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Ground State Destabilization from a Positioned General Base in the Ketosteroid Isomerase Active Site

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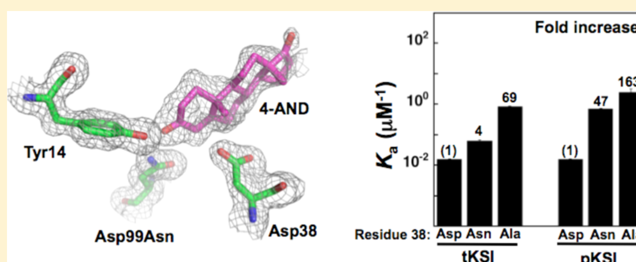
Supporting Information

ABSTRACT: We compared the binding affinities of ground state analogues for bacterial ketosteroid isomerase (KSI) with a wild-type anionic Asp general base and with uncharged Asn and Ala in the general base position to provide a measure of potential ground state destabilization that could arise from the close juxtaposition of the anionic Asp and hydrophobic steroid in the reaction's Michaelis complex. The analogue binding affinity increased ~1 order of magnitude for the Asp38Asn mutation and ~2 orders of magnitude for the Asp38Ala mutation, relative to the affinity with Asp38, for KSI from two sources. The increased level of binding suggests that the abutment of a charged general base and a hydrophobic steroid is modestly destabilizing, relative to a standard state in water, and that this destabilization is relieved in the transition state and intermediate in which the charge on the general base has been neutralized because of proton abstraction. Stronger binding also arose from mutation of Pro39, the residue adjacent to the Asp general base, consistent with an ability of the Asp general base to now reorient to avoid the destabilizing interaction. Consistent with this model, the Pro mutants reduced or eliminated the increased level of binding upon replacement of Asp38 with Asn or Ala. These results, supported by additional structural observations, suggest that ground state destabilization from the negatively charged Asp38 general base provides a modest contribution to KSI catalysis. They also provide a clear illustration of the well-recognized concept that enzymes evolve for catalytic function and not, in general, to maximize ground state binding. This ground state destabilization mechanism may be common to the many enzymes with anionic side chains that deprotonate carbon acids.



Enzymes catalyze biological transformations with extraordinary rate enhancements and exquisite specificities. Decades of research have shown that enzymes employ multiple strategies to attain their remarkable catalytic power (e.g., refs 1−10). Nevertheless, there is a dearth of experimental tests of certain potential catalytic mechanisms, including ground state destabilization.[9,11−13]

The concept of ground state destabilization had its beginnings in the idea that increased reaction rates could be obtained by activating substrates. More than seven decades ago, Bayliss suggested that increased reaction rates could be the result of "a rise in chemical potential of the reacting substrates",[14] and Quastel recognized that an enzyme's idiosyncratic electric field could induce effects on a substrate and so activate it.[15] Haldane suggested enzymatic interactions impart a potential strain on the substrate (later likened by Eyring and co-workers to the ancient torture device the "rack"), and Jencks further clarified and generalized this concept in terms of enzymatic binding interactions and binding energy.[11,16,17] Specifically, Jencks stated that "binding energy may be used to increase the rate of catalyzed reactions by destabilizing the bound substrate relative to the transition state" and noted that "in order for the mechanism of catalysis to be effective it is necessary that the destabilization be relieved at the transition state so that the free energy of activation that is required to reach the transition state is reduced".[11]

One form of ground state destabilization described by Jencks is ubiquitous in enzymes, an "entropic" or positioning destabilization from limiting the freedom of motion of the substrates with respect to one another and with respect to enzyme catalytic groups within the active site via binding interactions that increase the probability of reaction of the bound substrate(s) relative to free substrates and catalytic groups present in solution at a given concentration.[11,18−22] While the potential for rate enhancement from other ground state destabilization mechanisms has long been discussed (e.g., refs 12, 13, and 23−34), experimental tests have been limited.[7,35−39] Destabilizing interactions are often inferred from structure; however, energetics cannot be read from structure, so that functional experiments are needed. The simple and well-studied reaction of ketosteroid isomerase (KSI) provides a powerful system for further deepening our

