

Quantum delocalization of protons in the hydrogen-bond network of an enzyme active site

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Enzymes use protein architectures to create highly specialized structural motifs that can greatly enhance the rates of complex chemical transformations. Here, we use experiments, combined with ab initio simulations that exactly include nuclear quantum effects, to show that a triad of strongly hydrogen-bonded tyrosine residues within the active site of the enzyme ketosteroid isomerase (KSI) facilitates quantum proton delocalization. This delocalization dramatically stabilizes the deprotonation of an active-site tyrosine residue, resulting in a very large isotope effect on its acidity. When an intermediate analog is docked, it is incorporated into the hydrogen-bond network, giving rise to extended quantum proton delocalization in the active site. These results shed light on the role of nuclear quantum effects in the hydrogen-bond network that stabilizes the reactive intermediate of KSI, and the behavior of protons in biological systems containing strong hydrogen bonds.

enzyme | hydrogen bonding | nuclear quantum effects | proton delocalization | ab initio path integral molecular dynamics

Although many biological processes can be well-described with classical mechanics, there has been much interest and debate as to the role of quantum effects in biological systems ranging from photosynthetic energy transfer, to photoinduced isomerization in the vision cycle and avian magnetoreception (1). For example, nuclear quantum effects, such as tunneling and zero-point energy (ZPE), have been observed to lead to kinetic isotope effects of greater than 100 in biological proton and proton-coupled electron transfer processes (2, 3). However, the role of nuclear quantum effects in determining the ground-state thermodynamic properties of biological systems, which manifest as equilibrium isotope effects, has gained significantly less attention (4).

Ketosteroid isomerase (KSI) possesses one of the highest enzyme unimolecular rate constants and thus, is considered a paradigm of proton transfer catalysis in enzymology (5–11). The remarkable rate of KSI is intimately connected to the formation of a hydrogen-bond network in its active site (Fig. 1A), which acts to stabilize a charged dienolate intermediate, lowering its free energy by ~11 kcal/mol (1 kcal = 4.18 kJ) relative to solution (Fig. S1) (6). This extended hydrogen-bond network in the active site links the substrate to Asp103 and Tyr16, with the latter further hydrogen-bonded to Tyr57 and Tyr32, which is shown in Fig. 1A.

The mutant KSI^{D40N} preserves the structure of the wild-type (WT) enzyme while mimicking the protonation state of residue 40 in the intermediate complex (Fig. 1B), therefore permitting experimental investigation of an intermediate-like state of the enzyme (6, 12–14). Experiments have identified that, in the absence of an inhibitor, one of the residues in the active site of KSI^{D40N} is deprotonated (12). Although one might expect the carboxylic acid of Asp103 to be deprotonated, the combination of recent ¹³C NMR and ultraviolet visible spectroscopy (UV-Vis) experiments has shown that the ionization resides primarily on the hydroxyl group of Tyr57, which possesses an anomalously low pK_a of 6.3 ± 0.1 (12). Such a large tyrosine acidity is often associated with specific stabilizing electrostatic interactions (such

as a metal ion or cationic residue in close proximity), which is not the case here, suggesting that an additional stabilization mechanism is at play (15).

One possible explanation is suggested by the close proximity of the oxygen (O) atoms on the side chains of the adjacent residues Tyr16 (O16) and Tyr32 (O32) to the deprotonated O on Tyr57 (O57) (Fig. 1C) (16). In several high-resolution crystal structures, these distances are found to be around 2.6 Å (14, 16, 17), which is much shorter than those observed in hydrogen-bonded liquids such as water, where O–O distances are typically around 2.85 Å. Such short heavy-atom distances are only slightly larger than those typically associated with low-barrier hydrogen bonds (18–20), where extensive proton sharing is expected to occur between the atoms. In addition, at these short distances, the proton's position uncertainty (de Broglie wavelength) becomes comparable with the O–O distance, indicating that nuclear quantum effects could play an important role in stabilizing the deprotonated residue (Fig. 1C). In this work, we show how nuclear quantum effects determine the properties of protons in the active-site hydrogen-bond network of KSI^{D40N} in the absence and presence of an intermediate analog by combining ab initio path integral simulations and isotope effect experiments.

