Substituent Effects on the Binding of Phenols to the D38N Mutant of 3-Oxo-Δ^5-steroid Isomerase. A Probe for the Nature of Hydrogen Bonding to the Intermediate†

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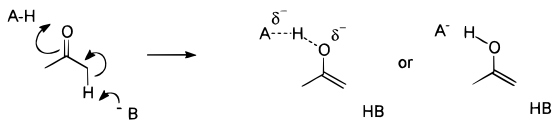
ABSTRACT: The nature of hydrogen bonding to the intermediate of the reaction catalyzed by 3-oxo-Δ^5-steroid isomerase (KSI) was investigated. Substituted phenols bind tightly to the active site of the D38N mutant of KSI, and are analogs of the intermediate dienol. These D38N−phenol complexes exhibit fluorescence, NMR, and UV spectral characteristics similar to D38N complexed with phenolic steroids. The binding of phenols to D38N is satisfactorily described by the modified Brønsted equation: $\log K_D = 0.85(pK_a) - 0.63\,\pi - 6.3$ ($n = 10$, $r = 0.967$), where K_D is the dissociation constant of the complex and π is the hydrophobicity parameter for the phenol substituent. The high value of the Brønsted α (0.85 ± 0.08) indicates that the negative charge in the D38N−phenol complex, and by implication in the KSI−intermediate complex, is localized almost exclusively on the bound ligand. It is concluded that stabilization of the anionic (dienolate) intermediate is provided by *ordinary* hydrogen bonds from the enzyme acids Tyr-14 and Asp-99, rather than low-barrier hydrogen bonds.

The importance of hydrogen bonding in enzymatic catalysis has been the subject of much recent activity. Of particular interest is the nature of the hydrogen bonds that act to stabilize intermediates and transition states in enzymes that catalyze the loss of a proton adjacent to either a carbonyl group or a carboxylic acid, such as triosephosphate isomerase (*1*), 4-oxalocrotonate isomerase (*2*), and mandelate racemase (*3*). These reactions involve abstraction of a proton by a base (B), with concomitant stabilization of the incipient anion either by hydrogen bonding to an electrophile (AH) or by concerted protonation of the oxygen (Scheme 1). Since the rate of loss of these protons in the absence of enzymatic catalysis is quite low (*4*), the nature of this catalysis has drawn much attention. The extraordinary catalytic activity of these enzymes has been attributed to the formation of low-barrier hydrogen bonds (*5*−*10*), but this interpretation is not universally accepted (*11*−*15*).

One of the most well−studied examples of enzymatic enolization is the reaction catalyzed by 3-oxo-Δ^5-steroid isomerase from *P. testosteroni* (3-ketosteroid isomerase, KSI; for reviews, see *16*−*18*). KSI catalyzes the sequential enolization/ketonization of 3-oxo-Δ^5-steroids to their Δ^4-conjugated isomers (Scheme 2) at nearly the diffusion-controlled rate (*19*). Asp-38 acts to transfer a proton from C-4 to C-6, and electrophilic stabilization of the intermediate is provided by Tyr-14 and Asp-99 (*20*−*24*). Although a concerted proton transfer to the carbonyl group of the substrate from Tyr-14 has been considered (*25*−*27*), more recent evidence suggests that stabilization is provided by hydrogen bonding to the anion (*24*, *28*−*31*). Several authors have postulated that stabilization of the intermediate is provided by a low-barrier hydrogen bond (LBHB) from Tyr-14 to the oxygen of the intermediate (*5*, *9*, *30*), and recently an 18.15 ppm signal in the ^{1}H NMR of the complex of KSI and an intermediate analog was interpreted as indicating a LBHB (*31*).

Scheme 1



In order to investigate the nature of hydrogen bonding in the intermediate (**2**), we probed the charge distribution in this complex by analyzing the binding of substituted phenols to the D38N mutant of KSI. The D38N mutant mimics the protonated Asp-38 in the intermediate complex, while the phenols are analogs of the intermediate dienol, and provide a way in which to systematically vary the pK_a. Our results indicate that the negative charge in the intermediate is localized almost exclusively on the steroid oxygen, with very little charge transfer to the hydrogen bonding groups of the enzyme.

