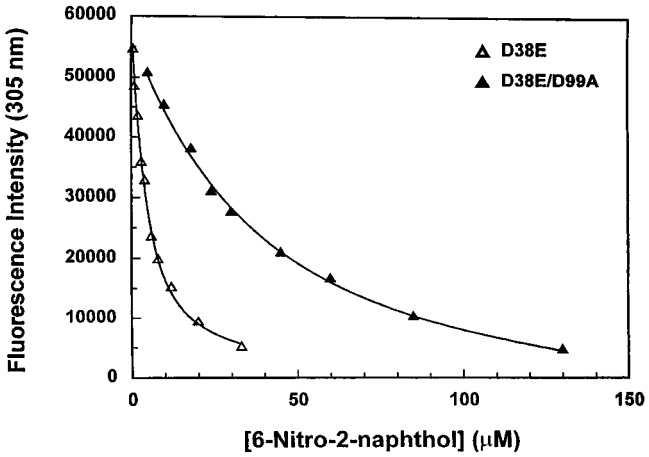


FIGURE 2: Fluorescence quenching titrations of D38E and D38E/D99A by 6-nitro-2-naphthol at pH 7.0 [34 mM potassium phosphate buffer, 1.7% (v/v) methanol]. The K_D values are 3.9 ± 0.3 and 34 ± 3 μM, respectively. Enzyme concentrations were 2.5 μM for D38E and 9 μM for D38E/D99A. Emission was measured at 305 nm with the excitation wavelength set at 277 nm.

Table 2: Dissociation Constants for the Binding of 2-Naphthols to the D38E Mutants[a]

substituent	pK_a[b]	$K_{D(D38E)}$, μM	$K_{D(D38E/Y14F)}$, μM	$K_{D(D38E/D99A)}$, μM
6-NO_2	8.36 ± 0.04	3.9 ± 0.3	61 ± 6	34 ± 3
5-NO_2	8.90 ± 0.03	9.6 ± 1.7	102 ± 9	51 ± 7
6-Br	9.42 ± 0.02	21 ± 3	127 ± 17	58 ± 10
H	9.70 ± 0.02	65 ± 9	152 ± 17	79 ± 9
6-CH_3O	9.89 ± 0.03	82 ± 13	168 ± 35	85 ± 15
6-OH	10.10 ± 0.05	112 ± 19	195 ± 47	95 ± 12

[a] All measurements were made at 25.0 ± 0.1 °C in 34 mM potassium phosphate, pH 7.0 [1.7% (v/v) methanol]. Errors are internal standard deviations within a run. [b] 1.7% (v/v) methanol, μ = 0.05 M (KCl), 25.0 ± 0.1 °C.

D99A. Thus, all three D38E mutants exhibit a comparable affinity for naphthol.

Upon binding to the D38E mutants, substituted 2-naphthols quench the tyrosine fluorescence (λ_{max} = 305 ± 1 nm) in a concentration-dependent manner (~95% quenching at saturation is obtained for D38E and D38E/D99A and ~90% for D38E/Y14F). Therefore, the dissociation constants for the binding of naphthols to the D38E mutants (K_Ds) were also determined by fluorescence quenching titration at pH 7.0 (1.7% methanol) (*20*). Representative fluorescence titration curves are shown in Figure 2 and the calculated K_D values are listed in Table 2. There is reasonable agreement between the K_i and K_D values for the 2-naphthol−KSI complexes (given the different amounts of methanol used in those determinations).[2] Dissociation constants of the complexes formed from each enzyme with 5-nitro-2-naphthol and with 2,6-dihydroxynaphthalene were also determined at pH 8.0; the K_D values are virtually identical to those at pH 7.0. For comparison, the K_D for the complex formed between the D38N KSI mutant and 2-naphthol at pH 7.0 was 0.40 ± 0.08 μM (3.3% methanol). The ca. 300-fold tighter binding[2] of naphthol to D38N relative to D38E (Table 2; 1.7% methanol) is consistent with the much tighter binding of equilenin to D38N relative to D38E [>800-fold; (*19*)]. The

[2] Increasing concentrations of methanol result in a decrease in the affinity of ligands for KSI (*34*). K_D values determined in 1.7% methanol are approximately doubled in 3.3% methanol.

