

Proton Affinity of the Oxyanion Hole in the Active Site of Ketosteroid Isomerase[†]

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ABSTRACT: The absorption spectra of a series of inhibitors bound at the active site of Δ^5 -3-ketosteroid isomerase from *Pseudomonas putida* were found to exhibit substantial variations in the contributions of the protonated and deprotonated forms. Systematic variation of the inhibitor solution pK_a combined with a method of quantifying the contributions of each protonation state showed the oxyanion hole in the active site of wild-type Δ^5 -3-ketosteroid isomerase to have a proton affinity equal to a solution pK_a of 10.05 ± 0.03 , which is similar to the measured pK_a (10.0) of the reaction intermediate. This observation supports the prediction of Cleland, Kreevoy, Frey, Gassman, and Gerlt that an enzyme utilizing a strong hydrogen bond for catalysis matches the proton affinity of the protein to the intermediate [Cleland, W. W., and Kreevoy, M. M. (1994) *Science* 264, 1887–1890; Frey, P. A., Whitt, S., and Tobin, J. (1994) *Science* 264, 1927–1930; Gerlt, J. A., and Gassman, P. G. (1993) *Biochemistry* 32, 11934–11952]. As the difference in proton affinity decreases, the strength of the hydrogen bond increases, and the closely matched proton affinity between the active site and the reaction intermediate supports the possibility that a short, strong hydrogen bond is catalytically relevant in Δ^5 -3-ketosteroid isomerase.

The origin(s) of catalytic rate enhancement in enzymes remains an open and important question. In some enzymes, short, strong hydrogen bonds (SSHBs)¹ between the substrate and protein have been proposed to produce a rate enhancement by stabilizing high-energy reaction intermediates (1–4). To form a SSHB, the proton affinity of the substrate must approximate the proton affinity of its hydrogen-bonding partner. In this paper, we present evidence of a matched proton affinity between a reaction intermediate and its hydrogen-bonding partner in the active site of Δ^5 -3-ketosteroid isomerase (KSI) from *Pseudomonas putida* (*pKSI*) and *Comamonas testosteroni* (*tKSI*). The observation of a matched proton affinity suggests that the formation of a SSHB may be possible and catalytically relevant in KSI.

KSI catalyzes a C–H bond cleavage and formation through an enolate intermediate at a diffusion-limited rate as depicted in Figure 1 (5). The general base Asp40 abstracts a proton from position 4 of the steroid ring to form an enolate that is stabilized by the hydrogen bond donating Tyr16 and Asp103 (all residue numbers use the *P. putida* sequence unless specifically describing *tKSI*). Tyr16 and Asp103 are positioned deep within the hydrophobic active site and form a so-called oxyanion hole. Protonated Asp40 then transfers its proton to position 6 of the steroid ring to complete the reaction. The hydrogen bonds from Tyr16 and Asp103 are known to significantly affect the rate of catalysis in KSI. Mutation of either Tyr16 or Asp103 in *pKSI* to nonpolar residues reduces the catalytic efficiency by $10^{3.4}$ or $10^{2.1}$, respectively (6). In *tKSI*, mutation of Tyr14 (homologous to Tyr16 in *pKSI*) results in a $10^{4.7}$ (7) reduction in catalytic efficiency, while

the mutation of Asp99 (homologous to Asp103 in *pKSI*) reduced the catalytic efficiency by $10^{3.6}$ (8). We note, however, that all amino acid residues in the active site interact strongly and contribute to catalysis, so site-directed mutagenesis can produce secondary effects that may lead to misinterpretation.

Gerlt and Gassman 15 years ago proposed the formation of unusually short, strong hydrogen bonds between the KSI oxyanion hole and the reaction intermediate as a means of catalytic rate enhancement (1, 2). In their model, high-energy states along the reaction coordinate are specifically stabilized by the formation of these bonds. Since then, the catalytic role of SSHBs has been debated (9, 10). Crystal structures of phenol, a reaction intermediate analogue, bound to the active site of *pKSI* show the O–O distance between phenol and the oxyanion hole residues to be 2.49 Å for Tyr16 and 2.61 Å for Asp103 (11). Also, far downfield shifts of ~16–18 ppm associated with the oxyanion hole protons in NMR studies have been interpreted as evidence of SSHB activity (12–15). The term “short, strong hydrogen bond” is general but is sometimes used interchangeably with “low-barrier hydrogen bond” (LBHB). As originally conceived, the LBHB is a specific physical state in which the proton involved in a hydrogen bond becomes delocalized between heavy atom centers; that is, the barrier between hydrogen bonding partners is low (16, 17). The resulting potential energy surface is broader than that for a normal hydrogen bond; the zero-point vibrational energy is lowered, and this produces a dramatically stabilized hydrogen bond. LBHBs have been proposed to be present in KSI on the basis of NMR (12–14) and crystallographic evidence (18). In a related system, photoactive yellow protein, where such data likewise support the presence of a LBHB (19, 20), neutron scattering experiments have more directly identified a LBHB via observation of a proton located equidistant from the two hydrogen-bonding oxygen centers, not localized to either heavy atom (21).