Isotope Substitution Experiments Reveal Large Isotope Effect on Acidity

To assess the impact of nuclear quantum effects on the anomalous acidity of Tyr57, we measured the isotope effect on the acid dissociation constant on substituting hydrogens (H) in the hydrogen-bond network with deuterium (D). Because tyrosinate absorbs light at 300 nm more intensely than tyrosine, titration curves were generated by recording UV spectra of KSI^{D40N} at different pL values (where L is H or D) (15). These experiments

Significance

Because of the low mass of the proton, nuclear quantum effects can dramatically alter the properties of hydrogen-bond networks, especially when short and strong hydrogen bonds occur. Here, we combine experiments and state-of-the-art simulations that include the quantum nature of both the electrons and nuclei to show that the enzyme ketosteroid isomerase contains a hydrogen-bond network in its active site that facilitates extensive quantum proton delocalization. This leads to a 10,000-fold increase in the acidity of an active-site residue compared with the limit where the nuclei are classical particles. This work opens up new avenues for understanding the interplay between quantum effects and hydrogen bonding in biological systems containing strong hydrogen bonds.

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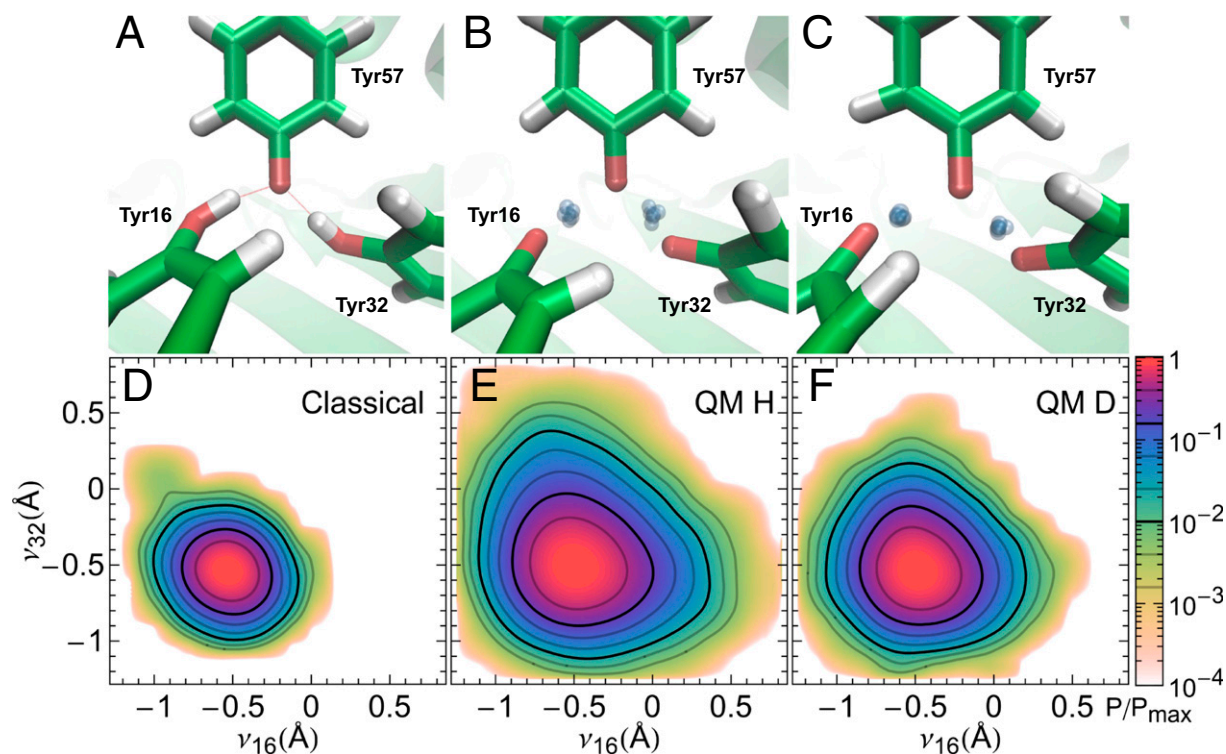


Fig. 3. Delocalized protons in the active site of KSI^{D40N} from AIMD and AI-PIMD simulations. Snapshots of (A–C) the active site of KSI^{D40N} and (D–F) probability distribution along the proton-sharing coordinates ν_{16} and ν_{32} when the nuclei are treated classically (Classical) or quantum mechanically for H (QM H) and D (QM D). In A–C, green, red, and white represent C, O, and H atoms, respectively. The blue-gray spheres in the QM snapshots show uncertainty in the delocalized protons positions. For clarity, all other particles are represented by their centroids. In D–F, probabilities are shown on a log scale and normalized by their maximum values.

isotope effects in water, validating such a combination for the simulation of isotope effects in hydrogen-bonded systems (37).