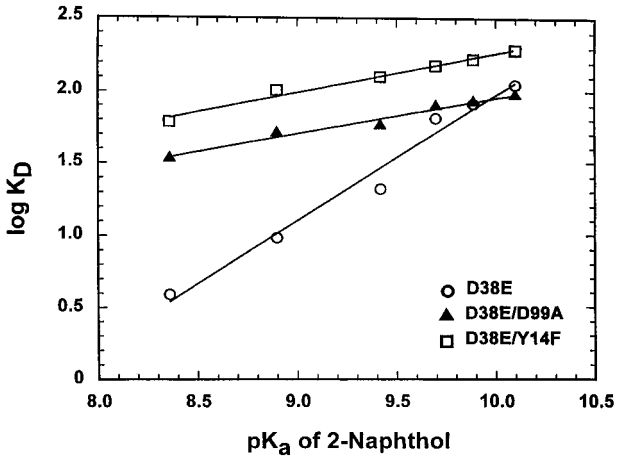


FIGURE 3: Brønsted plots for the binding of 2-naphthols to D38E, D38E/Y14F, and D38E/D99A at pH 7.0. The slopes of the lines, α, through the data are 0.87 ± 0.06 for D38E, 0.28 ± 0.02 for D38E/Y14F, and 0.25 ± 0.02 for D38E/D99A. K_D values are from Table 2.

stoichiometry of binding of 5-nitro-2-naphthol to the D38E mutants was determined by construction of Scatchard plots (*33*). One naphthol binding site per enzyme monomer was obtained for all three mutants.

Brønsted Analysis of the Binding of 2-Naphthols to the D38E Mutants. Logarithmic plots of the dissociation constants of the naphthol complexes with the D38E mutants are linear (Figure 3) and were fit to a modified Brønsted equation (eq 7). The values of α from the least-squares fit are 0.87 ± 0.06 for D38E (r = 0.989), 0.28 ± 0.02 for D38E/Y14F (r = 0.990), and 0.25 ± 0.02 for D38E/D99A (r = 0.988). For all D38E mutants, there appears to be no hydrophobic contribution to binding; correlations of K_D with pK_a are not improved by including the hydrophobic parameter π to the correlation. Thus, in contrast to the binding of phenols to D38N (*20*), electronic effects are the sole determinants of the K_D values for the binding of naphthols to the D38E mutants.

$$\log K_D = \alpha(pK_a) + \text{constant} \quad (7)$$

Effects of the D38E Mutants on the UV Spectrum of 5-Nitro-2-naphthol. At pH 7.0, the UV spectrum of the neutral naphthol shows two absorption maxima at 255 and 340 nm (Figure 4). Ionization of 5-nitro-2-naphthol in basic solution results in red shifts of both maxima to 276 and 353 nm, respectively, and the appearance of a shoulder at 430 nm. Complexation of 5-nitro-2-naphthol with D38E gives changes similar to those observed in base. In contrast, the UV spectrum of 5-nitro-2-naphthol bound to D38E/Y14F (data not shown) or D38E/D99A (Figure 4) resembles that of the neutral naphthol.

DISCUSSION

Kinetic Parameters for the D38E, D38E/Y14F, and D38E/D99A Mutant KSIs. Elimination of one of the two electrophilic groups of KSI (Tyr-14 or Asp-99) results in a substantial decrease in activity for the D38E/Y14F and D38E/D99A double mutants compared to D38E (ca. 4000-fold in k_{cat} and ca. 700-fold in k_{cat}/K_m for D38E/D99A, and ca. 20 000-fold in k_{cat} and ca. 15 000-fold in k_{cat}/K_m for D38E/Y14F; Table 1). Similar rate decreases have been reported