Several conditions must be met to allow formation of a SSHB. First, the dielectric constant of the medium surrounding the

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¹Abbreviations: DMSO, dimethyl sulfoxide; KSI, ketosteroid isomerase; LBHB, low-barrier hydrogen bond; NMR, nuclear magnetic resonance; *pKSI*, ketosteroid isomerase from *P. putida*; *tKSI*, ketosteroid isomerase from *C. testosteroni*; SSHB, short, strong hydrogen bond.

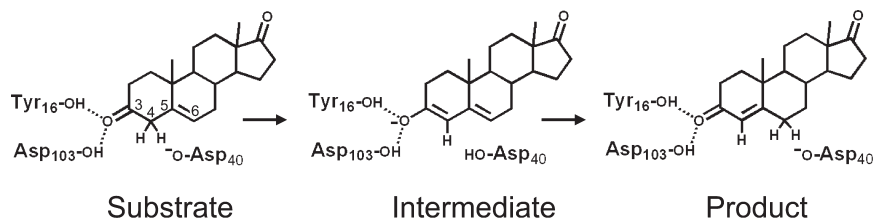


FIGURE 1: Schematic of the enzymatic isomerization of a Δ^5 -3-ketosteroid to a Δ^4 -3-ketosteroid as observed in KSI from *P. putida* and *C. testosteronei*. Residues are numbered according to the *P. putida* sequence. In *C. testosteronei*, Tyr16 is Tyr14, Asp103 is Asp99, and Asp40 is Asp38.

hydrogen bond must be low. In the gas phase, the heat of formation of hydrogen bonds can reach -25 to -30 kcal/mol (22). As the dielectric constant of the medium increases to ~ 6 , the maximum strength of the hydrogen bonds decreases to approximately half the gas phase value (23). The KSI active site around Tyr16 and Asp103 is often considered a low-dielectric medium because the site is largely composed of hydrophobic amino acids, and intermediate analogues have been shown to bind with increasing affinity as analogue hydrophobicity is increased. For example, equilenin binds approximately 3 orders of magnitude tighter than substituted phenols (11), and as shown below, the K_d for substituted naphthols binding to KSI falls between that for the substituted phenols and equilenin (the larger analogues sample a much larger area of the active site than just the oxyanion hole which complicates simple interpretations of binding data). Other researchers have observed Tyr14 in *t*KSI has a perturbed pK_a and enhanced fluorescence quantum yield compared to the tyrosine solution values, and these observations have been interpreted as evidence of a low dielectric constant in the active site (24). However, the pK_a of Tyr14 is probably perturbed by its immediate hydrogen bonding partners as well as from the local dielectric (25). A more precise and local measure of the dielectric is provided by dynamic Stokes shift experiments, which are presented in a separate paper (26). The dynamic Stokes shift experiments demonstrate only a very limited response of the oxyanion hole to a sudden change in the pK_a of a bound fluorophore, indicating a limited capacity for solvating a charge shift, which can be interpreted as a low-dielectric environment.

Another important condition for SSHB formation and the role of SSHBs in enzymatic catalysis is the matching of proton affinities between the hydrogen bond donor and acceptor groups. Formation of SSHBs to the high-energy species along the reaction coordinate contributes to catalysis by stabilizing the formation of these species. SSHB formation is governed by the changing proton affinities between the reaction ground state and high-energy intermediate during the catalytic cycle. If SSHBs are active in KSI, then the SSHB must be formed to the intermediate, not the starting substrate. In this work, we compare the proton affinity of the oxyanion hole to that of the reaction intermediate and demonstrate that they are well matched.