Quantum Delocalization of Protons in KSI^{D40N}

The excess isotope effect, $\Delta\Delta pK_a$, obtained from our simulations (*SI Materials and Methods, section C*) was 0.50 ± 0.03 , which is in excellent agreement with the experimental value of 0.57 ± 0.16 . The average distances between O57 and the adjacent O16 and O32 atoms obtained in our simulations were 2.56 and 2.57 Å, with standard deviations in both cases of 0.09 Å. The distribution of distances between O16 and O57 explored in the simulation is shown in Fig. 4A, *Inset*. These average O–O distances are slightly smaller than (and within the margin of error of) those in the starting crystal structure (~ 2.6 Å) (16). As we will discuss below, the close proximity of the neighboring O16 and O32 groups plays a crucial role in the origins of the observed isotope effect.

Fig. 3A–C shows snapshots from AIMD simulations, in which the nuclei are treated classically (Fig. 3A) or quantum mechanically using the path integral formalism (AI-PIMD) (Fig. 3B and C), whereas *Movies S1–S3* show the simulation trajectories. For the quantum simulations, the H16 and H32 protons are shown as their full ring polymers, which arise from the path integral quantum mechanics formalism. The spread of the ring polymer representing each proton is related to its de Broglie wavelength (quantum mechanical position uncertainty) (38, 39). The uncertainty principle dictates that localization of a quantum mechanical particle increases its quantum kinetic energy. The protons will thus attempt to delocalize (i.e., spread their ring polymers) to reduce this energetic penalty. The resulting proton positions in Fig. 3B arise from the interplay between the chemical environment, such as the covalent O–H bond, which acts to localize the proton and the quantum kinetic energy penalty that

must be paid to confine a quantum particle. Inclusion of nuclear quantum effects, thus, allows the protons to delocalize between the hydroxyl oxygens to mitigate the quantum kinetic energy penalty (Fig. 3B), which is not observed classically (Fig. 3A). Confinement of D, which because of its larger mass has a smaller position uncertainty, leads to a much less severe quantum kinetic energy penalty and hence less delocalization (Fig. 3C).

To characterize the degree of proton delocalization, we define a proton-sharing coordinate $\nu_X = d_{\text{OX},\text{HX}} - d_{\text{O57},\text{HX}}$, where $d_{\text{OX},\text{HX}}$ is the distance of proton HX from oxygen atom OX, and $X = 16$ or 32. Hence, $\nu_X = 0$ corresponds to a proton that is equidistant between the oxygen atoms of TyrX and Tyr57, whereas a positive value indicates proton transfer to Tyr57 from TyrX. Fig. 3D–F show the probability distribution along the proton-sharing coordinates ν_{16} and ν_{32} for classical and quantum nuclei for H and D, respectively. The free energies along ν_{16} and ν_{32} are provided in Fig. S3. In the classical AIMD simulation, H16 and H32 remain bound to their respective oxygens throughout the simulation (ν_{16} and ν_{32} are negative), with Tyr57 ionized 99.96% of the time (Fig. 3D). However, on including nuclear quantum effects (AI-PIMD simulations), there is a dramatic increase in the range of values that ν_{16} and ν_{32} can explore (Fig. 3E). In particular, the probability that Tyr57 is protonated ($\nu_X > 0$) increases by about 150-fold for H after including quantum effects (Fig. 3E), with the proton hole equally shifted onto the adjacent Tyr16 or Tyr32 residues. Proton transfers between the residues are observed frequently (*Movies S2* and *S3*), with site lifetimes on the order of 60 and 200 fs in the H and D simulations, respectively. Although path integral molecular dynamics simulations exactly include nuclear quantum effects for calculating static properties, they do not allow rigorous extraction of time-dependent properties; nevertheless, they offer a crude way to assess the timescale of the

proton motion. The frequent transfers observed are also consistent with Fig. 3 *E* and *F*, which shows a monotonic decrease in the probability along both ν_{16} and ν_{32} [i.e., although the proton-transferred state is lower in probability, the proton-transfer process along each of the proton-sharing coordinates contains no free-energy barrier (Fig. S3) and thus, is kinetically fast].