MATERIALS AND METHODS

Materials. Phenols were the best available commercial preparations from Aldrich or Fisher and were used without

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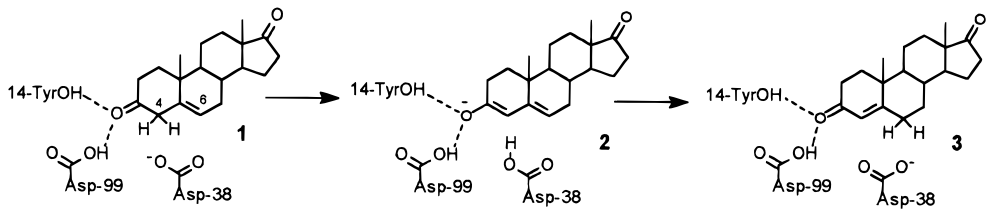
Scheme 2



further purification. All gave a single spot on thin-layer chromatography developed with hexane/ethyl acetate (2:1). CD_3OD (99.95 atom % D) was purchased from Aldrich. All other reagents were reagent grade or better. Water was purified as previously described (*32*). D38N was prepared and purified to electrophoretic homogeneity according to published procedures (*32*). Concentrations of D38N were determined by UV spectroscopy using a value of 0.343 for the absorbance at 280 nm in a 1.0-cm light path of a 1.00 mg/mL solution (*22*).

Measurement of the Binding Constants between Phenols and the D38N Mutant. Dissociation constants for the binding of phenols to D38N were determined by fluorescence quenching titration (*33*). Fluorescence spectra were recorded at 25 °C using a SLM 8000C spectrofluorometer equipped with single photon counting. Emission intensity as a function of wavelength was expressed as a ratio to the intensity of a rhodamine B internal standard, thereby canceling any effect of light fluctuation. Measurements were made in 10 mM acetate (pH 5.0), MES (pH 6.0), phosphate (pH 7.0), and Tris (pH 8.0) buffers, adjusted to $\mu = 0.05$ M with KCl. The final content of methanol in the cuvette was 10% (v/v). Slit widths of 2.0 and 4.0 nm were used for the excitation and emission beams, respectively. The emission spectra of D38N−phenol complexes were recorded from 300 to 350 nm with an excitation wavelength of 277 nm, and were corrected for the fluorescence of blank buffers or buffers containing various concentrations of phenols. The spectra of the final solutions remained unchanged after 30 min. Most titrations were performed in duplicate.

The fluorescence intensity, F, of D38N at the emission maximum (305−306 nm) at each phenol concentration was then used to calculate the dissociation constant for binding of the respective phenol to D38N according to eq 1. The experimental data were fit to eq 1 using FigP, a nonlinear least-squares program based on the Marquardt algorithm.

The stoichiometry of binding of *p*-nitrophenol to D38N was determined in 10 mM potassium phosphate, $\mu = 0.05$ M, pH 7.0, 10% methanol (v/v) by fluorescence quenching titration. The data were analyzed by (a) determination of the equivalence point for saturation of D38N with *p*-nitrophenol (*34*) and (b) construction of Scatchard plots in which the [bound ligand]/[free ligand] was plotted against [bound ligand] assuming one, two, and three phenol binding sites per enzyme monomer (*35*).

Measurement of the Octanol/Water Partition Coefficients. The octanol/water partition coefficient for *p*-hydroxybenzaldehyde, P_{HBZ}, was determined according to the method of Leo (*36*). The π value for the *p*-CHO substituent ($\pi_{p-CHO} = -0.25$) was calculated using the relationship: $\pi_{p-CHO} = \log P_{HBZ} - \log P_{phenol}$, where $\log P_{phenol}$ was taken to be 1.46 (*37*). All other π values were taken from Fujita et al. (*37*).

UV Spectral Study of the D38N Complex with m-Iodophenol. Ultraviolet absorption measurements were made at 25 °C with a Cary 1 Bio spectrophotometer in matched 1.0-cm cuvettes with a total volume of 1.0 mL. The UV spectrum of *m*-iodophenol (100 μM) was recorded under neutral (10 mM potassium phosphate, $\mu = 0.05$ M, pH 7.0, 10% methanol) and basic conditions (0.1 M NaOH, 10% methanol), and in the presence of D38N (50 μM in 10 mM potassium phosphate, $\mu = 0.05$ M, pH 7.0, 10% methanol). The spectrum of the bound *m*-iodophenol was derived by subtracting the absorption of the enzyme and of the residual free phenol, which was calculated from the dissociation constant (51.7 μM).