Proton affinity is often described by a pK_a value; however, pK_a is a term defined for bulk solvent. Comparing the proton affinities by comparing pK_a values of two small molecules titrated in the same solvent is entirely reasonable; however, the oxyanion hole is deep within a hydrophobic pocket where the environment cannot be described as aqueous. Therefore, titrating the oxyanion hole may provide a pK_a for the hydrogen bonding residues, but that pK_a is not comparable to the solution values obtained for the reaction intermediate or its analogues. Instead of titrating the active site residues by changing the solution pH, we have compared the proton affinity of the oxyanion hole to that of substituted

naphthols by determining the degree to which the naphthol is protonated while bound to the enzyme. This is quantified by measuring the contributions of protonated and deprotonated substituted naphthols whose electronic absorption spectra are sufficiently different to be observed directly. The proton affinity of the oxyanion hole is reported in terms of the solution pK_a that would bind as a 50:50 mixture of protonated and deprotonated states. Wild-type KSI, especially *p*KSI, is found to have a proton affinity matched to the reaction intermediate, and the matched proton affinity between the intermediate and oxyanion hole is consistent with enzymatic rate enhancement utilizing SSHBs.

METHODS

Protein expression and purification followed standard methods that have been described in detail previously (11). Electronic absorption measurements were conducted with either a PerkinElmer Lambda 25 or Cary 6000i spectrophotometer. Unless otherwise specified, absorption spectra of *p*KSI–naphthol complexes were recorded with 1 mM protein and 0.5 mM naphthol in 40 mM potassium phosphate buffer (pH 7) and 1% DMSO, with protein-alone solutions serving as a blank. Experiments with *t*KSI were performed with 1.6 mM protein and 62.5 μ M naphthol under conditions otherwise identical to those of *p*KSI. High-pH basis spectra were recorded in a 0.5 M NaOH/20% DMSO solution, and a 0.5 M HCl/20% DMSO solution was used for low-pH basis spectra for all naphthols except equilenin, which required 50% DMSO for solubility. All spectra were recorded in a 1 mm quartz cuvette.

Protein concentrations were determined by absorption at 280 nm. As determined by the Scripps protein calculator (27, 28), *p*KSI and *t*KSI have molar extinction coefficients at 280 nm of 16500 and 3840 $M^{-1} cm^{-1}$, respectively.

Binding constants were determined by monitoring changes in the fluorescence emission spectra of naphthols upon binding to KSI. Fluorescence emission spectra (330 nm excitation for all samples except 320 nm for 6-cyano-2-naphthol) were recorded for naphthols free in solution [16.5 μ M substituted naphthol (pH 7) and 40 mM potassium phosphate buffer with 20% DMSO] and bound to *p*KSI D40N [16.5 μ M substituted naphthol (pH 7), 40 mM potassium phosphate buffer, and 2 mM *p*KSI D40N]. The D40N mutation is commonly used to improve binding of the intermediate analogue to *p*KSI. Nearly complete binding of the substituted naphthol by *p*KSI under the previously described conditions was guaranteed by demonstrating that the emission line shape from the 16.5 μ M substituted naphthol was independent of *p*KSI D40N concentration from 2 mM to at least 0.5 mM. To determine K_d values, spectra were then recorded under conditions where both bound and unbound populations could be observed [16.5 μ M substituted naphthol, 25 μ M *p*KSI D40N (pH 7), and 40 mM potassium phosphate buffer with 20% DMSO and 25 μ M substituted naphthol, 25 μ M *p*KSI D40N

(pH 7), and 40 mM potassium phosphate buffer with 20% DMSO]. The spectra showing both populations were fit as a linear combination of the bound and free fluorescence spectra. The resulting fits yielded the concentration of bound and unbound naphthol, and the concentrations were used directly to determine the K_d by simple equilibrium theory.

As described below, some of the protein-bound electronic absorption spectra of naphthols bound at the active site of *p*KSI are clearly a mixture of the protonated and deprotonated form of the naphthol that depends on the particular naphthol. The contributions from these forms cannot be obtained simply by taking a linear combination of the protonated and deprotonated basis spectra obtained in aqueous solution due to additional shifts in the absorption bands of both the neutral and anionic forms of the ligands when they are bound in the protein active site. To deal with the shifts and still obtain quantitative information about the populations of protonated and deprotonated naphthols, we start with a linear combination of the aqueous solution basis spectral line shapes and band positions, and then the basis spectra are allowed to shift in energy in 10 wavenumber steps, retaining their line shape to determine if shifting the spectra produces a better fit. If a better fit is found, then a new linear combination is determined with the shifted basis spectra. The process is repeated until the algorithm converges, usually ~ 10 iterations. Thus, each fit yields the ratio of the protonated and deprotonated forms, which are used to describe the proton affinity of the oxyanion hole, and two band shifts. These shifts reflect the interaction of the polarizable chromophore with the protein environment, which is expected to be substantially different from the aqueous environment used to obtain the basis spectra; however, they do not bear on the protonation state of the naphthol, and we do not attempt to interpret these shifts.