As an experimental counterpart, we used chemical shifts of $^{13}\text{C}_\alpha$ -Tyr-labeled KSI D40N as a measure of fractional ionization of each Tyr residue (*SI Materials and Methods*, section B and Fig. S4) (14). This analysis yielded values of 79% for the Tyr57 ionization for H and 86% for D (Table S3) compared with simulated values of 94.2% and 98.3% ($\pm 0.3\%$), respectively. This comparison represents good quantitative agreement, because the population difference amounts to a difference in the relative free energy between experiment and theory of 0.7 kcal/mol—an error that is within the expected accuracy of the electronic structure approach used. In addition, the change in the ionization of Tyr57 obtained experimentally on exchanging H for D (7%) is in good agreement with the value predicted from our simulations (4.1%). The underprediction of the isotope effect on fractional ionization from our simulations is in line with the slightly low value of the simulated excess isotope effect, which is consistent with recent observations that the B3LYP-D3 density functional slightly underestimates the degree of proton sharing and hence, isotope effects in hydrogen-bonded systems after including nuclear quantum effects (37).

The large degree of proton sharing with the deprotonated Tyr57 residue upon including nuclear quantum effects can be elucidated by considering the potential energy required, $\Delta E_{\nu=0}$, to move a proton in the KSI tyrosine triad from its energetic minimum to a perfectly shared position between the two tyrosine groups ($\nu = 0$). $\Delta E_{\nu=0}$ depends strongly on the positions of the residues comprising the triad and in particular, the separation between the proton donor and acceptor oxygen atoms. Fig. 4*A* shows $\Delta E_{\nu=0}$ computed as a function of the distance between O16 and O57, R_{OO} , for the tyrosine triad in the absence of the protein environment. Removing the protein environment allows us to examine how changes in the triad distances from their positions in the enzyme affect the proton delocalization behavior without introducing steric overlaps with other active-site residues (*SI Materials and Methods*, section D).

Fig. 4*A* shows that, for the range of oxygen distances observed in the tyrosine triad ($R_{OO} = 2.50$ – 2.65 Å), $\Delta E_{\nu=0}$ for H16 is 3–6 kcal/mol. This energy is 6–12 times the thermal energy ($k_B T$) available at 300 K, leading to a very low thermal probability of the proton-shared state (lower than $e^{-6} = 2 \times 10^{-3}$). However, upon including nuclear quantum effects, the system possesses ZPE, which in this system is ~ 4 kcal/mol. The ZPE closely matches $\Delta E_{\nu=0}$ and thus floods the potential energy wells along the proton-sharing coordinate (Fig. 4*B*), allowing facile proton sharing (Fig. 3) (i.e., inducing a transition to a low-barrier hydrogen bond-type regime where the protons are quantum mechanically delocalized between the hydrogen-bonded heavy atoms) (18–20). This energy match leads to qualitatively different behavior of the protons in the active site of KSI D40N : from classical hydrogen bonding to quantum delocalization. The proton delocalization between the residues allows for ionization to be shared among three tyrosines to stabilize the deprotonation of Tyr57, leading to the large observed pK_a shift relative to the value in the classical limit (Fig. S2). This change in proton behavior gives rise to the large excess isotope effect, because an O–D stretch possesses a ZPE of ~ 3 kcal/mol, which is no longer sufficient to fully flood the potential energy well in the proton-sharing coordinate ν . As the O–O separation is decreased below the values observed in the tyrosine triad of KSI, $\Delta E_{\nu=0}$ becomes negligible compared with the thermal energy (~ 0.6 kcal/mol at 300 K). Hence, at very short distances (< 2.3 Å), thermal fluctuations alone permit extensive proton sharing between the residues, and the ZPE plays