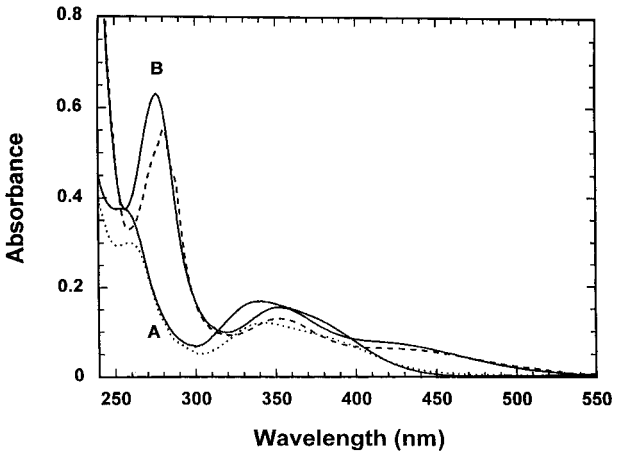


FIGURE 4: UV absorption spectra of 5-nitro-2-naphthol. Solid line A: 36 μM 5-nitro-2-naphthol in 34 mM potassium phosphate buffer, pH 7.0, 1.7% (v/v) methanol. Solid line B: 36 μM 5-nitro-2-naphthol in 0.1 M NaOH, 1.7% methanol. Dashed line: Calculated spectrum of 32 μM 5-nitro-2-naphthol bound to D38E. The spectrum was calculated based on a K_D of 9.6 μM by subtracting the spectra of D38E and free 5-nitro-2-naphthol (18 μM) from the spectrum of a solution of 50 μM D38E and 50 μM 5-nitro-2-naphthol in 34 mM potassium phosphate, pH 7.0, 1.7% methanol. Dotted line: Calculated spectrum of 29 μM 5-nitro-2-naphthol bound to D38E/D99A. The spectrum was calculated on the basis of $K_D = 51$ μM by subtracting the spectra of D38E/D99A and free 5-nitro-2-naphthol (21 μM) from the spectrum of a solution of 100 μM D38E/D99A and 50 μM 5-nitro-2-naphthol in 34 mM potassium phosphate, pH 7.0, 1.7% methanol.

for the Y14F and D99A single mutants relative to WT [ca. 3000-fold in k_{cat} and ca. 2200-fold in k_{cat}/K_m for D99A (*31*), and ca. 45 000-fold in k_{cat} and ca. 16 000-fold in k_{cat}/K_m for Y14F (*14*)]. Therefore, the kinetic properties of the D38E/Y14F and D38E/D99A double mutants parallel those of the analogous single mutants from WT. For all three D38E mutants, the possibility that significant structural changes have occurred is unlikely since (1) all mutants catalyze the isomerization of **1** to **3** (Table 1); (2) all mutants bind equilenin (Table 1); and (3) the UV spectra of D38E and D38E/D99A at pH 7.0 are identical to that of WT, whereas the spectrum of D38E/Y14F is similar to that of Y14F (*14*). The observed activity for the mutant enzymes is not due to contaminating WT activity since the K_m values for **1**−**3** are significantly different for the D38E mutants and WT [$K_m \approx 200$ μM; (*37*)].

Binding of 2-Naphthols to D38E, D38E/Y14F, and D38E/D99A KSIs. Our previous studies (*20*) on the binding of substituted phenols to the D38N mutant of KSI showed that there is a significant dependence of the dissociation constant (K_D) both on the pK_a of the phenol and on a hydrophobic term (eq 1). We interpreted the dependence on the pK_a ($\alpha = 0.85 \pm 0.08$) in terms of substantial charge on the phenolic oxygen in the complex, and by extension on the oxygen of the bound dienolate intermediate (**2**) in the catalytic reaction. These results, however, were obtained with an enzyme that lacks the natural carboxylate at position 38. The present study uses the D38E mutant, which preserves the carboxylate at position 38, as well as inhibitors that mimic both the A and B rings of equilenin. D38E was used rather than wild-type KSI since steroids bind approximately 4-fold better to this mutant (*19*). The binding of naphthols to D38E is sufficiently tight so that the nature of the enzyme-bound complex can be investigated when one of the two electrophilic components of KSI is removed in the D38E/Y14F and D38E/D99A mutants.

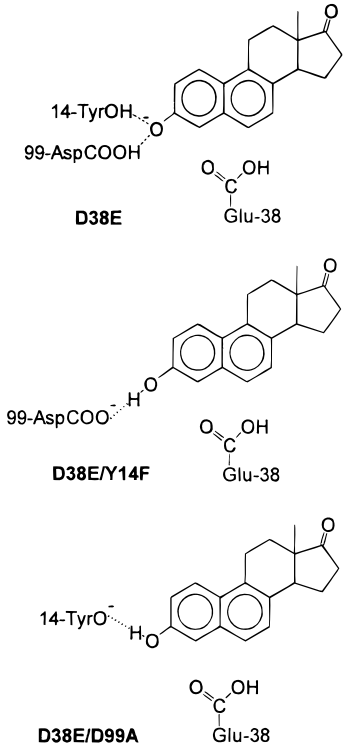
Several lines of evidence suggest that naphthols are suitable probes for the interaction of the dienol intermediate with KSI. (1) They are competitive inhibitors and thus bind at the active site of the D38E mutants, and there exists one naphthol binding site per enzyme monomer. (2) The pK_a of naphthol (pK_a 9.7) is very similar to those of both the dienolate intermediate [pK_a 10.0; (*38*)] and equilenin (*39*). (3) Quenching of the intrinsic fluorescence of the D38E mutants by naphthol binding is virtually quantitative, in agreement with earlier reports on the interactions of steroidal intermediate analogues with KSI (*40, 41*).