RESULTS AND DISCUSSION

Binding Constants for Substituted Naphthols. Substituted naphthols bind well to *p*KSI D40N and less well to the wild type. The D40N mutation is commonly used as it improves intermediate analogue binding (29); for example, in *p*KSI D40N, the K_d of equilenin is < 1 nM (11). The K_d values for the other naphthols were determined as described above to be 20 ± 6 μ M for 2,6-dihydroxynaphthalene, 4 ± 1 μ M for 6-bromo-2-naphthol, 10 ± 5 μ M for 6-methoxy-2-naphthol, and 2 ± 1 μ M for 6-cyano-2-naphthol (data not shown). Solvent conditions were similar to those used in the absorption experiments (pH 7 and 40 mM potassium phosphate) except a 1:5 mixture of DMSO and 40 mM potassium phosphate buffer (pH 7) was used to facilitate naphthol solubility. As DMSO increases the solubility of the naphthols in solution, we regard the measured K_d values to be upper limits for the solvent conditions used in the absorption experiments. Even for the poorest binding, 2,6-dihydroxynaphthalene, we calculate greater than 96% of the naphthol is bound to *p*KSI at the concentrations used for the absorption experiments.

Electronic Absorption Spectra of Substituted Naphthols Bound to *p*KSI. Figure 2 shows the absorption spectra of equilenin, an intermediate analogue with a solution pK_a of 9.7, at high and low pH (the basis spectra), and the spectrum of equilenin bound to the active site of *p*KSI D40N. The absorption spectrum of equilenin bound in the protein is clearly different from either of the basis spectra, and the three poorly resolved peaks observed in the protein-bound spectrum are consistent with both protonation states being present.

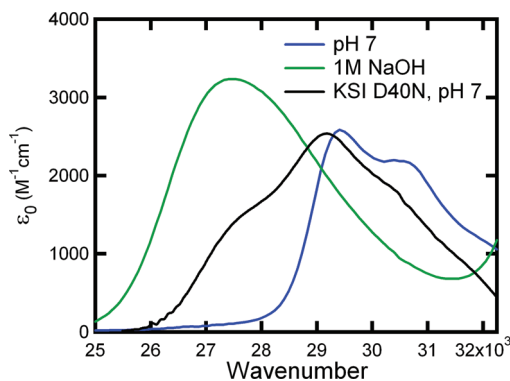


FIGURE 2: Absorption spectra of protonated equilenin in a 1:1 dimethyl sulfoxide/40 mM potassium phosphate buffer (pH 7) mixture (blue), deprotonated equilenin in a 1:1 dimethyl sulfoxide/1 M sodium hydroxide solution (green), and equilenin bound to *p*KSI D40N (black). The protein-bound equilenin is a nearly equal mixture of the protonated and deprotonated forms (see the text and Table 1).

There are conflicting data in the literature about the protonation state of substituted naphthols bound to *t*KSI. Pollack and co-workers argue that the naphthols are completely anionic when bound to *t*KSI (30), while others have observed a mixture of protonation states (31). We show below that the protonation state of the bound naphthol depends on its solution pK_a . Also, many of the previous experiments designed to determine the protonation state of the reaction intermediate used *t*KSI with its common D38N mutation for enhanced binding affinity. However, as discussed below, the proton affinity of the *t*KSI D38N oxyanion hole is significantly higher than in wild-type *t*KSI or *p*KSI. As a result, observations of a fully anionic substrate in *t*KSI D38N may not reflect the protonation state of the intermediate during catalysis.

