

Uncovering the Determinants of a Highly Perturbed Tyrosine pK_a in the Active Site of Ketosteroid Isomerase

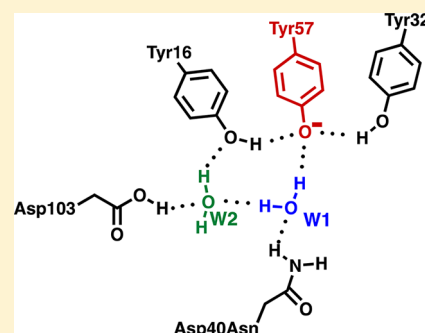
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S Supporting Information

ABSTRACT: Within the idiosyncratic enzyme active-site environment, side chain and ligand pK_a values can be profoundly perturbed relative to their values in aqueous solution. Whereas structural inspection of systems has often attributed perturbed pK_a values to dominant contributions from placement near charged groups or within hydrophobic pockets, Tyr57 of a *Pseudomonas putida* ketosteroid isomerase (KSI) mutant, suggested to have a pK_a perturbed by nearly 4 units to 6.3, is situated within a solvent-exposed active site devoid of cationic side chains, metal ions, or cofactors. Extensive comparisons among 45 variants with mutations in and around the KSI active site, along with protein semisynthesis, ¹³C NMR spectroscopy, absorbance spectroscopy, and X-ray crystallography, was used to unravel the basis for this perturbed Tyr pK_a . The results suggest that the origin of large energetic perturbations are more complex than suggested by visual inspection. For example, the introduction of positively charged residues near Tyr57 raises its pK_a rather than lowers it; this effect, and part of the increase in the Tyr pK_a from the introduction of nearby anionic groups, arises from accompanying active-site structural rearrangements. Other mutations with large effects also cause structural perturbations or appear to displace a structured water molecule that is part of a stabilizing hydrogen-bond network. Our results lead to a model in which three hydrogen bonds are donated to the stabilized ionized Tyr, with these hydrogen-bond donors, two Tyr side chains, and a water molecule positioned by other side chains and by a water-mediated hydrogen-bond network. These results support the notion that large energetic effects are often the consequence of multiple stabilizing interactions rather than a single dominant interaction. Most generally, this work provides a case study for how extensive and comprehensive comparisons via site-directed mutagenesis in a tight feedback loop with structural analysis can greatly facilitate our understanding of enzyme active-site energetics. The extensive data set provided may also be a valuable resource for those wishing to extensively test computational approaches for determining enzymatic pK_a values and energetic effects.



The numerous examples of ionizable groups with highly perturbed pK_a values within enzyme active sites demonstrate that proteins can manipulate local environments, a property that is likely integral to the catalytic power of enzymes (e.g., refs 1–18). Understanding the physical basis for perturbed pK_a values in enzymes has been a longstanding goal in the study of enzyme action and in the computational prediction and design of new enzymes and ligand-binding interactions (e.g., refs 1 and 19–36).

Structural analysis has shown residues with highly perturbed pK_a values buried in hydrophobic pockets or positioned near charged residues, cofactors, or substrates, and a role for these surrounding groups is supported in several instances by effects of mutations on pK_a values (e.g., refs 1, 6, 7, 10, 11, and 36–42). Nevertheless, energetics within protein interiors and active sites are complex.^{30,43–52}

Surprisingly, a tyrosine residue with an unusually low pK_a of 6.3 has been identified in the active site of a mutant of bacterial ketosteroid isomerase (KSI) from *Pseudomonas putida*, a solvent-exposed site devoid of metal ions, cofactors, and cationic amino acids.^{17,18} Multiple computational pK_a pre-

diction engines predicted that the active-site Tyr pK_a 's are substantially increased by their environment, not decreased, relative to the solution pK_a .¹⁷ Herein, we provide strong evidence that the ionizing residue is Tyr57, which has no nearby positive charges and is solvent exposed. Extensive site-directed mutagenesis in conjunction with structural studies provides evidence for the stabilization of anionic Tyr57 via three positioned hydrogen bonds, two from neighboring positioned Tyr residues and one from a water molecule engaged in a hydrogen-bond network with another water molecule and active-site side chains.

EXPERIMENTAL PROCEDURES

Materials. All reagents were of the highest purity commercially available ($\geq 97\%$). All buffers were prepared with reagent grade materials or better.

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KSI Mutagenesis, Expression, and Purification. Quik-Change site-directed mutagenesis was used to introduce the mutations into the pKSI and tKSI genes encoded on pKK22-3 plasmids or pET21 plasmids. The mutations were confirmed by sequencing miniprep DNA from DH5 α cells. Proteins were expressed and purified as previously described.^{44,53}

KSI Kinetics. Reactions to evaluate the effect of the R15K/D21N/D24C mutations on KSI activity were performed using 5(10)-estrene-3,17-dione and were monitored continuously at 248 nm in a PerkinElmer Lambda 25 spectrophotometer. Reactions were conducted at 25 °C in 40 mM potassium phosphate, pH 7.2, 1 mM sodium EDTA, and 2 mM DTT with 2% DMSO added as a cosolvent for substrate solubility. The values of $k_{\text{cat}}/K_{\text{M}}$ were determined by plotting the values of the observed rate constant (k_{obs}) measured under subsaturating substrate concentrations as a function of enzyme concentration.

Construction of the His-Tagged SUMO-D24C-131 KSI Plasmid. The sequence encoding residues 24–131 was PCR amplified out of the pKK22-3 plasmid containing KSI using a forward primer containing an *Asc*I site followed by the KSI sequence starting at isoleucine 25 and a reverse primer containing the terminal KSI sequence, a stop codon, and a *Pac*I site. Following digestion with the appropriate restriction enzymes, this PCR product was cloned between the *Asc*I and *Pac*I sites of a vector containing a His6-tag and ubiquitin-like (UBL) protein, SUMO (gift from Aaron Straight). Quik-Change site-directed mutagenesis was used to mutate the residue at position 24 to a cysteine, generating a His-tagged SUMO-D24C-131 construct. The product was confirmed by sequencing miniprep DNA from DH5 α cells.

Peptide Synthesis and Purification. A peptide comprising the N-terminal 23 amino acids of KSI with a C-terminal thioester for ligation was synthesized manually on β -mercaptopropionyl-Leu-PAM resin using BOC in situ neutralization protocols.^{54,55} The peptide was deprotected using trifluoromethanesulfonic acid and thioanisole.^{54,55} The peptide was purified by reverse-phase HPLC using a gradient elution between A (water, 0.1% TFA) and B (9:1 acetonitrile/water, 0.09% TFA). Fractions containing the peptide product were pooled and lyophilized. The product mass was confirmed by electrospray mass spectrometry.

Expression and Purification of the Recombinant Fragment Containing an N-Terminal Cysteine ¹³C_α-Tyr-Labeled at Y32, Y57, and Y119. The ¹³C-Tyr-labeled fusion protein with Y32, Y57, and Y119 ¹³C_α-labeled was expressed in BL21(DE3) cells grown in M9 minimal media supplemented with L-tyrosine (50 mg/L phenol-4-¹³C, 95–99%; Cambridge Isotope Laboratories, Inc.) and the remaining 19 unlabeled amino acids. Cells were grown at 37 °C to an OD of ~0.6 followed by the addition of 0.4 mM IPTG and a further 10 h of growth at 25 °C. Cells were harvested and resuspended in 20 mM sodium phosphate, pH 7.2, and 150 mM NaCl (lysis buffer) and lysed by passage through a French pressure cell. Inclusion bodies containing the fusion protein were isolated by the solubilization of membranes by the addition of 1% Triton X-100, 20 mM sodium phosphate, pH 7.2, and 150 mM NaCl followed by centrifugation at 8000g. The inclusion bodies were then washed several times in 20 mM sodium phosphate, pH 7.2, 150 mM NaCl to remove detergent.

Inclusion bodies were resolubilized in 7 M urea, 20 mM sodium phosphate, pH 7.2, 150 mM NaCl. The samples were centrifuged to remove aggregated protein. The supernatant was loaded on a Ni-NTA column pre-equilibrated with 7 M urea, 20

mM sodium phosphate, pH 7.2, and 150 mM NaCl. The column was washed with 7 M urea, 20 mM sodium phosphate, pH 7.2, and 150 mM NaCl until the A₂₈₀ dropped to ~0 (~10 column volumes). The product was eluted in one step using 250 mM imidazole, 7 M urea, 20 mM sodium phosphate, pH 7.2, and 150 mM NaCl.

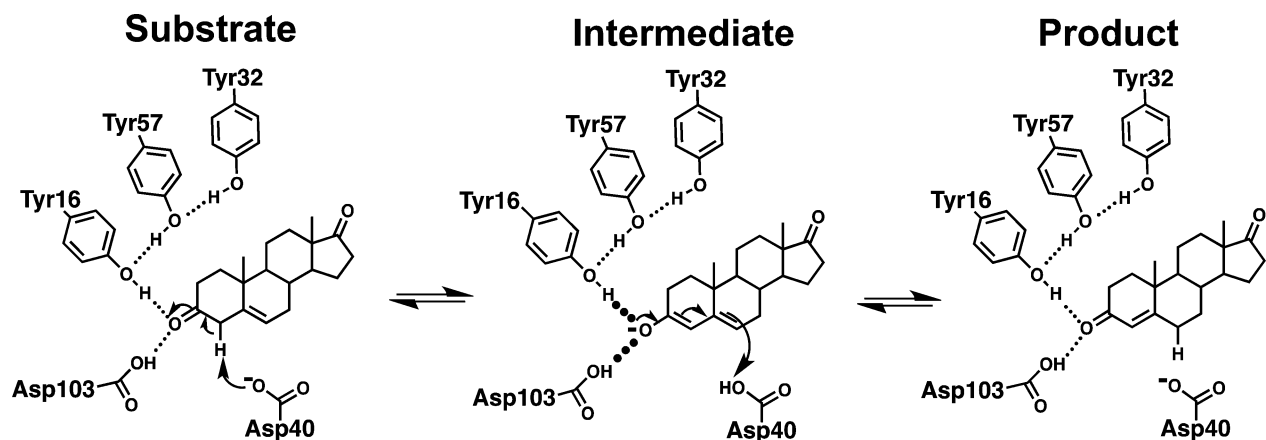
The eluted material was diluted at 4 °C by the dropwise addition of 20 mM sodium phosphate, pH 7.2, and 150 mM NaCl to a final urea concentration of 2 M to allow refolding of the SUMO protein. The material was concentrated using an Amicon centrifugal filter unit and then buffer-exchanged by passing through a HiPrep 26/10 (GE Healthcare Life Sciences) desalting column pre-equilibrated with 2 M urea, 50 mM Tris-HCl, pH 8.0, and 150 mM NaCl. The purity of the fusion protein was >95%, as determined by SDS-PAGE.