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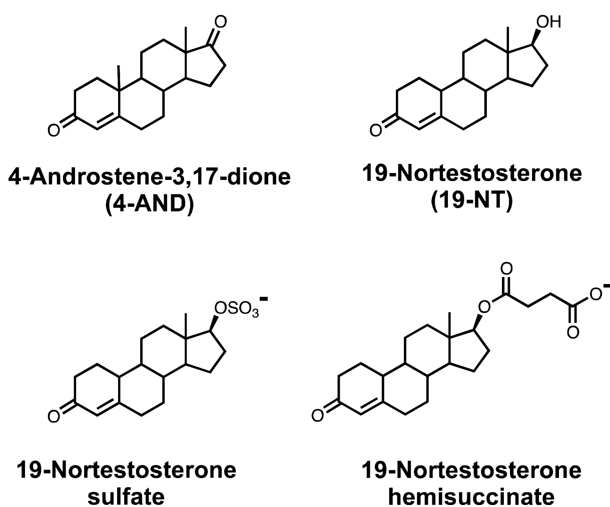
understanding of fundamental features of enzymatic catalysis.[40,41] Herein we combine structural and functional studies of KSI to investigate the potential catalytic contribution from ground state destabilization arising from interactions of the enzyme's general base and substrate. The results are consistent with a modest contribution in the KSI reaction, and other enzymes with similar potential for ground state destabilization will be amenable to analysis via the approaches outlined herein.

■ EXPERIMENTAL PROCEDURES

Materials. All reagents were of the highest purity commercially available (≥97%). 4-Androstene-3,17-dione (4-AND), 19-nortestosterone (19-NT), 19-nortestosterone sulfate (19-NT-sulfate), and 19-nortestosterone hemisuccinate (19-NT-hemisuccinate) were purchased from Steraloids (Newport, RI) (steroid structures are given in Scheme 1). All buffers were prepared with reagent grade materials or better.

Scheme 1. Structures of the Ground State Analogues Used Herein



KSI Mutagenesis, Expression, and Purification. QuikChange (Stratagene) site-directed mutagenesis was used to introduce the mutations into pKSI and tKSI genes encoded on pKK223-3 plasmids, which were confirmed by sequencing miniprep DNA from DH5α cells on an ABI3100 capillary sequencer (Stanford Protein and Nucleic Acid sequencing facility). Proteins were expressed and purified as previously described.[42] The final purity was >99% as estimated on the basis of a Coomassie-stained sodium dodecyl sulfate−polyacrylamide gel electrophoresis gel. The protein concentration was determined using the calculated molar extinction coefficient in 6 M guanidinium hydrochloride according to the method of Gill et al.[43]

Measuring the Affinity of Steroid Ligands for KSI. Fluorescence measurements were performed at 20 °C on a Fluorolog-3 spectrofluorometer (Horiba Jobin Yvon, FL3-22) using 45 μL microcuvettes from Starna Cells (Atascadero, CA) with excitation at 277 nm and recording emission at 345−355 and 307 nm for pKSI and tKSI, respectively. Band passes were 2 nm (excitation) and 4 nm (emission). Measurements were taken using 40 mM buffer and 1 mM sodium·EDTA. The following buffers were used: potassium acetate (pH 4.0−4.7), sodium citrate (pH 4.5−6.6), potassium phosphate (pH 6.7−8.5), and potassium glycine (pH 8.5−9.1). Binding of 4-AND, 19-NT, 19-NT-sulfate, and 19-NT-hemisuccinate to pKSI and tKSI was monitored by the decrease in fluorescence at 355 and 307 nm, respectively, as the steroid concentration was increased from 0 to 250 μM. The enzyme concentrations used (0.1−0.15 μM) were below the K_d for the ligand. To determine an observed binding affinity, the observed fluorescence as a function of steroid concentration was fit to a quadratic binding isotherm as previously described.[42]