Proton Affinity of the Oxyanion Hole. The acid–base equilibrium between the protonation of the oxyanion hole, A, and the substrate, B, is described by eq 1:



and the modified Boltzmann distribution by eq 2.

$$\frac{[\text{A}^- \cdots \text{BH}]}{[\text{AH} \cdots \text{B}^-]} = \exp\{-2.3[\text{p}K_a(\text{B}) - \text{p}K_a(\text{A})]\} \quad (2)$$

where A is the titratable protein group, which may be influenced by contributions from several residues in the oxyanion hole. We expect that the proton transfer originates from either of the residues that form hydrogen bonds to the substrate hydroxyl, Tyr16 or Asp103; however, we need not be more specific about which of these two centers transfers a proton to the substrate, and the data presented here do not directly address this question (see ref 25 for more discussion of the identity of the proton donor). The left side of eq 2 can be rearranged as shown in eq 3, where $[\text{C}]_T$ is the total concentration of the complex and $\%A^-$ is the percent of A deprotonated at equilibrium.

$$\frac{[\text{A}^- \cdots \text{BH}]}{[\text{AH} \cdots \text{B}^-]} = \frac{[\text{C}]_T \times \%A^-}{[\text{C}]_T(1 - \%A^-)} = \frac{\%A^-}{1 - \%A^-} \quad (3)$$

Rearranging eqs 2 and 3 leads directly to eq 4.

$$\%A^- = \frac{1}{1 - \exp\{-2.3[\text{p}K_a(\text{B}) - \text{p}K_a(\text{A})]\}} \quad (4)$$

Figure 3 shows the absorption spectra of substituted naphthols with varying pK_a values bound to the KSI active site.

The protein-bound absorption spectrum of each naphthol is fitted to a combination of high- and low-pH solution spectra. As

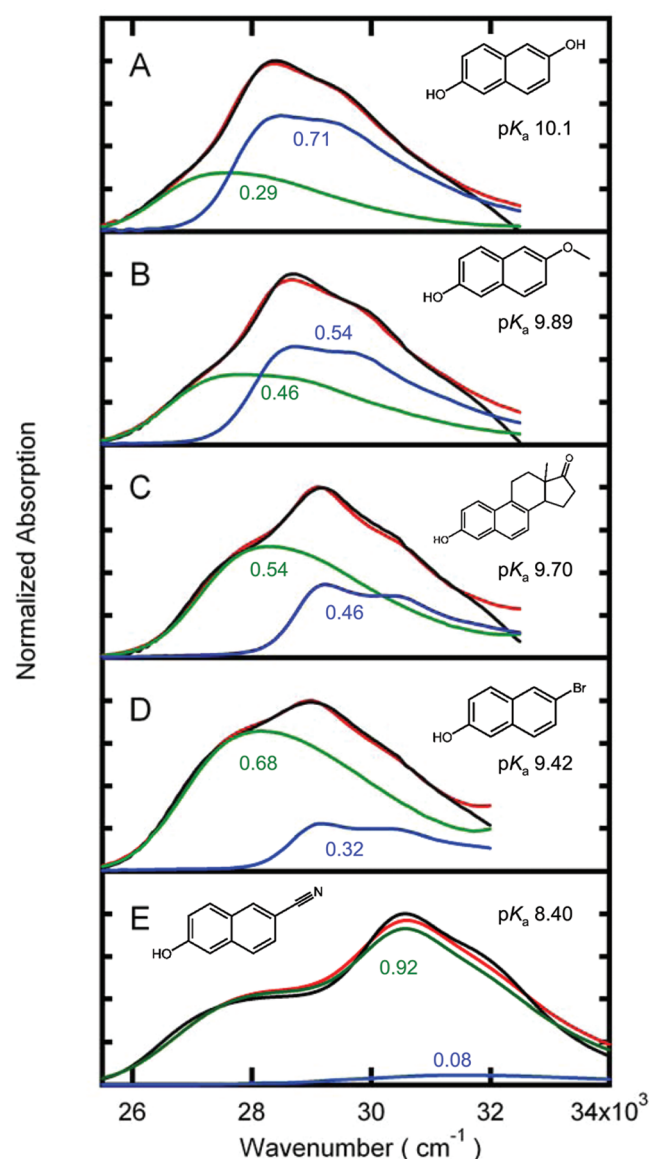


FIGURE 3: Absorption spectra of substituted naphthols (black, normalized at the absorption maximum) fit to a linear combination (red) of the protonated (blue) and deprotonated (green) species, each shifted as described in the text and in Table 1. The protonated and deprotonated spectra are scaled by their relative contributions to the fit (Table 1). The scaling factors are shown in the color corresponding to the curve they describe: (A) 2,6-dihydroxynaphthalene, solution pK_a of 10.1; (B) 6-methoxy-2-naphthol, solution pK_a of 9.89; (C) equilenin, solution pK_a of 9.7; (D) 6-bromo-2-naphthol, solution pK_a of 9.42; and (E) 6-cyano-2-naphthol, solution pK_a of 8.4.

described in the Methods, fitting the high- and low-pH spectra to the protein-bound spectrum requires a shift in the transition energies of the solution spectra. The fraction of protonated form bound to KSI and the transition energy shifts are listed in Table 1.