SUMO protease was added to cleave the fusion protein. The cleavage reaction was carried out in 2 M urea, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 2 mM DTT at 30 °C for 2 h. To minimize aggregation of the cleaved products, the concentration of the fusion protein in the cleavage reaction was below 100 μ M. Cleavage efficiency was typically >95%, as determined by SDS-PAGE. Following reaction, solid urea was added directly to the mixture to a final concentration of 8 M in the reaction mixture. The mixture was centrifuged to remove aggregated material. Cleavage products were purified by loading the mixture on a Superose-12 gel-filtration column pre-equilibrated with 7 M urea and 20 mM sodium phosphate, pH 7.2. Fractions containing the KSI fragment were identified by SDS-PAGE, pooled, concentrated to a final concentration of ~2 mM using a 3 kDa cutoff centrifugal filter unit, and stored at 4 °C.

Native Chemical Ligation. The peptide containing a C-terminal thioester was ligated to the ¹³C_α-Tyr-labeled recombinant fragment containing an N-terminal cysteine using native chemical ligation.⁵⁴ The lyophilized peptide was dissolved in 7 M urea and 20 mM sodium phosphate, pH 7.2, to a concentration of ~4 mM. The peptide and recombinant fragments were combined to give final concentrations of ~4 and ~2 mM, respectively, in 7 M urea and 20 mM sodium phosphate, pH 7.2. Sodium 4-mercaptophenylacetic acid was added to a final concentration of 1 M.⁵⁶ The ligation was allowed to proceed for 2 h at 25 °C. The ligation mixture was then refolded by a 20-fold dilution into 40 mM potassium phosphate, pH 7.2, 1 mM EDTA, and 2 mM DTT followed by stirring for 1 h at 4 °C. The refolded protein was purified by deoxycholate affinity chromatography followed by buffer exchange into 40 mM potassium phosphate, pH 7.2, 1 mM EDTA, and 2 mM DTT in a 10 kDa cutoff concentrator. The final purity was >99% on a Coomassie-stained SDS-PAGE gel. Protein concentration was determined using a calculated molar extinction coefficient at 280 nm. The yield relative to the limiting recombinant fragment was ~40%, and 4.2 mg of pure KSI was recovered.

Absorbance Spectra. Absorbance spectra of unliganded KSI were acquired in a 150 μ L microcuvette with a PerkinElmer Lambda 25 absorbance spectrophotometer. The spectra of 10–50 μ M enzyme were recorded at 25 °C in 10 mM buffer. The buffers used were the following: sodium acetate, pH 3.7–4.8; sodium phosphate, pH 4.7–9.1; and sodium glycine, pH 8.7–11.0; sodium carbonate, pH 8.3–11.2. The ionic strength was held constant at 0.1 M with sodium chloride.

Scheme 1. Mechanism of KSI-Catalyzed Isomerization



Determining Difference Absorbance Spectra. Absorbance spectra were recorded from 320 to 220 nm at each pH. Difference absorbance spectra were obtained by subtraction of the absorbance spectra at the low pH. The change in extinction coefficient at 300 nm as a function of pH was fit to a titration curve to determine an apparent pK_a . The change in extinction coefficient agreed with the expected change for ionization of a single tyrosine ($\Delta\epsilon = 2300 \text{ M}^{-1} \text{ cm}^{-1}$).⁵⁷ Fits using the extinction coefficient change at 244, 290, 295, and 305 nm gave values in good agreement with the value determined using the extinction coefficient change at 300 nm. Reported pK_a values are the average of two or more independent determinations. To evaluate if pH-dependent changes in the absorbance spectra independent of tyrosine ionization affect the determined pK_a , individual spectra were normalized at 267 or 278 nm, the isosbestic points for tyrosine/tyrosinate, and the change in extinction coefficient determined at 290–305 nm was fit to a titration curve. The results were in good agreement with the values determined by normalizing at 320 nm.

Changes in Trp absorbance (maximum at 280 and 288 nm) from the two Trp residues in KSI could affect the observed absorbance change and limit accurate determination of tyrosyl pK_a values and number of tyrosines ionizing.^{57–59} Although differences in Trp absorbance could complicate interpretation of absorbance differences near 290 nm, absorbance differences at 244 nm have been suggested to be unaffected by changes in Trp absorbance.^{57–59} We therefore compared the change in extinction coefficient at 244 and at 295–305 nm to ensure minimal interference from Trp absorbance changes. Tyr pK_a values and the number of ionized Tyr residues determined by the change in extinction coefficient at 244 nm were in good agreement with values determined at 295, 300, and 305 nm ($\pm 0.2 \text{ p}K_a$ units; extinction coefficient change consistent with one Tyr ionized), again suggesting that observed absorbance differences corresponded to ionization of a single Tyr and that changes in extinction Trp absorbance do not contribute significantly to the absorbance difference spectra at 295–305 nm.

NMR Spectroscopy. ¹³C NMR spectra of KSI were acquired at the Stanford Magnetic Resonance Laboratory on a 600 MHz Varian UNITYINOVA spectrometer running VNMR v6.1C. NMR samples consisted of 0.4–1.0 mM KSI in 40 mM potassium phosphate buffer, pH 7.2, 1 mM EDTA, 2 mM DTT, and 10% (v/v) D₂O in a 10 mm Shigemi symmetrical microtube at 25 °C. Data were collected with 39 000 points and

a 1.0 s recycle delay for 4308–17 232 scans and processed using a 10 Hz line broadening. Chemical shifts were referenced externally to a sample of sodium 3-trimethylsilylpropionate-2,2,3,3-*d*₄ (0 ppm) under the same buffer conditions.

KSI X-ray Crystallography. Single-crystal diffraction data were collected at the SSRL beamline BL9-1 using a wavelength of 0.98 Å.⁶⁰ The reflections were indexed and integrated with the programs XDS;⁶¹ the intensities were scaled, merged, and converted to amplitudes with SCALA and TRUNCATE.⁶² The phases were derived from PDB entry 3CPO and refined with REFMAC5.^{63,64} Manual model building was carried out with COOT.⁶⁵ Crystallographic refinement statistics are given in the Supporting Information.

RESULTS AND DISCUSSION

Uncovering a Tyr Residue with a Perturbed pK_a in the KSI Active Site. Ketosteroid isomerase (KSI) catalyzes the double-bond isomerization in steroid substrates via formation of a dienolate intermediate within an active site composed of an Asp general acid/base, an ‘oxyanion hole’ containing two hydrogen-bond donors, a Tyr and an Asp residues, a network of adjoining hydrogen-bonding residues, and neighboring hydrophobic residues that participate in substrate binding (Scheme 1).^{66–68}

When studying a mutant of this enzyme with the Asp general base mutated to Asn (Asp40Asn), Fafarman et al. used ¹³C NMR to identify a Tyr residue with a perturbed pK_a and UV absorbance to measure its pK_a value of 6.3 ± 0.1 .¹⁷ The ¹³C NMR spectrum of Asp40Asn KSI from *Pseudomonas putida* (pKSI) containing isotopically enriched Tyr residues showed four peaks at pH 7, with one peak shifted downfield near the chemical shift expected for ionized Tyr.¹⁷ In the simplest scenario, the four distinct peaks corresponded to the four tyrosine residues in pKSI: Tyr16, Tyr32, Tyr57, and Tyr119, and the one downfield peak corresponded to one tyrosine ionized at pH 7. ¹³C NMR experiments using a Tyr119Phe mutant indicated that Tyr119 is not the ionizing residue, as expected because this is a surface residue.¹⁷ Of the three remaining tyrosines, results with site-specific vibrational probes could be most simply accounted for if Tyr57 is the ionizing residue,¹⁷ but alternative assignments could not be ruled out. We used protein semisynthesis to generate KSI bearing ¹³C-labeled Tyr at specific Tyr residues to probe the identity of the ionizing Tyr.