NMR Spectroscopy. ^{1}H NMR spectra of free D38N (0.55 mM) and several D38N−phenol complexes (0.55 mM) were obtained at 4 ± 0.5 °C with a GE Omega HR 500 MHz spectrometer. NMR samples were prepared in 10 mM potassium phosphate, 20 mM KCl, and 10% (v/v) CD_3OD, in H_2O at pH 7.2. Conditions for NMR spectroscopy were similar to those described by Zhao et al. (*31*) except that the delays between the pulses were adjusted for maximum excitation at 17.4 ppm.

RESULTS

Dissociation Constants of Complexes between Phenols and D38N. The intrinsic fluorescence of KSI arises almost entirely from its three tyrosine residues (Tyr-14, Tyr-55, and Tyr-88). Tyr-14, which has the highest quantum yield (*38*), and Tyr-55 are located in the hydrophobic active site while Tyr-88 is more distant and at least partially exposed to the aqueous environment (*24*, *39*). Upon binding to KSI, phenolic steroids (e.g., equilenin) and 3-ketosteroids (e.g., 19-nortestosterone, 5α-estrane-3,17-dione) have been shown to quench the tyrosine fluorescence (λ_{max} 305 nm) in a concentration-dependent manner (*33*, *40*, *41*).

The fluorescence emission spectrum of D38N also shows a maximum at 305 ± 1 nm. Addition of simple substituted phenols to D38N significantly decreases the intensity of this peak, indicating that these phenols bind to D38N. Thus, the fluorescence spectrum of D38N was determined in the presence of varying concentrations of phenols at pH values ranging from 5.11 to 7.95. A typical plot for the quenching of D38N fluorescence by *p*-hydroxyacetophenone is shown in Figure 1.

The stoichiometry of binding of *p*-nitrophenol to D38N at pH 7.2 was determined by fluorescence titration. The results were analyzed by (a) equivalence point determination and (b) construction of Scatchard plots. At the equivalence point for saturation of D38N with the ligand, the amount of *p*-nitrophenol bound to the enzyme was calculated to be 0.9 mol/mol of enzyme subunit. Scatchard plots were constructed in which [bound ligand]/[free ligand] was plotted

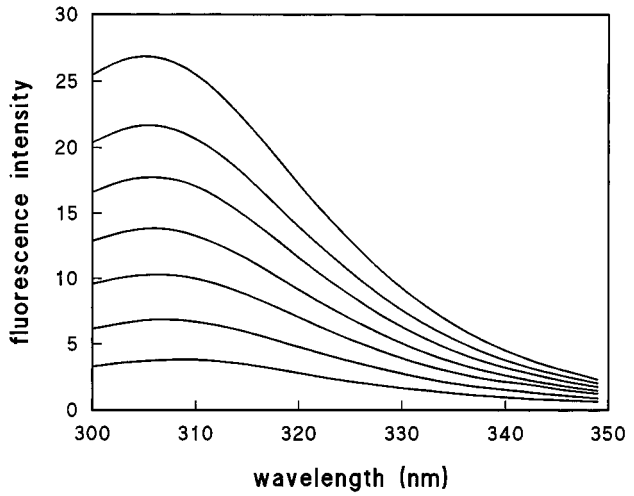


FIGURE 1: Fluorescence emission spectra of 2 μM D38N as a function of concentration of added *p*-hydroxyacetophenone at pH 5.96 (10 mM MES, μ = 0.05 M, 10% methanol). The *p*-hydroxyacetophenone concentrations were (in order of decreasing fluorescence) 0, 2, 4, 7, 11, 18, and 30 μM. The excitation wavelength was 277 nm.