It is evident from the spectra in Figure 3 that substituted naphthols with different solution pK_a values bind to the $pKSI$ D40N active site with varying degrees of protonation. A comparison of the degree of protonation while bound to $pKSI$ (Table 1) with the solution pK_a of each substituted naphthol yields a proton affinity curve (analogous to a titration curve) for the $pKSI$ D40N active site, as shown in Figure 4. This proton affinity curve cannot be used to determine the actual pK_a of titratable groups in the oxyanion hole. The pK_a of any titratable group is a solvent-defined quantity; usually, the solvent is water, and the pK_a values listed for the naphthols are the aqueous values. However, the active site constitutes a very specific solvent environment highly perturbed from water. By challenging the active site of $pKSI$ with naphthols whose solution pK_a values are quite different, we can compare the proton affinity of the oxyanion hole to that of the substituted naphthol. Therefore, the value determined by the proton affinity curve in Figure 4 is not the pK_a of the oxyanion hole; rather, the oxyanion hole has a proton affinity equal to that of a naphthol with a solution pK_a of that value.

The proton affinity of the oxyanion hole can be found with eq 4 via variation of the inhibitor solution pK_a and measurement of the degree of protonation. As shown in the fit in Figure 4, we observe the proton affinity of the oxyanion hole in $pKSI$ D40N to match that of a naphthol with a solution pK_a of 9.76 ± 0.03 . The high precision of this measurement is possible because the fit of

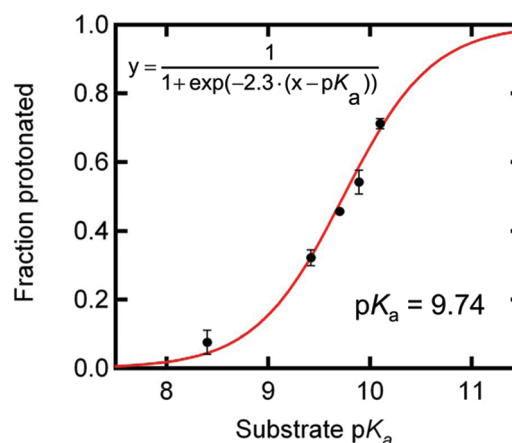


FIGURE 4: Fraction of substrate bound as the protonated species (Table 1) vs the substrate pK_a (black dots) fit to eq 4 (red line) to determine the proton affinity of the active site. Error bars indicate one standard deviation.

Table 1: Shifts in Absorption Band Maxima Relative to Those in Solution Resulting from the Binding of Naphthols in the $pKSI$ Active Site and the Fraction of the Neutral Species Observed when Bound^a

ligand	protein	neutral shift (cm^{-1})	anionic shift (cm^{-1})	fraction neutral
equilenin	wild type	-280 ± 17	446 ± 28	0.31 ± 0.02
equilenin	D40N	-180 ± 14	730 ± 56	0.456 ± 0.005
6-bromo-2-naphthol	D40N	-475 ± 7	190 ± 28	0.32 ± 0.02
2,6-hydroxynaphthalene	D40N	-405 ± 63	1230 ± 212	0.71 ± 0.01
6-cyano-2-naphthol	D40N	-1480 ± 280	275 ± 7	0.08 ± 0.03
6-methoxy-2-naphthol	D40N	-390 ± 14	235 ± 7	0.54 ± 0.03

^aErrors indicate one standard deviation.

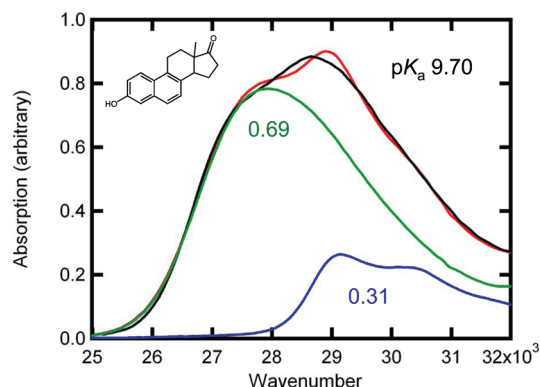


FIGURE 5: Absorption spectrum of equilenin bound to wild-type *pKSI* (black) fit to a linear combination (red) of the protonated (blue) and deprotonated (green) species. The protonated and deprotonated spectra are scaled by their relative contributions to the fit, and the scaling factors are shown below each curve.

eq 4 to the data in Figure 4 requires only one adjustable parameter. The shape of the sigmoid is determined by acid–base theory, as described above in the derivation of eq 4 with the end points set to 0 and 100% protonated by definition.