Protein Semisynthesis to Probe the Identity of the Ionizing Tyrosine. Three mutations were introduced in KSI to facilitate protein semisynthesis: Arg15Lys, Asp21Asn, and Asp24Cys. The Arg15Lys mutation allowed the use of trifluoromethanesulfonic acid deprotection of all side-chain protecting groups following solid-phase peptide synthesis (SPPS); the Asp21Asn mutation eliminated potential aspartimide formation during SPPS, and the Asp24Cys mutation introduced a cysteine residue for native chemical ligation. The combined mutations had a less than 2-fold effect on the activity of the WT enzyme (Figure S1 of the Supporting Information), and the ^{13}C NMR spectrum of recombinant ^{13}C -Tyr-labeled Arg15Lys/Asp21Asn/Asp24Cys was nearly identical to the spectrum of ^{13}C -Tyr-labeled Asp40Asn (Figure S2 of the Supporting Information).¹⁷

Semisynthetic KSI Asp40Asn bearing the mutations introduced for protein semisynthesis was prepared by ligating a synthetic 23-mer peptide containing natural abundance Tyr16 to a 108-residue recombinant peptide fragment containing $^{13}\text{C}_\alpha$ -labeled Tyr57, Tyr32, and Tyr119 and refolding out of urea (Figure 1A). Although semisynthetic Asp40Asn containing ^{13}C labels at all four Tyr residues gave a ^{13}C NMR spectrum

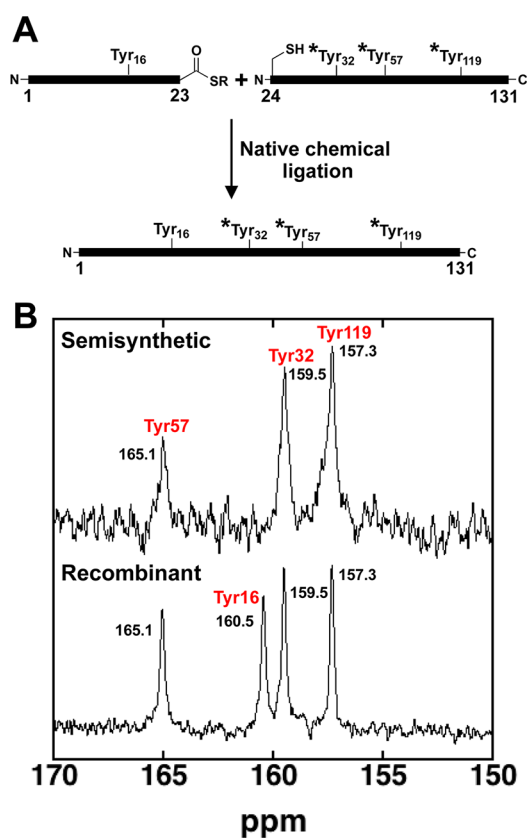


Figure 1. ^{13}C NMR spectra of site-specifically labeled semisynthetic R15K/D21N/D24C/D40N KSI to assign the identity of the ionizing Tyr residue. (A) Synthetic fragment (1–23) bearing unlabeled Tyr16 is ligated to a recombinant fragment (24–131) bearing $^{13}\text{C}_\alpha$ -Tyr at residues 32, 57, and 119 (*Tyr). (B) ^{13}C NMR spectrum for fully recombinant R15K/D21N/D24C/D40N KSI with $^{13}\text{C}_\alpha$ -Tyr residues and semisynthetic R15K/D21N/D24C/D40N KSI with unlabeled Tyr16. The spectrum of semisynthetic KSI lacks the peak at 160.5 ppm, allowing assignment of this peak to Tyr16. The peak at 157.3 ppm was previously assigned to Tyr119, and the peak at 165.1 was attributed to Tyr57, as described in the text.

identical to that previously observed for fully recombinant Asp40Asn (Figures 1B and S2 of the Supporting Information), semisynthetic enzyme containing unlabeled Tyr16 results in a ^{13}C NMR spectrum lacking a peak at 160.5 ppm but still containing the most downfield peak at 165.1 ppm (Figure 1B). With Tyr16 not ^{13}C labeled and Tyr119 previously assigned as the 157.3 ppm peak, the only remaining options for the peaks at 159.5 and 165.1 ppm are Tyr32 and Tyr57.¹⁷ Tyr32 is in a deep enzyme pocket surrounded by hydrophobic groups, with Tyr57 as the only apparent hydrogen-bond donor that could stabilize an anionic Tyr32. In contrast, Tyr57 is situated between two potential hydrogen-bond donors, Tyr16 and Tyr32, and is solvent exposed (Figure S3 of Supporting Information). These features are expected to result in much greater stabilization of ionized Tyr57 than ionized Tyr32. Shortened distances between Tyr residues observed from X-ray crystallography are also consistent with deprotonation of the central Tyr57 above pH 7 (see Table S1 of the Supporting Information). We therefore attribute the tyrosine ionization to Tyr57 in unliganded Asp40Asn, consistent with the prediction of Fafarman et al.,¹⁷ and we then set out to understand the origin of the large and unexpected pK_a perturbation. The results obtained provide additional support for Tyr57 as the ionized Tyr residue, and we assume that this is the case for the remainder of this article.

Developing a Model for the Stabilized Tyr57 Anion from Structural Analysis.

To assess potential determinants underlying the low Tyr pK_a in pKSI Asp40Asn, we first evaluated published KSI X-ray structures. Unliganded wild-type KSI (PDB ID 1OPY) shows Tyr57 positioned in a solvent accessible active site and not adjacent to any cationic side chains (Figure 2A). Previously identified cases of stabilized tyrosinates have nearby positive charges,^{1,12,69} but a structural feature or features other than a cationic group must be responsible for this unusually low Tyr pK_a . Hydrogen bonds from neutral hydrogen-bond donors have been suggested to perturb the pK_a values of functional groups in proteins (e.g., refs 1 and 39). In particular, the unusually low pK_a for aspartic acid in turkey ovomucoid third domain, RNase Sa, RNase T1, and α -lytic protease have been attributed to hydrogen bonds from neutral donors that stabilize the ionized carboxylate relative to its neutral form (pK_a values of 2.4, <2.3, 0.6, and <1.5, respectively).^{31,39,70–72}

Consideration of KSI structures leads to a model involving three hydrogen-bond donors to the Tyr57 anion (Figure 2). As noted above, Tyr57 has two Tyr residues adjacent to it, Tyr16 and Tyr32. The short O–O distances between Tyr57 and each of these Tyr residues suggests the presence of hydrogen bonds, and these hydrogen bonds appear to shorten upon ionization of Tyr57 (Supporting Information Table S1). A crystal structure of the Met116Ala mutant of KSI described below shows electron density consistent with two water molecules within the active site (Wat1 and Wat2, Figure 2B). Although Asp40 was present in this structure, it suggests a possible extended hydrogen-bonding network composed of Asp40Asn, Asp103, and a distinct crystallographically observed water molecule in the Asp40Asn mutant (Wat2, Figure 2B–D).^{73,74} Further support for the placement of Wat1 and Wat2 comes from the observation of Wat2 in the wild-type KSI structure (PDB ID 8CHO) and from the observation of Wat1 in the Asp40Asn/Asp103Asn structure with bound ligand (PDB ID 3FZW). Although the electron density map for the wild-type structure is not available to evaluate if multiple water molecules are

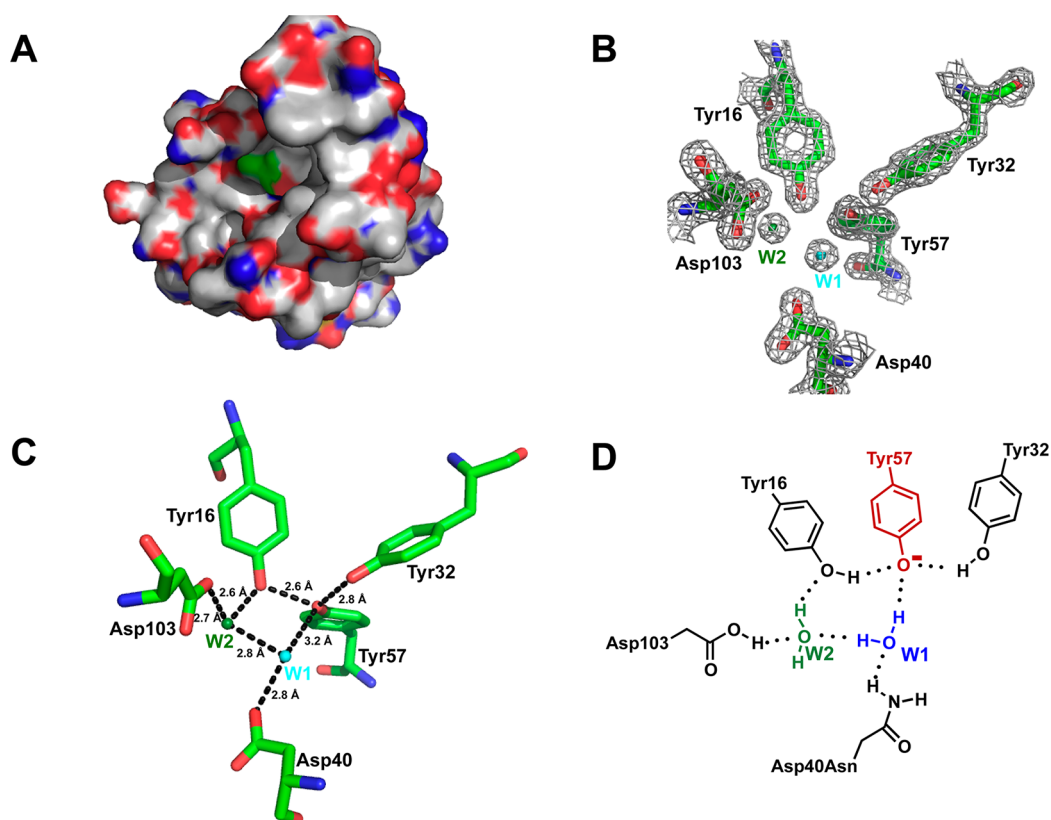


Figure 2. Structural features of the KSI active site. (A) Space-filling representation of the KSI monomer shows that Tyr57 (green) is solvent accessible in the unbound wild-type active site (PDB ID 8CHO). Red is oxygen, blue is nitrogen, and white is carbon. Tyr57 carbon atoms are shown in green. (B) $2F_0 - F_c$ electron density map for the Met116Ala KSI structure shows density for two ordered water molecules in the active site (contoured at 1.0σ). (C) Structural model of the active-site residues in Met112Ala KSI. (D) Model for hydrogen-bond stabilization of the Tyr57 anion in the Asp40Asn mutant that is tested herein. Ionized Tyr57 is red. Crystallographically observed water molecules are colored as in panel B. The figure was generated using Pymol (Schrödinger, LLC).

positioned in the unliganded wild-type KSI active site, the structural data support the model from the Met116Ala mutant of KSI.