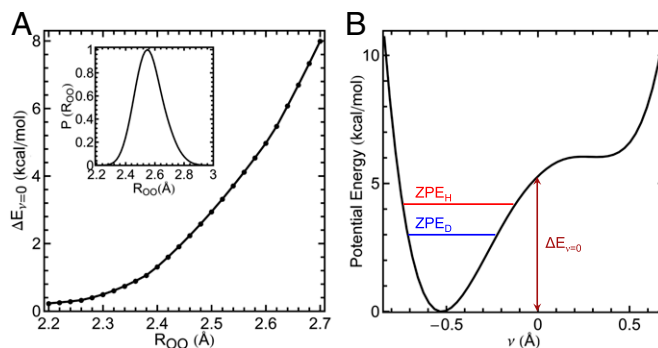


Fig. 4. Comparison of the energy required to share a proton between residues ($\Delta E_{\nu=0}$) as a function of the hydrogen-bond donor-acceptor O–O distance (R_{OO}) compared with the ZPE. (A) $\Delta E_{\nu=0}$ as a function of the O–O distance (R_{OO}) between O16 and O57 using the tyrosine triad geometry from a crystal structure (details in *SI Materials and Methods*, section D). A, Inset shows the probability distribution of R_{OO} obtained from the AI-PIMD simulation of KSI D40N with ionized Tyr57. The probability is normalized by the maximum value. (B) Potential energy as a function of the proton transfer coordinate ν for $R_{OO} = 2.6$ Å, indicating values for H and D (O–H and O–D) ZPEs (ZPE_H and ZPE_D , respectively). The position of Tyr32 is fixed as the proton H16 is scanned along R_{OO} .

a negligible role in determining the protons positions. Thus, one would expect a small isotope effect. However, at bond lengths in excess of 2.7 Å, $\Delta E_{\nu=0}$ becomes so large (> 8 kcal/mol) (Fig. 4*A*) that the ZPE is not sufficient to flood the barriers, also resulting in a small expected isotope effect (40). The large excess isotope effect in KSI D40N , thus, arises from the close matching of the ZPE and the depth of the energetic well ($\Delta E_{\nu=0}$), which is highly sensitive to the O–O distance. Hence, although proton delocalization can occur classically at short O–O distances (< 2.3 Å), nuclear quantum effects allow this to occur for a much wider range of O–O distances (up to ~ 2.6 Å), making delocalization feasible without incurring the steep steric costs that would be associated with bringing oxygen atoms any closer. The distances in the active-site triad motif of KSI, thus, maximize quantum proton delocalization, which acts to stabilize the deprotonated residue.

Quantum Delocalization Stabilizes an Intermediate Analog by Sharing Ionization

Finally, we considered the role of nuclear quantum effects when an intermediate analog participates in the active-site hydrogen-bond network. Recent experiments have investigated how the binding of intermediate analogs to KSI D40N affects the sharing of ionization along the extended hydrogen-bond network that is formed (Fig. 1*B*) (14). These experiments identified that ionization sharing is maximized when phenol, whose solution pK_a of 10 equals that of the actual intermediate of KSI (Fig. 1*A*), is bound (14, 41, 42). We thus performed AI-PIMD simulations of the KSI D40N -phenol complex. The protons were observed to be delocalized across the network (Fig. 5), with partial ionizations of Tyr57, Tyr16, and phenol calculated to be 18.5%, 56.7%, and 22.5% ($\pm 0.7\%$), compared with estimates from previous experiments using ^{13}C NMR of 40%, 40%, and 20% (14). Hence, simulation and experiment are in good agreement that the ionization is shared almost equally among the three residues [i.e., that there is almost no difference ($< 2 k_B T$) in the free energy after shifting the ionization among any of the three groups]. Therefore, the ability of the protons to delocalize within the KSI D40N tyrosine triad, initially found in the enzyme in the absence of the intermediate analog and manifested as a strongly perturbed pK_a , extends on incorporation of the intermediate analog, which shifts the center of the ionization along the network from Tyr57 to Tyr16. In both cases, proton delocalization