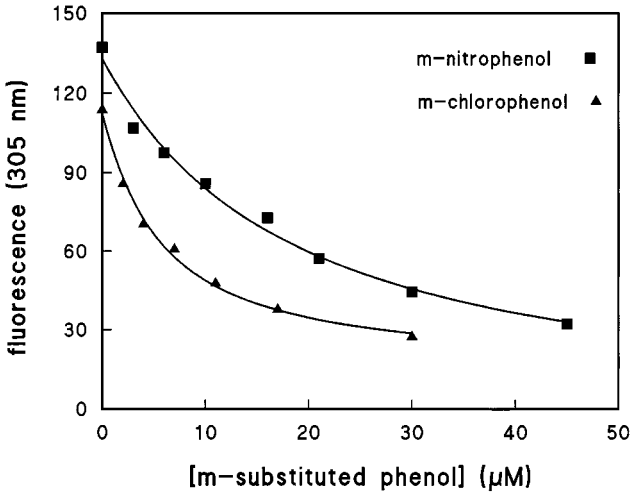


FIGURE 2: Fluorescence quenching titration of D38N by *m*-nitrophenol at pH 5.96 (10 mM MES, μ = 0.05 M, 10% methanol; K_D = 17 ± 3 μM) and *m*-chlorophenol at pH 7.21 (10 mM potassium phosphate, μ = 0.05 M, 10% methanol; K_D = 6.2 ± 0.6 μM). The D38N concentrations were 3 and 1.5 μM, respectively. Emission was measured at 305 nm with the excitation wavelength set at 277 nm.

against [bound ligand] assuming one, two, or three bindings sites per enzyme monomer (*35*). Negative values for [free ligand] were obtained using the assumption of two or three binding sites, whereas the assumption of one binding site per monomer gave satisfactory linearity of the Scatchard plots. Thus, both methods of data analysis are consistent with one binding site per enzyme monomer.

Fluorescence intensities at 305−306 nm were analyzed as a function of phenol concentration to give the apparent dissociation constant (K_D) of the D38N−phenol complexes according to eq 1, where F_0 is the intensity in the absence of phenol, F_{inf} is the intensity extrapolated to infinite phenol concentration, K_D is the dissociation constant, and $[E]_t$ is the concentration of D38N. Equation 1 may be derived from the relationship $[D38N]_{free}/[D38N]_{bound} = (F - F_{inf})/(F_0 - F)$, and is similar to that used previously (*32*). The fit of the titration curve to the data was optimized to yield values

Table 1: Dissociation Constants of D38N−Phenol Complexes as a Function of pH[a]

substituted phenol	pK_a[b]	π[c]	pH	K_D (μM)[d]
p-NO_2	7.14	0.50	5.96	2.6 ± 0.2
			5.96	2.6 ± 0.5
p-CHO	7.66	−0.25	5.96	15 ± 2
			5.96	9.4 ± 1.1
			5.96	9.3 ± 0.5
p-CN	7.95	0.14	5.96	15 ± 4
			5.96	14 ± 3
p-CH_3CO	8.05	−0.11	5.96	13 ± 2
			5.96	11 ± 2
			5.96	7.8 ± 0.2
m-NO_2	8.35	0.54	5.11	62 ± 2
			5.96	19 ± 3
			5.96	17 ± 3
			7.21	3.1 ± 0.7
			7.21	3.4 ± 0.5
m-Cl	9.02	1.04	7.21	6.2 ± 0.6
			7.21	6.8 ± 1.0
m-I	9.17	1.47	5.96	10.0 ± 1.1
p-I	9.31	1.45	5.96	23 ± 3
			7.21	4.0 ± 0.7
			7.21	3.9 ± 0.6
p-Cl	9.38	0.93	7.21	21 ± 3
			7.95	7.9 ± 1.3
H	9.95	0.00	7.95	61 ± 10

[a] 10 mM buffer adjusted to μ = 0.05 M with KCl; buffers were acetate (pH 5.0), MES (pH 6.0), phosphate (pH 7.0), and Tris (pH 8.0). Methanol concentration was 10% by volume. [b] From Jencks and Regenstein (1976). [c] From Fujita et al. (1964), except for the π value for *p*-hydroxybenzaldehyde determined in this work. [d] Errors are standard deviations within a run.