Although it was reasonably straightforward to develop this structural model for stabilization of the Tyr57 anion, testing it and understanding its underlying energetics required multiple cycles of site-directed mutagenesis comparisons and structural data. The results described in the following sections with 45 KSI variants support the above model, with its multiple interactions contributing to stabilization of the Tyr57 anion, but they also reveal complex energetic and structural effects.

Energetic and Structural Analysis of Mutational Effects. *UV Absorbance Spectroscopy to Determine Tyr pK_a Values.* Although NMR is powerful in identifying and assigning the ionized residue, we could not accurately determine pK_a values by this method for two reasons. The NMR spectra exhibit peak broadening at low pH, presumably from exchange, and KSI is less soluble at low pH values so that obtaining NMR data across an entire titration range is challenging given the limited sensitivity of NMR. These traits prevented a simple determination of chemical shift as a function of pH.¹⁷ We therefore turned to UV-vis spectra as a function of pH to evaluate the effect of mutations on the Tyr pK_a because the tyrosinate ion has an absorbance peak at 295 nm that is absent for neutral tyrosine and is not subject to complications from exchange (Figure 3).¹⁷ This assay had the additional advantage of allowing rapid determination with

modest amounts of sample, thereby facilitating the extensive analyses involving 45 KSI variants carried out herein.

Figure 3A shows the tyrosine absorbance spectra at pH 4.7 (red line) and pH 14 (blue line), and Figure 3B shows the difference spectrum (black line). At low pH, the absorbance maximum is centered near 280 nm, and at high pH, ionized tyrosine gives maxima at 244 and 295 nm.^{57,75} To determine KSI tyrosine pK_a values, we recorded absorbance spectra across a pH series and calculated difference spectra relative to the pH 4 spectrum for that KSI variant. Figure 3B shows an overlay of the absorbance difference spectrum of Asp40Asn KSI (green line). An increase in absorbance with maxima centered near 244 and 295 nm is observed with extinction coefficient differences of 1.4×10^4 and $2.6 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, respectively, consistent with ionization of one tyrosine (see the Experimental Procedures section).

Effect of Mutations of the Hydrogen-Bond Donors Directly Adjacent to Tyr57 on the Tyr pK_a . We first tested the effect from mutation of Tyr16 and Tyr32, the potential hydrogen-bond donors directly adjacent to Tyr57 (Figure 2B–D). A simple expectation would be that groups closest to the ionizing hydroxyl group would have the largest pK_a effects.

To eliminate potential complications from ionization of the active-site Asp103 in the Tyr mutants (see below), we first mutated the Asp103 to Asn; we then determined the effects of the Tyr mutations in the Asp40Asn/Asp103Asn background. The Asp103Asn mutation has only a small effect on the Tyr57 pK_a , as described below, and, remarkably, Tyr57 ionizes at a

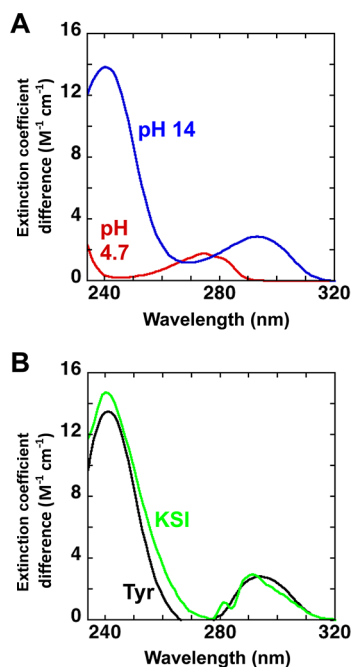


Figure 3. Evaluating tyrosine ionization by UV spectroscopy. (A) Absorbance spectra of tyrosine measured in 10 mM sodium acetate, pH 4.7 (red), and 1 M NaOH, pH 14 (blue). (B) Difference absorbance spectra for tyrosine (pH 14 – pH 4.7, black) and Asp40Asn KSI (pH 10 – pH 4, green). The difference spectra show maxima for tyrosine at 244 ($\Delta\epsilon = 1.3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) and 295 nm ($\Delta\epsilon = 2.8 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) and for Asp40Asn KSI at 244 ($\Delta\epsilon = 1.4 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) and 293 nm ($\Delta\epsilon = 2.6 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$). The similar spectra shape and extinction coefficient differences provide support for the ionization of one tyrosine. The dashed line in Figures 4 and 10 represent the expected extinction coefficient change for the ionization of one tyrosine (green).

lower pH than Asp103, which resides within 6 Å of Tyr57 and is also exposed to solvent within the active-site pocket.^{3,73} (The solution pK_a of the Asp side chain is ~ 4 , whereas that for a Tyr side chain is 10.⁷⁶ Despite this difference of over 5 orders of magnitude in the stability of their respective anions in aqueous solution, Tyr is observed to deprotonate instead of Asp103.¹⁷) Figure 4 shows the absorbance spectra for Asp40Asn/

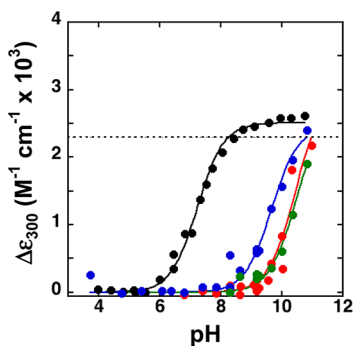


Figure 4. Changes in extinction coefficients at 300 nm relative to pH 4 for D40N/D103N (black), Y16F/D40N/D103N (red), Y32F/D40N/D103N (blue), and Y57F/D40N/D103N (green). Absorbance spectra were collected as described in the Experimental Section. The lines are a fit of the data to a titration curve and give pK_a values of 7.2 for D40N/D103N, >9 for Y32F/D40N/D103N, >10 for Y16F/D40N/D103N, and >10 for Y57F/D40N/D103N.

Asp103Asn as a function of pH (black line). Like the Asp40Asn mutant, a simple titration corresponding to the formation of a single tyrosinate is observed with highly perturbed pK_a of 7.2 ± 0.2 . In contrast, mutation of Tyr57 or of either adjacent Tyr residues increased the pK_a such that there was no stabilized tyrosine anion: its pK_a is >10 (Figure 4 and Table 1) and thus is at least as high as that of Tyr in an unstructured peptide (pK_a 10.2⁷⁷) (see also Figure S4 of the Supporting Information).

Table 1. Effects of Tyr Mutations on the Tyr pK_a

mutant	pK_a	ΔpK_a
none ^a	7.2	(0)
Tyr16Phe	>10	>3
Tyr32Phe	>9	>2
Tyr57Phe	>10	>3
Tyr16Ala	>10	>3
Tyr32Ala	>10	>3
Tyr57Ala	>10	>3

^aRefers to Asp40Asn/Asp103Asn.

One model to account for the complete loss of a pK_a perturbation upon mutation of either adjacent Tyr would be hindered solvation of Tyr57 as a result of the Phe residues replacing the neighboring Tyr residues. However, mutation of these Tyr residues to Ala similarly eliminated the highly perturbed pK_a value (Figure S5 of the Supporting Information and Table 1). Although it is possible that other properties of the site render it a highly non-aqueous-like environment, Tyr57 is solvent accessible, and there is evidence from prior studies of Tyr16 to Ala mutants for an ability of solvent to access the newly created space.^{44,45}

A more likely model for the effect of the Tyr16 mutations is conformational rearrangement of Tyr57 upon mutation of Tyr16 to disrupt the hydrogen bonds potentially remaining with Tyr32 and with Wat1. Indeed, a superposition of the previously determined Tyr16Ser/Asp40Asn and Asp40Asn structures shows that although the overall structures are superimposable, with a root-mean square deviation of 0.4 Å for the backbone atoms, the Tyr32 and Tyr57 side chains are displaced by 0.8 and 0.7 Å upon removal of Tyr16 (Figure 5). A rearrangement is also observed in the Tyr16Phe mutant (Figure S6A of the Supporting Information).

Although a similar rearrangement is not observed in the Tyr32Phe mutant (Figure S6B of the Supporting Information), Tyr32 is buried deep in a hydrophobic pocket that might exclude water. A structure of the Tyr32Ala mutant is not available, where greater water access is expected, but there is evidence for rearrangements in this more severe mutant from its 30-fold rate decrease compared to the only 3-fold effect from mutation to Phe (ref 78 and unpublished results).

Effect of Mutations of the Distal Active-Site Hydrogen-Bond Donors on the Tyr pK_a . The model of Figure 2 invokes a hydrogen-bond network that includes the side chains at positions 40 and 103 and two water molecules. To learn about the nature and contributions of this presumed network, we perturbed the hydrogen-bonding capability of the distal hydrogen-bonding groups Asp103 and Asp40Asn. We first determined the change in apparent Tyr pK_a upon mutation of Asp103 to groups that either ablate (Ala, Leu) or perturb (Asn) the hydrogen-bonding ability of residue 103. Measurements were made in the Asp40Asn background because this latter

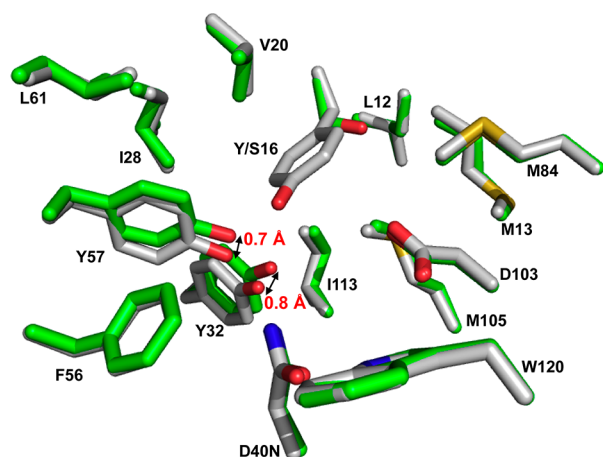


Figure 5. Crystal structure of Tyr16Ser/Asp40Asn shows that Ty32 and Tyr57 are displaced 0.8 and 0.7 Å, respectively, relative to Asp40Asn. Superposition of the previously determined 1.6 Å Tyr16Ser/Asp103Asn structure (PDB ID 3IPT, carbon atoms colored green) and the previously determined 2.0 Å Asp40Asn structure (PDB ID 1OGX, carbon atoms colored white). The bound equilenin is omitted from both structures for clarity. The overall root-mean-square deviation between the two structures for backbone atoms is 0.4 Å. The pK_a comparison in the text uses the Ala mutant, whereas this structural comparison uses the Tyr16Ser version of KSI; prior studies show that the mutation of Tyr16 to Ala or Ser has the same functional effect within 2-fold.⁴⁴

mutation is required to observe the perturbed Tyr57 pK_a , as noted above and further assessed below.