Absorption spectra of equilenin bound to wild-type *pKSI* also show a mixture of protonation states, as shown in Figure 5. Wild-type *pKSI* binds the substituted naphthols more weakly than *pKSI* D40N, and this weaker binding significantly limits our ability to probe the active site with multiple substituted naphthols as most bind with a K_d of > 1 mM. However, equilenin binds to wild-type *pKSI* with a K_d of $1.8 \mu\text{M}$ (32), which is sufficient to ensure nearly 100% binding under the conditions of the experiment. On the basis of only the contributions from the protonated and deprotonated forms of equilenin, the proton affinity of the oxyanion hole of wild-type *pKSI* is observed to match a solution pK_a of 10.05 ± 0.03 . As discussed below, the solution pK_a of the reaction intermediate has been measured to be 10.0 (33), and the matched proton affinity between the protein and reaction intermediate suggests SSHBs may form during the catalytic cycle.

Electronic Absorption Spectra of Equilenin Bound to *tKSI*. If the matched proton affinity found in *pKSI* is observed in *KSI* from other species, then the matched proton affinity is likely an important, conserved feature of the active site, and the proton affinity would not be expected to vary much from species to species that share the same natural substrate. There is insufficient evidence in the literature to guarantee that *tKSI* and *pKSI* utilize exactly the same catalytic mechanism; however, they share 34% sequence identity, and the active site of *tKSI* has analogues of the most catalytically relevant residues, Asp40, Asp103, Tyr16, and Tyr57, in *pKSI*. The structural similarities between *tKSI* and *pKSI* suggest at least a similar catalytic mechanism takes place in both enzymes.

Electronic absorption spectra of substituted naphthols bound to *tKSI* with the active site D38N mutation (analogous to D40N in *pKSI*) show more of the anionic species than when bound to *pKSI*. The most basic of the naphthols, 2,6-dihydroxynaphthalene, bound as only $\sim 30\%$ protonated to *tKSI* D38N (data not shown), while in *pKSI* D40N, 2,6-dihydroxynaphthalene was found to be 71% protonated. Because most of the substituted naphthols fall near 0% protonated on the proton affinity curve, the proton affinity of the *tKSI* D38N oxyanion hole cannot be measured as accurately as in *pKSI* D40N. Nonetheless, from this

much more limited data, a proton affinity curve was created for *tKSI* and the proton affinity was estimated to be equal to a solution pK_a of ~ 10.5 for *tKSI* D38N. Like wild-type *pKSI*, wild-type *tKSI* does not bind the substituted naphthols sufficiently well to construct a proton affinity curve. However, equilenin binds to *tKSI* with a K_d of $3 \mu\text{M}$ (29), and experiments with equilenin like those described above for wild-type *pKSI* show a proton affinity of wild-type *tKSI* to be equal to a solution pK_a of 10.3 ± 0.1 .

The proton affinity observed in wild-type *tKSI* is similar but not identical to that of wild-type *pKSI*. The 0.3 pK_a unit difference may indicate the two enzymes utilize hydrogen bonds differing in strength during catalysis or, possibly, *tKSI* has been optimized to catalyze the isomerization of steroids with a slightly different proton affinity compared to that of the canonical steroid shown in Figure 1. However, the D40N (*pKSI*) and D38N (*tKSI*) mutations indicate that a small number of mutations can affect the oxyanion hole proton affinity, and the similarity of the two wild-type proton affinities suggests a proton affinity near 10 is functionally relevant. As described above, if the proton affinity were not functionally relevant, then the two proteins could have oxyanion hole proton affinities that diverge over time.

Connection between Active Site Proton Affinity and Catalytic Efficiency. The proton affinity of the oxyanion hole in *pKSI* and *tKSI* is similar to the proton affinity of the intermediate shown in Figure 1. The intermediate in Figure 1 is widely considered to be representative of intermediates produced in vivo. Zeng and Pollack synthesized the intermediate shown in Figure 1 and determined the solution pK_a to be 10.0 (33). We have presented evidence that the proton affinity of the oxyanion hole is equal to a solution pK_a of 10.05 ± 0.03 in *pKSI* and 10.3 ± 0.1 in *tKSI*. A matched proton affinity between the oxyanion hole and reaction intermediate has previously been proposed by Cleland, Kreevoy, Frey, Gassman, and Gerlt (1–4) to enhance the catalytic rate in *KSI*, and the observation of the matched proton affinity supports this claim.