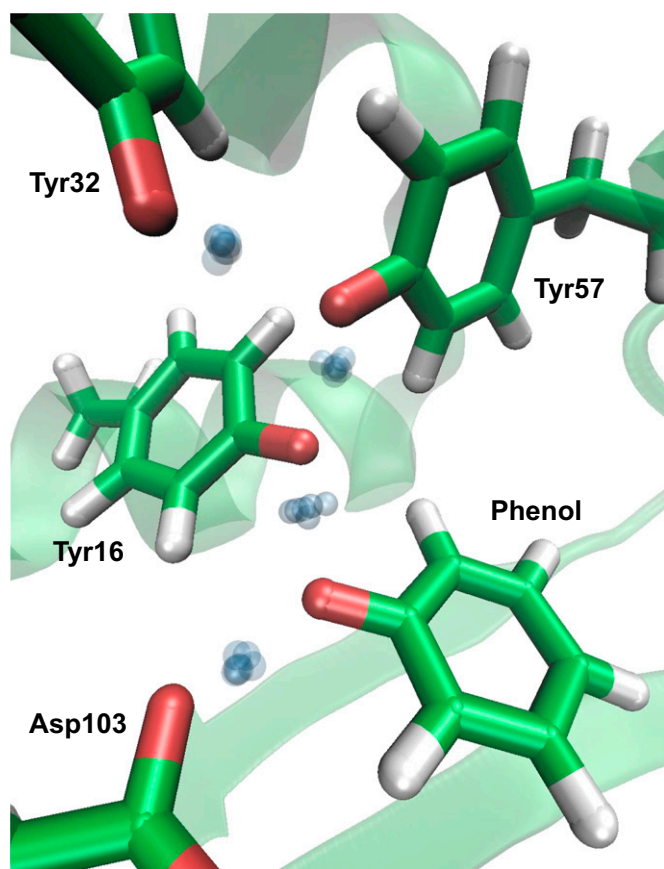


Fig. 5. Simulation snapshot of the KSI^{D40N} active site with the bound intermediate analog (phenol) that gives rise to an extended delocalized proton network. Green, red, and white represent C, O, and H atoms, respectively. The blue spheres represent uncertainty in the delocalized proton position in the hydrogen-bond network. For clarity, all other particles are represented by their centroids.

acts to share the ionization of a negatively charged group, which suggests that KSI could use quantum delocalization in its active-site hydrogen-bond network to distribute the ionization arising in its intermediate complex (Fig. 1*A*) to provide energetic stabilization.

Conclusion

In conclusion, KSI^{D40N} exhibits a large equilibrium isotope effect in the acidity of its active-site tyrosine residues arising from a highly specialized triad motif consisting of several short O–O distances, whose positions that enhance quantum delocalization of protons within the active-site hydrogen-bond network. This delocalization manifests in a very large isotope effect and substantial acidity shift. Our simulations, which include electronic quantum effects and exactly treat the quantum nature of the nuclei, show qualitatively and quantitatively different proton behavior compared with conventional simulations in which the nuclei are treated classically, and provide good agreement with experiment. The ability to perform such simulations thus offers the opportunity to investigate in unprecedented detail the plethora of systems in which short-strong hydrogen bonds occur, where incorporating both nuclear and electronic quantum effects is crucial to understand their functions.

Materials and Methods

Expression and Purification of KSI. WT and KSI^{D40N} from *Pseudomonas putida* were overexpressed in BL-21 A1 cells (Invitrogen), isolated by affinity

chromatography using a custom-designed deoxycholate-bound column resin, and purified by gel filtration chromatography (GE Healthcare) as described previously (43). For ¹³C NMR experiments, ¹³C_ε-tyrosine was incorporated into KSI according to the methods described previously (14).

UV-Vis Titration Experiments. A series of buffers were prepared with a *pL* between 4 and 10 by weighing portions of a weak acid and its sodium-conjugate base salt and adding the appropriate form of distilled deionized water [Millipore H₂O and Spectra stable isotopes sterile-filtered D₂O (>99% ²H)]. Buffers were prepared at 40 mM. Tyr57 is solvent-accessible, and therefore, the tyrosine residues in the active-site network are expected to be fully deuterated in D₂O solution.

The following buffer systems were used for the following *pL* ranges: acetic acid/sodium acetate, 4–5.25; sodium monobasic phosphate/dibasic phosphate, 5.5–8.25; and sodium bicarbonate/sodium carbonate, 8.5–10. Buffers were stored at room temperature with caps firmly sealed.