Mutation of Asp103 to Ala, Leu, and Asn increased the apparent Tyr pK_a by 0.6, 1.9, 0.9 pK_a units, respectively (Figures 6A and S7 of the Supporting Information and Table 2). These results suggest that the distal Asp103 hydrogen bond plays a role in lowering the pK_a of Tyr57, although, as expected, its removal does not eliminate the preferential stabilization of the tyrosinate anion in the active site. The larger effect from mutation of Asp103 to Leu rather than Ala is consistent with a perturbation from the introduction of the longer hydrophobic side chain (Figure 6B), and an X-ray structure of this mutant reveals that the localized active-site water molecule (Wat2, Figure 2B) is displaced by >1 Å (Figure 6B).^{73,78} Wat2 in its normal position may help orient and/or polarize the groups directly donating hydrogen bonds to the Tyr57 anion (Figure 2B,C).

Table 2. Effects of Asp103 Mutations on the Tyr pK_a ^a

residue 103	pK_a	ΔpK_a
Asp	6.3	(0)
Ala	6.9	0.6
Leu	8.2	1.9
Asn	7.2	0.9

^aMeasurements were made in the Asp40Asn background.

The similar effect of mutation of Asp103 to Ala and Asn was surprising because Asn can maintain hydrogen bonding (Figure 2D). However, the side-chain truncation to Ala could allow access of additional water molecules and maintenance of the hydrogen bond from Wat2. Alternatively, mutation of protonated Asp to Asn could disrupt a hydrogen-bond network involving Wat2, either by additional geometrical constraints of planarity of the amide hydrogen atoms or from steric effects or solvation requirements of an additional solvent molecule recruited to accept a hydrogen bond from the additional amide hydrogen atom.

We next turned to the effects of residue 40 on the Tyr pK_a . The low Tyr pK_a is only observed upon mutating Asp40, the general base in the KSI reaction (Scheme 1); thus, we use Asn at position 40 as our reference point, and we address the Asp40 effects in a later section (Effects of Introducing Charged Residues at Position 40 on the Tyr pK_a). We mutated Asn at position 40 to perturb (Ser, Gln) or ablate (Ala, Leu, Val, and Ile) the hydrogen-bonding capability at residue 40, and measurements were made in the Asp103Asn background as above. All position 40 mutations increased the Tyr pK_a relative to Asp40Asn. Mutation to Ala, Ser, and Gln increased the apparent Tyr pK_a by about one unit, to 7.9–8.3, and mutation to Leu increased the Tyr pK_a at least three units, to >10 (Figure 7 and Table 3). A downfield peak was observed in ¹³C NMR spectra at pH 10 for the Ala, Ser, and Gln mutants, consistent with an ionized Tyr, but it was not observed for the Leu mutant, consistent with loss of the tyrosinate anion in this mutant (Figure S8 of the Supporting Information). The increased pK_a upon mutation to Ser, Gln, or Ala provides evidence for a specific involvement of Asn40 in stabilizing the tyrosinate at position 57 (Figure 2C), and the absence of a direct interaction with Tyr57 and the presence of an intervening positioned water molecule suggest the involvement of Asn40 in the hydrogen-bond network depicted in Figure 2.

The mutations to Leu, Val, and Ile each ablate hydrogen-bonding capability at position 40 and introduce branched,

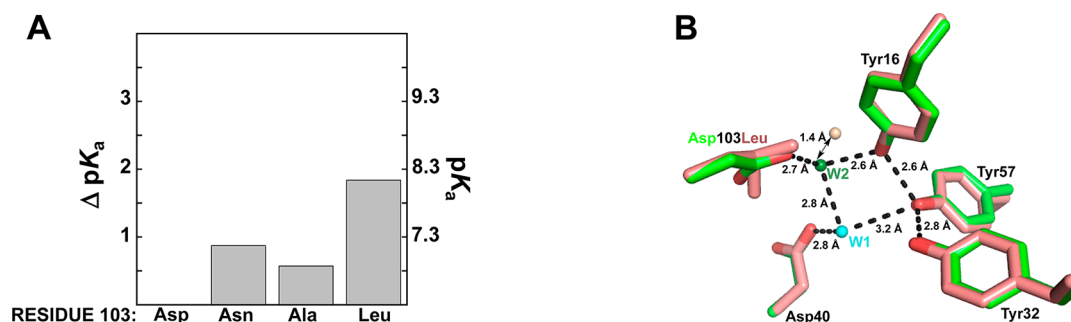


Figure 6. Effects of Asp103 mutations on the Tyr pK_a . (A) ΔpK_a values relative to the pK_a value of Asp40Asn. The values are from Table 2. (B) Superposition of structural models of Met116Ala (green, PDB ID 3RGR) and Asp103Leu (salmon, PDB ID 1W00) suggests the position of an organized water molecule in the oxyanion hole is perturbed in Asp103Leu (salmon sphere) compared to wild type (green sphere). Wat1 is not observed in the Asp103Leu structure.

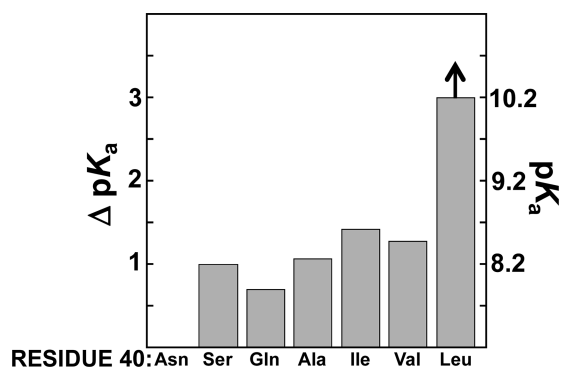


Figure 7. Effects of Asp40 mutations on the Tyr pK_a . The ΔpK_a values are relative to the pK_a value of Asp40Asn/Asp103Asn and are from Table 3.

Table 3. Effects of Asp40 Mutations on the Tyr pK_a ^a

residue 40	pK_a	ΔpK_a
Asp	7.2	(0)
Ala	8.3	1.1
Ser	8.2	1.0
Gln	7.9	0.7
Ile	8.5	1.3
Val	8.5	1.3
Leu	>10	>3

^aMeasurements were made in the Asp103Asn background.

hydrophobic side chains, but the Leu mutant shows a significantly higher Tyr pK_a than the others (Figure 7 and Table 3). Modeling Leu in the place of Asn40 shows that the Leu side chain would, in the absence of other rearrangements, clash with Phe56, which neighbors Tyr57, and may thus affect the position of Tyr57 and its ability to be stabilized as an anion. The larger Leu residue could also disrupt the position of one or both of the water molecules that help stabilize the Tyr57 anion (Wat1 and Wat2 in Figure 2C). In either case, removal of the Phe56 side chain might provide space for the Leu side chain and thereby prevent destabilization of the hydrogen-bond network. Introduction of the Asp40Leu mutation with Phe56 mutated to Ala increased the Tyr pK_a by only 0.6 pK_a units, similar to the effect of the smaller branched amino acids at position 40 and much smaller than the >3 unit increase with Phe56 intact (Figure S9 of the Supporting Information and Tables 4 and 5). (The Phe56Ala mutation alone had only a small effect on the Tyr pK_a (Table S4 of Supporting Information and see below)).

Table 4. Effects of Mutating Asp40 to Leu in the Phe56 Background on the Tyr pK_a

residue mutated	pK_a	ΔpK_a
Asp40Asn	6.3	(0)
Asp40Leu	>10	>3

Table 5. Effects of Mutating Asp40 to Leu in the Phe56Ala Background on the Tyr pK_a

residue mutated	pK_a	ΔpK_a
Asp40Asn/Phe56Ala	6.9	(0)
Asp40Leu/Phe56Ala	7.5	0.6

Double-Mutant Cycle to Test the Properties of the Putative Active-Site Hydrogen-Bond Network. Despite the absence of direct interaction of the side chains at positions 40 and 103 with Tyr57, the Tyr57 pK_a is increased upon mutation of these residues, as described in the previous section. These results, the observed structural perturbation of a localized water molecule in an Asp103 mutant (Figure 6B), and additional results presented below support the model of a hydrogen-bond network involving these side chains and the intervening water molecules shown in Figure 2. To learn more about this putative network, we next determined the consequences of mutating residues 40 and 103 together.

Whereas the Asp103Ala mutation increased the Tyr pK_a by 0.6 units when introduced in the Asp40Asn background, the same mutation in a background with residue 40 mutated to Ala had a 1.2 units larger effect (1.8 pK_a unit total effect; Figure 8A and Table 6). These results indicate that there is a functional interaction between the distal hydrogen-bonding groups, and the simplest model from the energetic and structural data is that the Asp40Asn and Asp103 side chains and positioned water molecules participate in a robust hydrogen-bond network that helps to stabilize ionized Tyr. The larger than additive effect supports the scenario depicted in Figure 8B in which the side chains are partially redundant such that the remaining side chain and water-mediated interactions are alone able to position the water molecules or Tyr hydrogen-bond donors upon ablation of one distal hydrogen-bonding group; however, ablation of both distal hydrogen-bonding groups destabilizes the network sufficiently so that it and its stabilizing effect is abolished. The loss of ~ 3 kcal/mol stabilization of the tyrosinate upon ablation of the hydrogen-bonding capability at position 40 and 103 provides additional support for a substantial role of the distal hydrogen-bonding groups in stabilizing the Tyr57 anion.