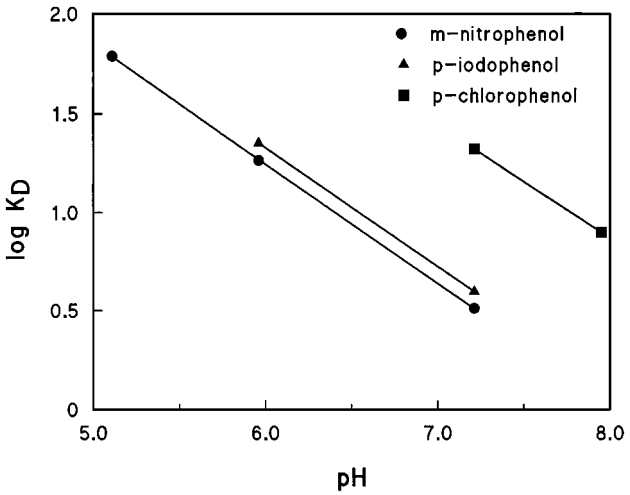


FIGURE 3: Plots of log K_D vs pH for *m*-nitrophenol, *p*-iodophenol, and *p*-chlorophenol. The slope of the parallel lines is −0.59 ± 0.02. K_D values are from Table 1.

for K_D, F_0, and F_{inf}. Values ranging from 92% to 95% quenching at saturation were obtained for the 10 substituted phenols examined. Representative fluorescence titration curves are shown in Figure 2, and calculated values of K_D are given in Table 1. For comparison, the K_D for the complex of *m*-nitrophenol and wild-type KSI was determined at pH 7.2 to be 98 ± 7 μM.

$$[\text{phenol}] = (F_0 - F)\{K_D/(F - F_{inf}) + [E]_t/(F_0 - F_{inf})\} \quad (1)$$

Due to the large variation in the dissociation constants of different substituted phenols, all K_D's could not be deter-

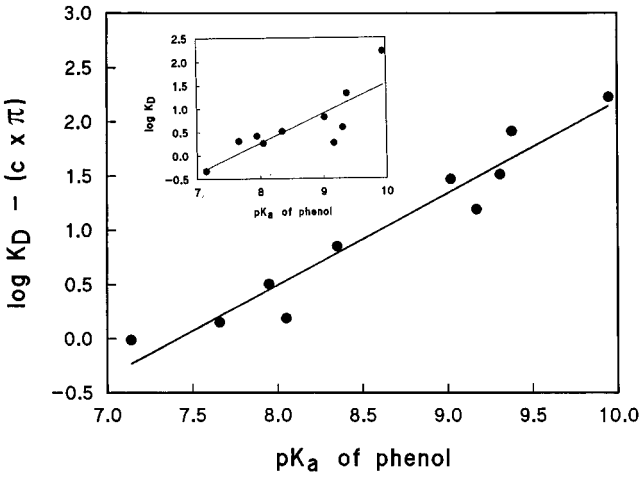


FIGURE 4: Brønsted-type plot for the binding of phenols to D38N at pH 7.2, log K_D corrected for the hydrophobic effect π vs pK_a of the phenol. The slope of the line, α, through the data is 0.85 ± 0.08. Inset: Plot of log K_D (uncorrected) vs pK_a of the phenol (*46*).

mined at a single pH. We took advantage of the pH dependence of binding of intermediate analogs to D38N (*32*), and extrapolated all K_D's to pH 7.2 by assuming that the pH dependence of log K_D vs pH is similar to that for *m*-nitrophenol (pK_a 8.35), *p*-iodophenol (pK_a 9.31), and *p*-chlorophenol (pK_a 9.38). Plots of log K_D vs pH for these phenols give parallel lines with slopes of −0.59 ± 0.02 (Figure 3). A somewhat steeper dependence of K_D upon pH in this pH region (slope ca. −1) was observed for the binding of equilenin to D38N (*32*).

Brønsted Analysis of the Binding of Phenols to D38N. The phenols examined in this work vary significantly in substituent type and position in the ring and their pK_as range from 7.1 to 10.0. We thus attempted to fit the data at pH 7.2 to the classical Brønsted equation (eq 2). Although a linear least-squares fit gives $\alpha = 0.64 \pm 0.16$ and constant = −4.8 ± 1.4 (correlation coefficient r = 0.817), the large errors in the values of the parameters and the poor linear correlation (Figure 4, inset) suggest that the pK_a of the phenols is not the sole determinant of the K_D values.