Matching the proton affinity between the reaction intermediate and oxyanion hole is believed to produce a catalytic rate enhancement by stabilizing the intermediate via the formation of strong hydrogen bonds. Other laboratories have examined the dependence of hydrogen bond strength versus the difference in proton affinity between the hydrogen bonding groups (34, 35). Decreasing the difference in proton affinity between two hydrogen bonding groups does lower the heat of formation for a hydrogen bond. The degree to which the heat of formation decreases depends on other factors as well, particularly the solvent. Shan et al. (34) have shown $\log K_{\text{HB}}$, where K_{HB} is the equilibrium constant of hydrogen bond formation, to vary linearly with ΔpK_a in both water (dielectric constant of 80) and dimethyl sulfoxide (dielectric constant of 47.2) with slopes of 0.05 and 0.73, respectively. Taking the value of 0.73 to be more relevant for the hydrophobic *KSI* active site, the hydrogen bond becomes 0.95 kcal/mol less stable for every pK_a unit mismatch between hydrogen-bonding partners. Ab initio calculations of LBHB systems in vacuum have determined a 1.8 kcal/mol penalty to hydrogen bond stability for every unit of pK_a mismatch between hydrogen-bonding partners (35). Either value suggests a large stabilization of the intermediate in *KSI*. Given the 0.95 kcal/mol value, the difference in ΔG° for hydrogen bond formation between the oxyanion hole and the ketone starting material with a conjugate acid pK_a of approximately -2 versus the intermediate with a matched pK_a would be 11.5 kcal/mol.

However, we note that the assumption of linearity for $\log K_{\text{HB}}$ versus ΔpK_a is not necessarily valid over the 12 pK_a unit range.

Regardless of whether the hydrogen bonds in KSI are of the low-barrier variety, there is a significant amount of stabilization energy available for producing a rate enhancement. Åqvist and Feierberg (36) have used empirical valence bond simulations to determine that stabilization of the intermediate accounts for 60% of the rate enhancement observed in p KSI, with electrostatic preorganization of the transition state accounting for the balance. However, they find no evidence of LBHB activity.

Hydrogen bonds exist between the oxyanion hole and substrate during the course of the reaction. We have shown the proton affinity of a reaction intermediate is similar to the proton affinity of the oxyanion hole. This observation suggests catalytic rate enhancement is at least partially achieved via variation of the proton affinity between the substrate and oxyanion hole. As the reaction intermediate is formed, the difference in proton affinity between the substrate and oxyanion hole decreases, and the increasingly stable hydrogen bonds stabilize the reaction intermediate. Our observations therefore support predictions by Cleland, Kreevoy, Frey, Gassman, and Gerlt (1–4), who theorized a hydrogen bond could produce a catalytic rate enhancement if the proton affinities between the protein and substrate are matched for the high-energy states along a reaction coordinate.

CONCLUSION

Substituted naphthols with solution pK_a values of ~ 10 bind to the active site of p KSI and t KSI as a mixture of protonation states. By quantifying the degree of protonation at the active site as a function of solution pK_a , we obtained a proton affinity curve for the oxyanion hole. We determined from this proton affinity curve that p KSI D40N from *P. putida* has a proton affinity equal to a solution pK_a value of 9.76 ± 0.03 and wild-type p KSI has a value of 10.05 ± 0.03 . The same experiment performed on t KSI from *C. testosteroni* yields proton affinities equal to ~ 10.5 and 10.3 ± 0.1 for the D38N mutant and wild type, respectively. The similarity of the wild-type KSI proton affinities to the measured solution pK_a of the reaction intermediate shown in Figure 1 supports theories of a catalytic rate enhancement via changes in hydrogen bond stabilization energy along the reaction coordinate.

Catalytic rate enhancement in KSI probably results from a combination of active site characteristics, including electrostatic preorganization, geometrical constraints, and SSHBs. We provide evidence that KSI is particularly well suited to forming SSHBs between the high-energy intermediate and oxyanion hole as they appear to have equal proton affinity. The formation of SSHBs results in a catalytically relevant amount of stabilization energy, which is available only to chemical species whose proton affinity matches that of the oxyanion hole. Such an oxyanion hole whose proton affinity is matched to the reaction intermediate allows KSI to stabilize the intermediate relative to the starting materials through a SSHB.

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