Effect of Neighboring Group Mutations on the Tyr pK_a . To delineate further the interactions important for the lowered Tyr pK_a , we determined the consequence of mutations to residues that surround the hydrogen-bond network (Figure 9A). As described above, Phe56 packs against Tyr57 and mutation to Ala had a small effect on the apparent Tyr pK_a (Table 7). We also mutated Phe86, Met105, and Met116. Phe86 packs against Asp103, and Met105 and Met116 pack against Tyr16 and Asp103 (Figure 9A). Despite these close interactions, the Phe86Ala and Met105Ala mutations had no measurable effect on the apparent Tyr pK_a , and the Met116Ala mutation unexpectedly decreased the apparent the Tyr pK_a , although by less than 0.5 units (Figure 9B and Table 7). An overlay of the crystal structures of the Met116Ala mutant (see Table S2 of the Supporting Information for statistics, PDB ID 3RGR) with wild-type KSI shows that the residues engaged in the hydrogen-bonding network are essentially superimposable (Figure 9C). A structure of the homologous KSI from *Comamonas testosteroni*, tKSI, similarly shows no rearrangement of active Tyr residues upon mutation of the neighbor Phe56 residue (Phe56Gly tKSI, Figure 9D).⁷⁹ These results indicate that subtractive mutations of individual surrounding residues are not sufficient to disrupt the positioning of neighboring Tyr hydrogen-bond donors, despite the clear need for the positioning of these residues to stabilize the Tyr57 anion. In general, important effects that are encoded in redundant structural networks are not revealed by single mutations.

Recreating the Hydrogen-Bond Network in tKSI. The homologous KSI enzymes, tKSI and pKSI, share 34% overall

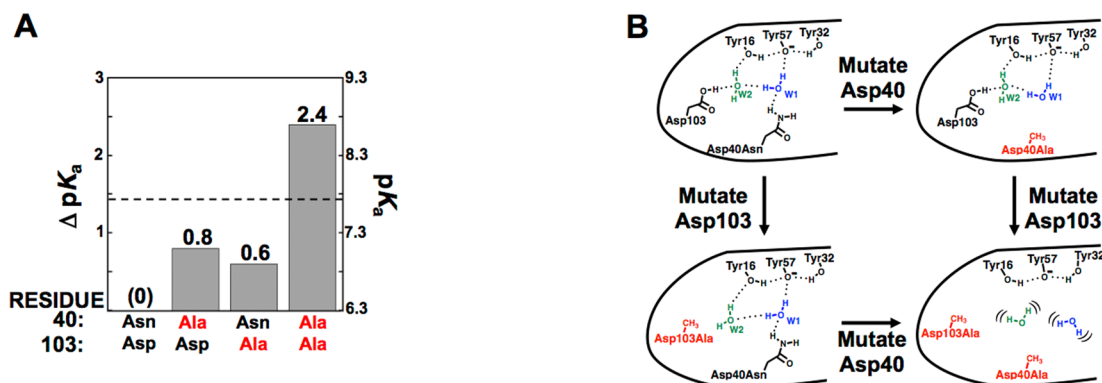


Figure 8. Probing the side chain and water hydrogen-bond network that helps stabilize the ionized Tyr. (A) Effects on the Tyr pK_a for the single Asp40Ala and Asp103Ala mutations and double Asp40Ala/Asp103Ala mutation. The dashed line represents the expected Tyr pK_a if the Ala mutations are additive. The values are from Table 6. (B) Model for the larger than additive effect for mutation of both Asp40 and Asp103 compared to the single mutations. In this model, the water molecules substantially rearrange or become fully mobile only after both mutations are introduced.

Table 6. Effects of Mutating Asp40 and Asp103 to Ala on the Tyr pK_a

residue mutated	pK_a	ΔpK_a
none ^a	6.3	(0)
Asp40	7.1	0.8
Asp103	6.9	0.6
Asp40/Asp103	8.7	2.4

^aRefers to Asp40Asn.

sequence identity.⁸⁰ The active-site hydrogen-bonding residues in tKSI are identical to those in pKSI, and their positioning is the same except that the residue corresponding to Tyr32 is replaced with Phe (Figure 10A; for simplicity, pKSI numbering is used throughout).⁸¹ If positioning of the active-site residues and water molecules, as depicted in Figure 2, is sufficient for stabilization of the Tyr57 anion in pKSI, then introduction of an analogous Tyr in tKSI should reproduce this positioned network and thus the stabilized Tyr57 anion equivalent.

As expected, because of the absence of the second Tyr hydrogen-bond donor, no perturbed pK_a was observed in Asp40Asn tKSI with the wild-type Phe32 present (Figure 10B). We recreated the potential active-site hydrogen-bond arrangement by mutating Phe32 to Tyr, and, to eliminate potential complications from Asp103 ionization, we measured the pK_a value in the Asp103Asn background as for pKSI above. Remarkably, introduction of Tyr at this position in tKSI gave a pK_a indistinguishable from that for pKSI (7.3 ± 0.2 and 7.2 ± 0.2 , respectively; Figure 10B and Table 8). These results provide further strong evidence for a role of this Tyr in reducing the pK_a of its neighbor. Furthermore, despite differences in the identity of the second-shell residues, no significant difference is observed in the Tyr57 pK_a , consistent with the observed absence of pK_a perturbations from mutation of the surrounding residues in pKSI (Figure 9).

Using the Perturbed Tyr Ionization to Explore the Effects of Nearby Charged Residues. Effects of Introducing Charged Residues at Position 40 on the Tyr pK_a . In the simplest scenario, cationic residues at position 40 would decrease the Tyr pK_a . Indeed, other Tyr residues with substantially decreased pK_a values are found near cationic groups (Table S3 of the Supporting Information).^{1,12,69,82,83}

As noted above, the presence of an anionic Asp residue at position 40 greatly increases the Tyr57 pK_a relative to Asp40Asn (and other neutral residues; Figure 7 and Table

3). However, the converse was not observed. Mutation of residue 40 to positively charged side chains (His, Lys, and Arg in the Asp103Asn background, as described above, to eliminate possible complications from its ionization) increased rather than decreased the Tyr pK_a relative to Asp40Asn, with effects of 1.0 to 2.0, pK_a units (Figure 11 and Table 9). These results indicate that the presence of a nearby positive charge alone is not enough to lower the Tyr57 pK_a value in the KSI active site and suggest that distinct factors can be involved and that the features responsible, which are present in Asp40Asn KSI, are disrupted upon introduction of positively charged residues at position 40.

The simplest model to account for these unexpected observations is that the positively charged mutations introduce active-site structural rearrangements relative to Asp40Asn that disrupt the position of the groups stabilizing the Tyr57 anion. A crystal structure of the Asp40His/Asp103Asn in the related tKSI shows that although the Tyr positions are unaffected, the Phe56 side chain is displaced relative to tKSI Asp40Asn/Asp103Asn (Figure 12A and Table S2, PDB ID 3MYT). As described above, Phe56 is directly adjacent to Tyr57 and to the crystallographically observed water molecule, Wat2 (Figure 9). We also compared the previously determined wild-type KSI and Asp40Asn structures to evaluate if a negatively charged residue at position 40 might also affect the arrangement of active site residues relative to uncharged Asp40Asn. The structural overlay shows that although the active-site Tyr residues are superimposable, the Phe56 side chain is displaced by 1.9 Å (Figure 12B). Mutations that affect the position of Phe56 may thus affect the positioned water molecule (or the position of Tyr57 in a manner too subtle to ascertain in the X-ray structures) and thereby destabilize the tyrosine anion.

Effects of Charged Mutations at Residue 40 with the Phe56 Side Chain Removed. Reversion of residue 40 from Asn to Asp in the Phe56 background increased the Tyr pK_a by ≥ 3 units (Figure 11 and Table 9), and a pK_a increase of ≥ 3 units was observed upon introducing anionic Glu in the Phe56 background (Table 9). In the Phe56Ala/Asp103Asn background, the Asp40Arg mutation decreased the Tyr pK_a by 1.5 units relative to Asn40Asn (Figure 11 and Table 10) in contrast to the increase observed with Phe56 intact (Figure 11A). Together with the above structural data, these results suggest that rearrangement of Phe56 upon mutation of residue 40 to positively charged residues destabilizes ionized Tyr, obscuring