$$\log K_D = \alpha(pK_a) + \text{constant} \quad (2)$$

Since the active site of KSI is highly hydrophobic, with a local dielectric constant near Tyr-14 in the free enzyme estimated to be 18 ± 2 (*33*), we reasoned that the hydrophobicity of the substituents may be important in determining the binding of phenols to D38N (*42*, *43*). The dissociation constants at pH 7.2 were fit to a modified form of the Brønsted equation (eq 3) with pK_a and π as independent variables. The parameter π for hydrophobicity is defined as $\pi = \log P_{X-\text{phenol}} - \log P_{\text{phenol}}$ where P is the octanol/water partition coefficient (*44*). The values from the least-squares fit are: $\alpha = 0.85 \pm 0.08$, $c = -0.63 \pm 0.11$, and constant = −6.3 ± 0.7 ($r = 0.967$). The substantially improved fit of the data to this equation suggests that both hydrophobicity and electronic effects are important in the binding of phenols to D38N. The effect of pK_a on the dissociation constants of D38N−phenol complexes is emphasized in Figure 4 which shows a plot of log $K_D - (c\pi)$ vs pK_a.

$$\log K_D = \alpha(pK_a) + c\pi + \text{constant} \quad (3)$$

D38N-Induced UV Spectral Shifts of m-Iodophenol. The protonation state of the phenolic hydroxyl group upon binding of phenols to D38N was examined by recording the ultraviolet absorption spectrum of *m*-iodophenol under various conditions. In neutral buffer, the spectrum of the protonated phenol shows two absorption maxima at 225 and 276.5 nm, whereas ionization of *m*-iodophenol in basic solution results in red shifts of both maxima to 239.5 and 295.5 nm, respectively (Figure 5). Complexation of *m*-iodophenol to D38N at pH 7.2 results in spectral changes similar to those in base; i.e., the peak at 225 nm is red-shifted by 13.5 nm to 238.5 nm, and the peak at 276 nm is red-shifted by 13 nm to 289.5 nm. In addition, both peaks are intensified by 1.7- and 2.3-fold, respectively.

^{1}H NMR Spectroscopy of D38N−Phenol Complexes. Recently, a highly deshielded ^{1}H NMR signal (18.15 ppm) was observed between KSI mutants and the intermediate analogs 17β-dihydroequilenin and estradiol-17β-hemisuccinate at −3.3 °C (*31*). This signal is absent in complexes with KSI mutants lacking Tyr-14 (Y14F/Y88F), and was attributed to a proton in an intermolecular low-barrier hydrogen bond between Tyr-14 and the anion of the intermediate analog.

We examined the ^{1}H NMR spectra of several D38N complexes at 4 °C and observed similar low-field resonances. As shown in Table 2, the D38N−phenol complexes, except for the D38N−*m*-chlorophenol complex, exhibit NMR spectral characteristics similar to D38N complexed with equilenin. Furthermore, the decrease in pK_a of the phenolic ligand results in a decrease of the deshielding effect; upfield shifting of the highly deshielded signal by 1.28 and 2.23 ppm is observed when *m*-iodophenol (pK_a = 9.17) is replaced by *p*-hydroxyacetophenone (pK_a = 8.05) and *p*-nitrophenol (pK_a = 7.14), respectively.

DISCUSSION

Phenols as Intermediate Analogs with KSI. Even though the generally accepted view has been that the whole steroid skeleton is essential for KSI−ligand interactions (*41*, *45*), we find that substituted phenols bind tightly to the active site of the D38N mutant of KSI, although they bind substantially less well to wild-type KSI (ca. 30-fold). The tighter binding of simple phenols to D38N than to wild type is analogous to the much tighter binding of equilenin to D38N than to wild type [ca. 10^3-fold; (*32*)]. Several lines of evidence are consistent with a similar type of binding for phenols and steroidal intermediate analogs. (1) Upon complexation of *m*-iodophenol to D38N at pH 7.2, the UV spectrum of the phenol undergoes changes in the position and intensity of the absorption maxima that resemble those upon ionization of the phenol in base (Figure 5). Similar KSI-induced spectral changes have been reported for the absorption spectrum of the phenolic steroid 17β-estradiol when bound to WT (*22*, *34*), as well as for the fluorescence spectra of equilenin and dihydroequilenin bound to WT and several mutants (*30*, *32*, *34*). (2) The D38N−phenol complexes exhibit highly deshielded protons in the ^{1}H NMR (Table 2). These signals are shifted upfield with an increase in the acidity of the phenolic ligand (δ: 15.94 and 14.99