After preparation of buffers, *pL* was recorded using an Orion2 Star glass electrode (Thermo) immediately after calibration with standard buffers at pH 4, pH 7, and pH 10. In H₂O, the pH of the buffer was taken as the reading on the electrode. In D₂O, the *pD* of the buffer was calculated by adding 0.41 to the operational pH* from the electrode reading (44). A series of samples for titration was prepared by combining 60 μL protein (100 μM stock in buffer-free L₂O), buffer (150 μL 40 mM stock), and extra L₂O. The final samples were 600 μL, 10 μM protein, and 10 mM buffer.

UV-Vis measurements were carried out on the samples on a Lambda 25 Spectrophotometer (Perkin-Elmer) that acquired data from 400 to 200 nm with a 1.0-nm data interval, a 960-nm/min scan rate, and a 1.00-nm slit width. For each measurement, a background was taken to the pure buffer of a given *pL* before acquiring on the protein-containing sample. Spectra were recorded in duplicate to control for random detector error.

The spectra were baselined by setting the absorption at 320 nm to zero, and the change in absorption at 300 nm was followed at varying *pL* values using a previously established method (15) to determine the fractional ionization of a tyrosine-tyrosinate pair. The error in A₃₀₀ from comparing duplicate spectra after baselining was generally between 0% and 2%. For each *pL*, the average A₃₀₀ was calculated and converted to an extinction coefficient (ϵ_{300}). The titration experiment was repeated on two independently prepared buffer stocks to control for error in buffer preparation.

Simulations. AI-PIMD and AIMD simulations were performed using a QM/MM approach of KSI^{D40N} with Tyr57 protonated, KSI^{D40N} with Tyr57 ionized, KSI^{D40N} with the intermediate analog bound, and tyrosine in aqueous solution. The simulations were carried out in the NVT ensemble at 300 K with a time step of 0.5 fs. The path integral-generalized Langevin equation approach was used, which allowed results within the statistical error bars to be obtained using only six path integral beads to represent each particle (34). The electronic structure in the QM region (Fig. S5) was evaluated using the B3LYP functional (28) with dispersion corrections (29). The 6–31G* basis set was used, because we found it to produce proton transfer potential energy profiles with a mean absolute error of less than 0.4 kcal/mol compared to using larger basis sets for this system (Fig. S6). Energies and forces in the QM region and the electrostatic interactions between the QM and MM regions were obtained using an MPI interface to the GPU-accelerated TeraChem package (35, 36). Atoms in the MM region were described using the AMBER03 force field (45) and the TIP3P water model (46). The simulations were performed using periodic boundary conditions with Ewald summation to treat long-range electrostatic interactions. The energies and forces within the MM region and the Lennard-Jones interactions between the QM and MM regions were calculated by MPI calls to the LAMMPS molecular dynamics package (47). The QM region of KSI^{D40N} contained the *p*-methylene phenol side chains of residues Tyr16, Tyr32, and Tyr57 (Fig. S5*A*). For KSI^{D40N} with the intermediate analog, residue Asp103 and the bound intermediate analog were also included in the QM region (Fig. S5*B*). The QM region of tyrosine in solution contained the side chain of the tyrosine residue and the 41 water molecules within 6.5 Å of the side-chain O–H group. All bonds across the QM/MM interface were capped with hydrogen link atoms in the QM region (26). These capping atoms were constrained to be along the bisected bonds and do not interact with the MM region.

The initial configuration of KSI^{D40N} was obtained from a crystal structure (16) (Protein Data Bank ID code 1OGX). For KSI^{D40N} with the intermediate analog, a crystal structure (14) (Protein Data Bank ID code 3VGN) was used with the ligand changed to phenol. The crystal structures were solvated in TIP3P water, and the energy was minimized before performing AI-PIMD simulations. The initial configuration for tyrosine in aqueous solution was obtained by solvating the amino acid in TIP3P (46) water using the AMBER03

force field (45) and equilibrating for 5 ns in the NPT ensemble at a temperature of 300 K and pressure of 1 bar. Each system was then equilibrated for 10 ps followed by production runs of 30 ps.

To calculate the excess isotope effect, $\Delta\Delta pK_a$ (SI Materials and Methods, section C), we used the thermodynamic free-energy perturbation path integral estimator (22). Combined with an appropriate choice of the integration variable to smooth the free-energy derivatives (22), this approach allowed us to evaluate the isotope effects in the liquid phase using only a single AI-PIMD trajectory. Simulations performed with D substitution showed no change within the statistical error bars reported.

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