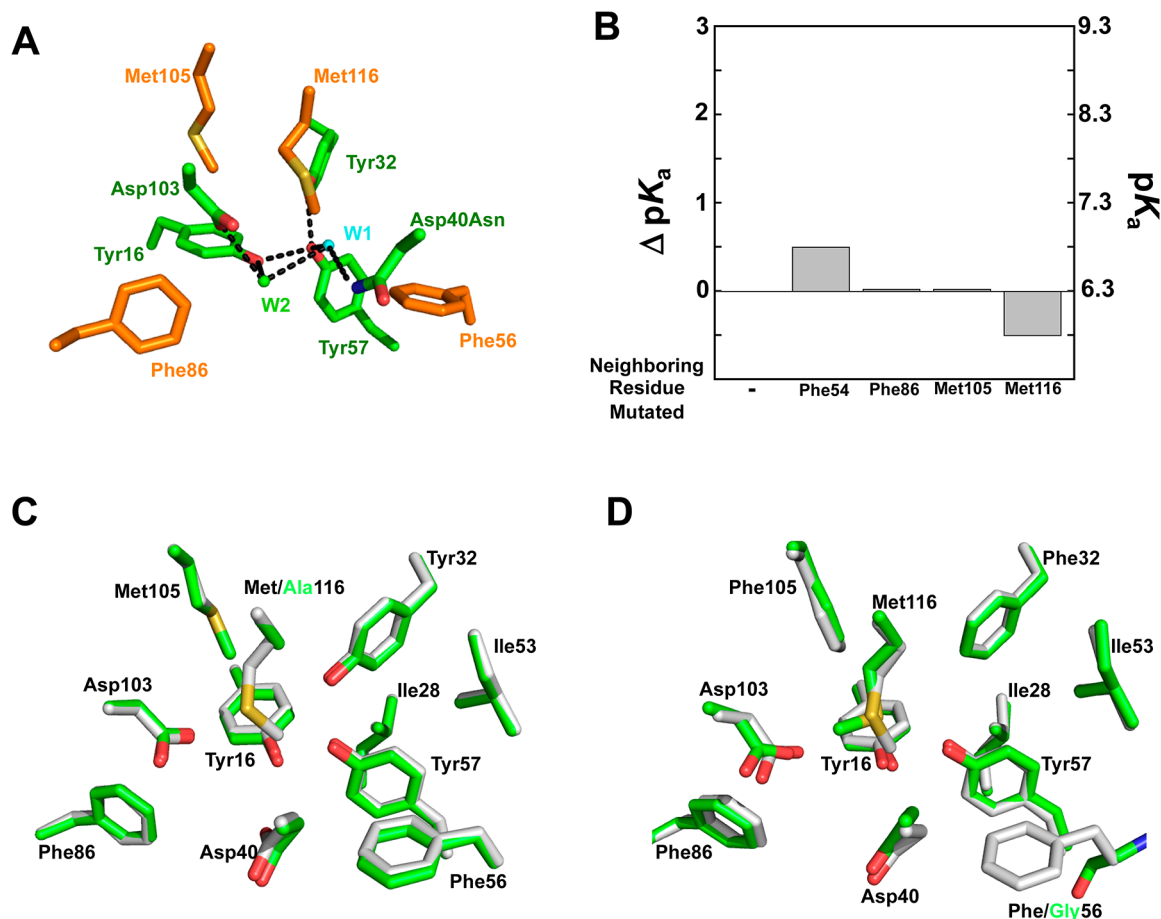


Figure 9. Effects of neighboring group mutations on the Tyr pK_a. (A) Structural model showing the positions of residues neighboring the active-site hydrogen-bond network that have been mutated. The same model as in Figure 2C is shown but with peripheral residues added (orange). (B) Changes in pK_a upon mutation of the peripheral residues to Ala relative to Asp40Asn KSI. “-” refers to the Asp40Asn variant. The values are from Table 7. (C) Structural overlays of the Met116Ala mutant (carbon atoms colored green) and wild-type pKSI (carbon atoms colored white). (D) Structural overlay of tKSI Phe56Gly mutant (carbon atoms colored green) with wild-type tKSI (carbon atoms colored white) showing that the residues engaged in the hydrogen-bonding network are essentially superimposable. pKSI numbering is used for tKSI for simplicity.

Table 7. Effects of Mutating Neighboring Residue to Ala on the Tyr pK_a

residue mutated	pK _a	ΔpK _a
none ^a	6.3	(0)
Phe56	6.9	0.6
Phe86	6.3	0
Met105	6.3	0
Met116	5.9	-0.4

^aNone refers to Asp40Asn.

any favorable effect from introducing a nearby positive charge. This residue also appears to rearrange in the presence of Asp40 (Figure 12B). Indeed, reversion of Asn40 to Asp40 in the Phe56Ala/Asp103Asn background gave a smaller destabilizing effect of 2.4 pK_a units, suggesting that factors in addition to the introduction of a negative charge at residue 40 contribute to the >3 pK_a unit increase for reverting Asn40 to Asp40 with Phe56 intact (Figures 11 and 13 and Table 9). The additional effect presumably arises from structural rearrangement of Phe56, consistent with effects noted above (Effects of Introducing Charged Residues at Position 40 on the Tyr pK_a).

Given that addition of the positively charged side chains at position 40 reduced the pK_a by ~2 units in the absence of the structurally rearranging Phe56 side chain, we tested whether the

remainder of the groups interacting with Tyr57 are used to stabilize the Tyr57 anion when an active-site positive charge is present. In other words, would a nearby positive charge alone be sufficient to stabilize ionized Tyr57 in an active site that likely does not suffer from structural rearrangements upon introducing charged groups. To eliminate potential complications from rearrangement of Phe56 and ionization of Asp103, Tyr16 and Tyr32 were individually mutated to Phe or Ala in the Asp40Arg/Phe56Ala/Asp103Ala background. These Tyr mutations eliminated the ionized Tyr (pK_a > 10; Table S4 and Figure S10 of the Supporting Information), suggesting that even with this nearby positive charge that can help stabilize the Tyr anion the adjacent Tyr residues are needed to stabilize the Tyr anion sufficiently to observe it below pH 10. The simplest model to account for these results is that the hydrogen bonds from the neighboring Tyr residues and possibly the positioned active-site water molecule (W2) contribute to Tyr anion stabilization such that the positively charged active-site residue at position 40 is not fully responsible for the pK_a perturbation.

Implications. Structural analysis has allowed us to develop a model for the substantial stabilization of the anionic form of an active-site Tyr residue, and extensive mutagenesis coupled with structural analysis has provided strong support for this model (Figure 2). Two neighboring Tyr residues are positioned to

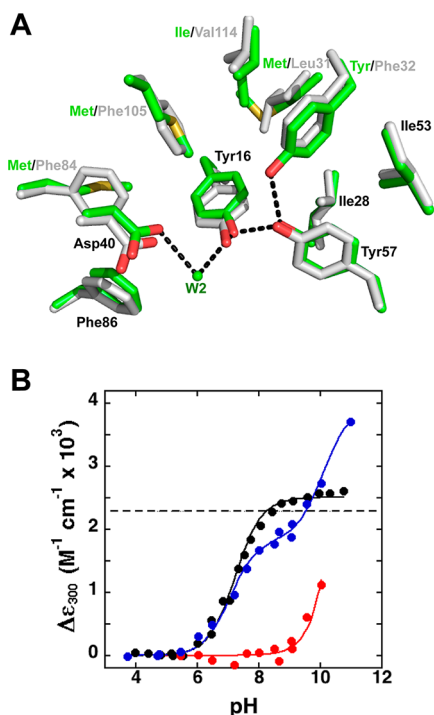


Figure 10. Recreating the potential hydrogen-bond network in tKSI. (A) Superposition of structural models of the wild-type pKSI (green, PDB ID 1OPY) and wild-type tKSI (gray, PDB ID 8CHO); although the active site residues are the same, the surrounding residues are different. (B) Relative extinction coefficients at 300 nm for pKSI D40N/D103N (black), tKSI D40N/D103N (red), and tKSI F32Y/D40N/D103N (blue) (pKSI numbering is used throughout). Absorbance spectra were collected as described in the Experimental Section. The lines are fits of the data to the ionization of one or two Tyr residues, and give Tyr pK_a values of 7.2 for pKSI D40N/D103N, >10 for tKSI D40N/D103N, and 7.3 for tKSI F32Y/D40N/D103N. The values are from Table 8.

Table 8. Effects of Tyr Mutations on the Tyr pK_a in tKSI^a

tKSI mutant	pK_a	ΔpK_a
D40N D103N	>10	(0)
F32Y D40N D103N	7.3	>-3

^aFor simplicity, pKSI numbering is used throughout.

donate hydrogen bonds to stabilize anionic Tyr57, and a third hydrogen bond is donated from a water molecule positioned within a hydrogen-bond network that involves a second

Table 9. Effects of Charged Asp40 Residues on the Tyr pK_a in the Asp103Asn Background

residue 40	pK_a	ΔpK_a
Asn	7.2	(0)
Asp	>10	>3
Glu	>10	>3
Arg	8.2	1.0
His	8.8	1.6
Lys	9.2	2.0

positioned water molecule, two distal side chains, and one of the Tyr hydrogen-bond donors. This model accounts for the ability of the KSI active site to lower the pK_a of Tyr57 from an expected solution value of 10.2 to 6.3, a stabilization of 5 kcal/mol that occurs without a nearby charged residue.

Whereas mutation of Tyr57 has only a small effect on KSI catalysis⁸⁴ and is not present in its anionic form in the normal reaction cycle, our multifaceted dissection of contributions to the 5 kcal/mol stabilization may have revealed properties that are also hallmarks of enzymatic catalysis. These include the positioning of residues, networks of interactions, and stabilization (here, of an anion and in catalysis of a transition state) via multiple features that contribute in a fundamentally nonadditive manner and can thus be difficult to uncover from individual mutations and without complementary structural information.^{43,85–92}

The high tractability of this system also allowed us to explore the effects from the presence of nearby charged residues. Whereas introduction of a nearby anion (the wild-type general base Asp40 for Asn at this position) gave the expected result of a large increase in the Tyr pK_a , the introduction of positively charged side chains at the same position also increased, rather than decreased, the pK_a . Structural analysis suggested a possible complication from a nearby Phe residue, Phe56, which alters its position in response to the introduction of either negatively or positively charged residues at position 40, leading to overestimation of the destabilizing effect from introduction of an anionic residue and obscuring the stabilizing effect from introduction of a cationic residue (Figure 12). Removal of the Phe56 side chain, and thus its disruptive reorientation, led to the expected simpler behavior: (i) introduction of the anionic Asp40 increased the Tyr57 pK_a (less than with Phe56 present) with the Tyr anion still experiencing net stabilization relative to solution because of the stabilizing features that remain surrounding it (Table 10; Figure 11) and (ii)

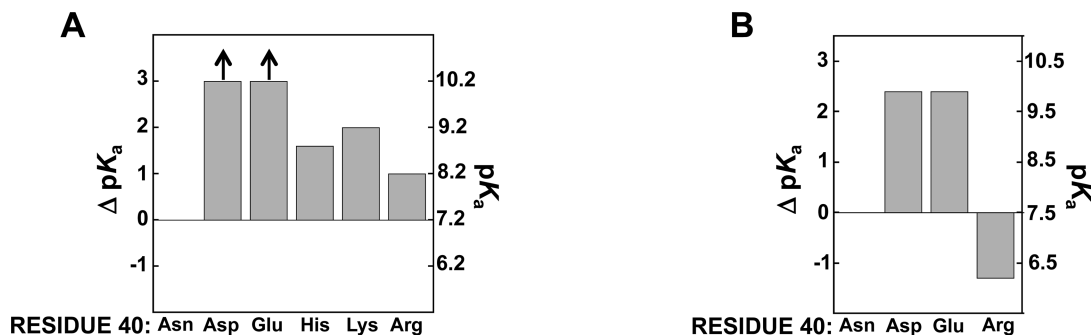


Figure 11. Effect of charged residues at position 40 on the Tyr pK_a . (A) Effects of charged residues in the Asn103 background. The ΔpK_a values are relative to the pK_a value for Asp40Asn/Asp103Asn KSI. The values are from Table 9. (B) Effects of charged mutations in the Phe56Ala/Asp103Asn background. The number on the right at pK_a 0 represents the pK_a for the parent form of KSI used in each comparison. The values are from Table 10.