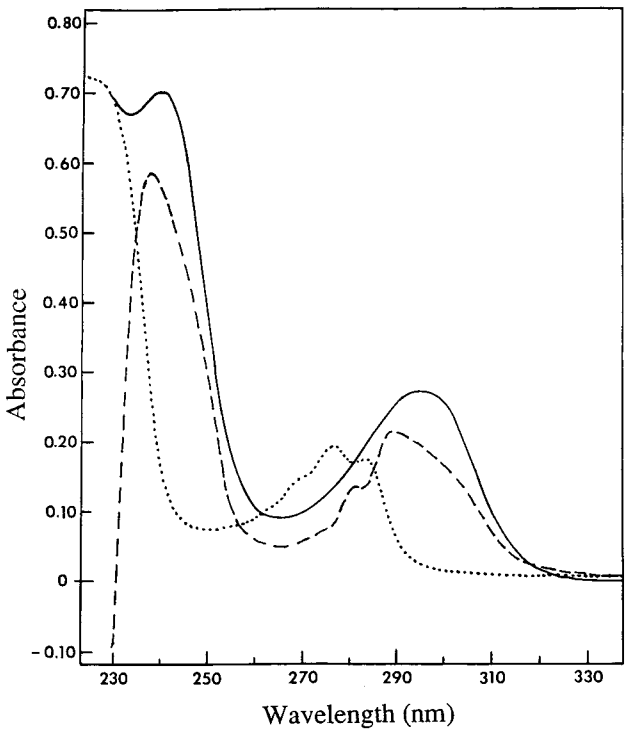


FIGURE 5: Ultraviolet absorption spectra of *m*-iodophenol. Dotted line: 100 μM *m*-iodophenol in 10 mM potassium phosphate, μ = 0.05 M, pH 7.0, 10% methanol (v/v). Solid line: 100 μM *m*-iodophenol in 0.1 M NaOH, 10% methanol (v/v). Dashed line: Calculated spectrum of 48.3 μM *m*-iodophenol bound to D38N. This spectrum was calculated on the basis of a K_D = 1.8 μM by subtracting the spectra of D38N and free *m*-iodophenol (51.7 μM) from the spectrum of a solution of 50 μM D38N and 100 μM *m*-iodophenol in 10 mM phosphate buffer (μ = 0.05, pH 7.0, 10% methanol, v/v).

Table 2: Low-Field ^{1}H NMR Resonances (in ppm) of D38N Complexes at 4 °C[a]

enzyme	pK_a of ligand[b]	downfield resonances	
free D38N		13.11	
D38N−equilenin	∼9[c]	13.11	17.49
D38N−*m*-iodophenol	9.17	12.93	17.22
D38N−*m*-chlorophenol	9.02	13.02	
D38N−*p*-hydroxyacetophenone	8.05	13.09	15.94
D38N−*p*-nitrophenol	7.14	13.16	14.99

[a] NMR samples (0.55 mM in free D38N or D38N complex) were prepared in 10 mM potassium phosphate, 20 mM KCl, and 10% (v/v) CD_3OD, in H_2O at pH 7.2. NMR spectra were obtained at 4 ± 0.5 °C using the 1331 pulse sequence to suppress the water signal. [b] From Jencks and Regenstein (1976). [c] From Davenport et al. (1986).

ppm for *p*-hydroxyacetophenone and *p*-nitrophenol, respectively). Similar observations have been made for D38N complexed with phenolic steroids (*31*). The complex of D38N with dihydroequilenin (pK_a ∼9) shows a downfield signal at 18.15 ppm, whereas the complex of D38N with 4-fluoroestradiol (pK_a 7.4) has a signal at 16.41 ppm. (3) Binding of phenols to D38N quenches the intrinsic fluorescence of the isomerase almost entirely (92−95%), in agreement with earlier fluorescence studies of the interactions of steroid ligands with the enzyme (*33*, *40*, *41*). The fact that phenol binding to D38N results in UV, NMR, and fluorescence spectral changes similar to those induced by phenolic steroids suggests that even though they lack the B, C, and D steroid rings, phenols are good probes for the interaction of the dienol intermediate with KSI. Also important is the similarity of the pK_a's of phenol [pK_a 10.0; (*46*)] and the dienol(ate) intermediate [pK_a 10.0; (*28*)].