Binding of naphthols to D38E, D38E/Y14F, and D38E/D99A at pH 7 is described by a modified Brønsted equation (eq 7). In all cases, there is a good correlation between the dissociation constants of the complexes and the aqueous pK_as of the naphthols (Figure 3). For the binding of substituted naphthols to D38E, there is a substantial dependence on the pK_a of the phenolic oxygen (Figure 3, $\alpha = 0.87 \pm 0.06$). However, unlike the binding of phenols to D38N, the correlation is not improved with the addition of a hydrophobic parameter (π), which ranged in value from −0.67 for OH to +0.86 for Br (*42*), showing that the hydrophobicity of the substituent is not an important contributor to the binding. Consistent with our earlier studies with D38N (*20*), the steep dependence of the K_D of the D38E−naphthol complex on the naphthol pK_a indicates that the negative charge in these complexes, and by extension in the D38E−intermediate complex, is localized almost exclusively on the bound ligand. Support for this conclusion comes from the spectra of either equilenin or 5-nitro-2-naphthol bound to D38E at neutral pH, which manifest spectral changes in the ligands that resemble those obtained when their phenolic hydroxyl groups are ionized in base (Figures 1 and 4). Xue et al. (*43*) have found that mixing **1** with the D38N mutant of KSI produces an intermediate with a spectrum that suggests a dienolate ion, with some contribution from dienol. We have reported a similar spectrum for the complex formed by directly mixing the dienol with D38N (*19*). These results are all consistent with the intermediacy of a dienolate ion in the KSI-catalyzed reaction, with little formal charge on either of the two hydrogen bonding groups of the enzyme (Tyr-14 or Asp-99).[3]

In contrast to the large dependence on pK_a for D38E, the Brønsted α values for the two double mutants are only 0.28 ± 0.02 for D38E/Y14F and 0.25 ± 0.02 for D38E/D99A, consistent with little negative charge on the phenolic oxygen of the bound naphthol. Extrapolation of this result to the intermediate in the catalytic reaction suggests that elimination of one of the electrophilic components of KSI in the D38E/Y14F and D38E/D99A mutants results in the enzyme−intermediate complex being a dienol rather than a dienolate. This interpretation is supported by the effects of these mutations on the excitation spectrum of equilenin and the absorption spectrum of 5-nitro-2-naphthol. Although the complexes with D38E show spectra that are similar to those of the ionized ligands in solution, the spectra of the

[3] The insensitivity of the dissociation constants for 2-naphthol to mutation of Tyr-14 and Asp-99 suggests that there is little difference in energy between the dienol and dienolate forms of this ligand for the D38E mutant.

Scheme 2



corresponding complexes with D38E/Y14F (not shown) and D38E/D99A are similar to those of the un-ionized ligands (Figures 1 and 4). The simplest interpretation of these results is that, although there is substantial negative charge on the oxygen of the naphthols when they are bound to D38E, the structure of the bound complex with D38E/Y14F has a negative charge on the COO^- of Asp-99, and that with D38E/D99A has the charge on the O^- of Tyr-14 (Scheme 2). The independence of the binding constants on pH (7 or 8) indicates no change in the total charge on going from protein plus ligand to complex. Since Glu-38 is ionized in the free enzymes, a negative charge likely remains at the active site in the complexes. However, alternative structures that involve ionization of Glu-38 or a water molecule trapped in a cavity generated by mutation of either Tyr-14 or Asp-99 are certainly possible. These observations are entirely consistent with the hydrogen bonding network that we have proposed (*15*) for the active site of KSI, in which both Asp-99 and Tyr-14 hydrogen bond directly to the oxygen of the ligand (Scheme 1). Removal of one of these hydrogen bonds could eliminate sufficient stabilization of the anion to cause the negative charge to reside elsewhere on the enzyme.

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