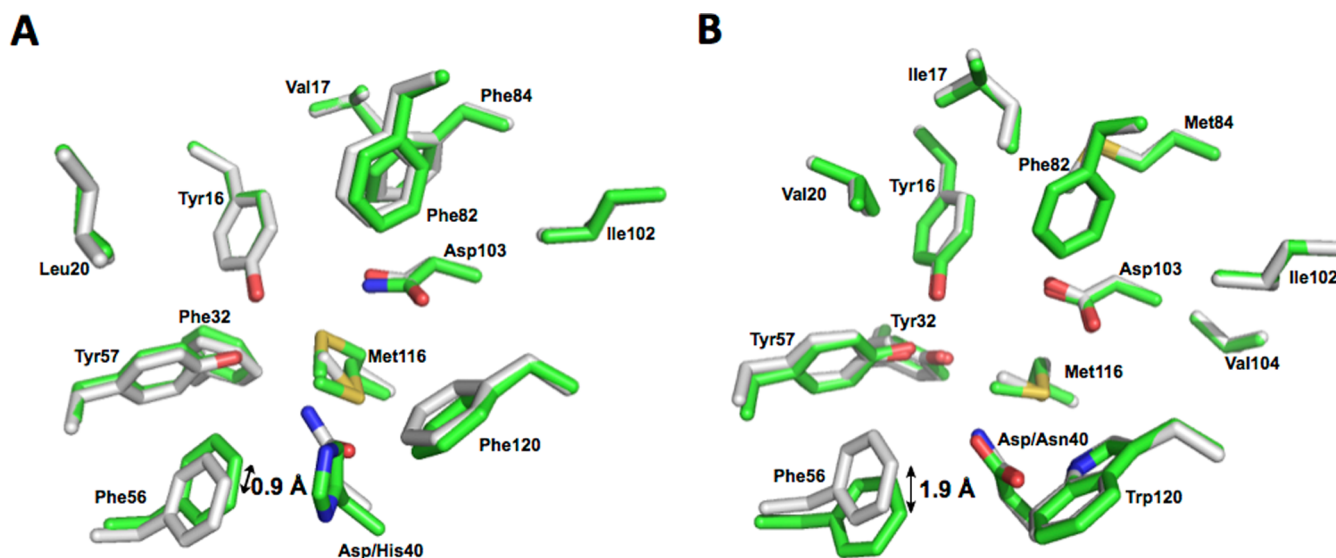


Figure 12. Introduction of charged residues at position 40 cause structural rearrangements of Phe56 relative to Asp40Asn. (A) Superposition of the 2.0 Å Asp40His/Asp103Asn structure determined herein (PDB ID 3MYT, carbon atoms colored green) and the previously determined 2.3 Å Asp40Asn structure (PDB ID 1QJG, carbon atoms colored white) showing that Phe56 is displaced 0.9 Å upon introduction of the His residue. (B) Superposition of the previously determined structures of the 1.1 Å wild-type KSI structure (PDB ID 1OH0, carbon atoms colored green) and the 2.0 Å Asp40Asn structure (PDB ID 1OGX, carbon atoms colored white) showing that the Phe56 side chain is displaced 1.9 Å upon changing residue 40 from Asp to Asn. pKSI numbering is used for simplicity.

Table 10. Effects of Charged Asp40 Residues on the Tyr pK_a in the Phe56Ala/Asp103Asn Background

residue 40	pK _a	ΔpK _a
Asn	7.4	(0)
Asp	9.8	2.4
Glu	9.8	2.4
Arg	5.9	-1.5

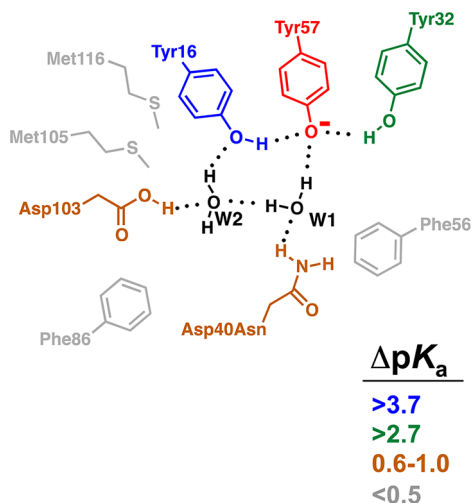


Figure 13. Summary of the effects of Ala mutations on the Tyr57 pK_a. Ionized Tyr57 is red, and the residues are colored according to the change in the Tyr pK_a value upon mutation of the residue to Ala (ΔpK_a, relative to Asp40Asn). The effects at residue 40 are for mutation from Asn to other neutral residues; mutation to charged residue can have larger effects (Tables 9 and 10). Crystallographically observed water molecules are colored black. Mutation of Tyr57 eliminates the observed pK_a, as expected (Table 1).

introduction of positively charged side chains at position 40 stabilizes the Tyr anion, as expected (Table 10; Figure 11).

Our ability to dissect these energetic contributions highlights the power of extensive mutagenesis carried out in a tight feedback loop with structure. For example, had we just mutated one or the other of the Tyr residues and saw the pK_a revert to >10 we might have concluded, incorrectly, that that a single Tyr was the origin of all of the 5 kcal/mol of stabilization and that this hydrogen bond was especially strong. Without extensive structural information, we might never have come upon the model of Figure 2 involving water molecules positioned within a hydrogen-bond network. Had we not identified the conformational rearrangement of Phe56 from X-ray structures, we might have incorrectly concluded that there was no significant electrostatic stabilization of the Tyr57 anion from introduction of a nearby positive charge (at position 40) in this system (Table 10; Figure 11).

Despite the strong structural and functional evidence for the model of Figure 2, we have not achieved a quantitative understanding of this system. For example, how much of the stabilization arises from the repositioning of the neighboring Tyr residues and Wat1, rendering formation of the anion less costly than in solution where conformational rearrangement of the hydrogen-bonding waters will be required? How much solvent rearrangement occurs beyond the first shell upon ionization in solution, and how much does the hydrogen-bonded network with Wat1 and Wat2 eliminate or reduce the need for such rearrangement? Are there three or only two hydrogen bonds, on average, from solvent to a tyrosinate anion in solution? Does the stronger hydrogen-bond donation ability of the Tyr hydroxyl groups relative to water (solution pK_a values of 10 vs 16) contribute substantially to the stabilization or are such effects small or negligible in the protein environment?

These quantitative questions pose a grand challenge to computational chemistry. We have presented 45 equilibrium values within a single environment, with substantial support from X-ray crystallographic structures. It is not surprising that the common, off-the-shelf pK_a prediction algorithms fail for this

system given the involvement of discrete water molecules.^{17,34,93–95} However, even the contributions from the neighboring Tyr residues in mutants with the water network disrupted are not accounted for.

We suggest that there is considerably more potential to unravel the energetic underpinnings of such complex systems such as KSI and similarly highly tractable systems in comparison to systems where only a limited number of measurements can be compared to computation or where multiple measurements are distributed over the entire protein rather than focused in a particular region. We believe that such studies must, as has occurred in the protein-folding community,^{96–99} make predictions (in this case, of new pK_a values and, ultimately, also of accompanying conformational and/or dynamic changes). We would be pleased to carry out experimental tests after such predictions are made to deepen the connection between experiment and computation and allow true blind tests of emerging methods, and we urge interested computational chemists to contact us.

■ ASSOCIATED CONTENT

■ Supporting Information

Effect of the R15K/D21N/D24C mutations on KSI activity; ¹³C NMR spectra of recombinant KSI bearing mutations for semisynthesis; space-filling representation of KSI showing the location of Tyr57 and Tyr32 in the active site; ¹³C NMR spectra of the Tyr mutants; change in extinction coefficient at 300 nm for the Tyr16Ala and Tyr32Ala mutations; superposition of the X-ray structures of the Tyr16Phe and Tyr32Phe mutants with wild-type KSI; change in extinction coefficient at 300 nm for the Asp103 mutations; ¹³C NMR spectra of the Asp40 mutants; effects of the Asp40Leu mutation on the Tyr pK_a in the Phe56 and Phe56Ala backgrounds; change in extinction coefficient at 300 nm for mutation of Tyr16 and Tyr32 to Phe and Ala in the D40R/F56A/D103A background; Tyr O–O distances in crystal structures bearing an expected neutral and anionic Tyr at position 57; crystallographic data and refinement statistics for the Met116Ala and tKSI Asp40His/Asp103Asn structures; summary of the previously reported Tyr residues with low pK_a values; and summary of effects of the mutations on the Tyr pK_a reported herein. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

KSI, ketosteroid isomerase; pKSI, KSI from *Pseudomonas putida*; tKSI, KSI from *Comamonas testosteroni*; PDB, Protein Data Bank; SPPS, solid-phase peptide synthesis; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SUMO, small ubiquitin-like modifier

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