Ionization State of Phenols Bound to D38N. Although the ultraviolet and fluorescence spectra of intermediate analogs bound to KSI indicate that these compounds are ionized at the active site, there has been no quantitative assessment of the charge distribution in this complex (*22*, *29*, *40*). In this work, we make use of simple phenols whose acidity can be varied to determine the effect of pK_a on binding to KSI. A simple Brønsted analysis (Figure 4, inset) reveals a trend toward better binding as the acidity of the phenol is increased, but the scatter in the data is considerable. However, a dual parameter analysis using the hydrophobicity parameter π (*44*) shows that the data are accommodated quite well by a model with pK_a and π as independent variables (eq 3; Figure 4). In this correlation, the pK_a values of phenols in aqueous solution are used even though the acidities of the phenolic ligands increase significantly at the active site of KSI [Figure 5; (*29*)]. The assumption is made that the pK_a's of all phenols are equally perturbed when they are bound to D38N (*47*). The absolute value of c (0.63 ± 0.11) which was determined by linear regression on eq 3 falls within the expected range of coefficients for the hydrophobicity parameter π (0.4−1.1) for quantitative biological structure−activity relationships (*44*).

The α value is a measure of the extent to which of proton transfer resembles the complete proton transfer in the reference ionization reaction (*48*). In order to provide evidence concerning the ionization state of phenols at the active site of D38N, it is necessary that the reference state (water) be an appropriate model for the active site. Of particular concern is the difference in dielectric constant of water [ϵ 78; (*49*)] and of the hydrophobic active site of KSI [ϵ 18; (*33*)]. Although the dependence of the acidity of phenols on substituent is greater in the gas phase and in dimethyl sulfoxide than in water, this difference appears primarily to be due to the greater hydrogen bonding ability of water, rather than a difference in the dielectric constant (*50*). In support of this conclusion, the ρ value for ionization of phenols varies only slightly (ca. 10%) in aqueous solutions of methanol [ϵ 32.7; (*49*)] and ethanol [ϵ 24.5; (*49*)] as the percentage of the alcohol is increased from 0 to 100% (*51*, *52*, *53*).

The high value of α (0.85 ± 0.08) indicates that stabilization of KSI-bound phenols and, by implication the dienol(ate) intermediate, is provided by interactions that do not involve substantial protonation of the anion. These results are consistent with ordinary hydrogen bond(s) that are

[1] The energy of the hydrogen bond between Tyr-14 and the intermediate has been estimated to be >7 kcal/mol (*56*) on the basis of a comparison of the rates of dissociation of the intermediate from the Y14F (>39 s^{-1}) and D38N (<10^{-4} s^{-1}) mutants of KSI. However, the rate of dissociation from D38N is a poor model for the rate of dissociation from wild type, since D38N binds the intermediate substantially stronger than does wild type (*32*). It is more appropriate to compare the rates of dissociation from Y14F and WT [∼2100 s^{-1}, calculated from the K_D of 2.4 μM (*32*) and an assumed association rate constant of 8.6 × 10^8 M^{-1} s^{-1}]. The similarity of the rates of dissociation of the intermediate from Y14F and WT makes it impossible to come to any conclusion about the energy of the interaction with the OH of Tyr-14.

asymmetric (double potential well) with the proton(s) closer to the enzyme acid(s) and are indicative of an anionic (dienolate) intermediate, rather than a neutral dienol or a low-barrier hydrogen bond to the intermediate. Although it has been postulated that enzymatic stabilization of enol(ate)s by hydrogen bonding results in partial transfer of the proton to the enol(ate) intermediate (*7*, *54*), in the case of KSI, this proton transfer is probably minimal.

The hydrogen bonds from Tyr-14 and Asp-99 are likely to be the main contributors to the enhanced stability of the bound dienolate ion compared to bound substrate (*24*). The free energy profile for KSI (*19*, *32*) shows that this intermediate is stabilized by ca. 11 kcal/mol relative to substrate at the active site of KSI. Two hydrogen bonds of ca. 5 kcal/mol, with some contribution to stability from F101 *(55*), would be sufficient to account for the decreased energy of this complex.[1]

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