To confirm that fluorescence quenching upon ligand addition did not arise from absorption of light by the ligand at the concentrations needed to saturate the enzyme, i.e., inner filter effects, similarly high concentrations of ligand were added to samples of tryptophan and tyrosine and the fluorescence spectra were recorded. The fluorescence spectra of tryptophan and tyrosine showed no decrease in fluorescence upon ligand addition, indicating that absorption of light by the ligand does not contribute to the observed decrease in fluorescence in the high-ligand concentration samples.

Activity Measurements. Reactions with 5(10)estrene-3,17-dione [5(10)-EST] were conducted at 25 °C in 50 mM buffer with 4% DMSO (v/v) added as a cosolvent for substrate solubility and were monitored continuously at 248 nm in a PerkinElmer Lambda 25 spectrophotometer. A constant ionic strength of 100 mM was maintained in all samples using NaCl. The following buffers were used: sodium formate (pH 3.0−4.9), sodium acetate (pH 4.2−5.5), sodium 2-(*N*-morpholino)-ethanesulfonate (MES) (pH 4.7−6.8), and sodium 3-(*N*-morpholino)propanesulfonate (MOPS) (pH 6.9−7.7). A molar absorptivity of 14800 M^{-1} cm^{-1} at 248 nm for the product 4-estrene-3,17-dione was used, as previously determined.[44]

Values of k_{cat}/K_M were determined by dividing the initial rate of product formation by enzyme and substrate concentration. The maintenance of a subsaturating level of substrate was tested by ensuring that initial rates doubled with a 2-fold change in substrate concentration (2.5 and 5 μM substrate). Values of k_{cat} were determined by dividing the initial rate by enzyme concentration. The maintenance of a saturating level of substrate was tested by ensuring that initial rates measured with 300 and 600 μM substrate were within 20%. Enzyme concentrations were typically varied over 5-fold at each pH. The subsaturating and saturating substrate concentrations used were chosen on the basis of prior measurements of K_M being between 20 and 50 μM with 5(10)-EST in Asp99Asn and several other oxyanion hole mutants.[44,45]

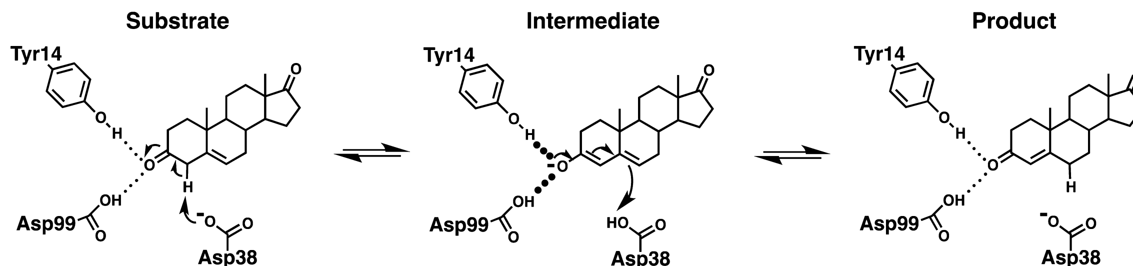
The observed rate constants as a function of pH were then fit to a single titration event in Kaleidagraph 4.04 (Synergy Software, Reading, PA) using eqs 1 and 2 for k_{cat}/K_M and k_{cat}, respectively.

$$(k_{cat}/K_M)_{obs} = \frac{(k_{cat}/K_M)_{max}}{1 + \frac{[H^+]}{K_a^{E}}} \quad (1)$$

$$(k_{cat})_{obs} = \frac{(k_{cat})_{max}}{1 + \frac{[H^+]}{K_a^{E\cdot S}}} \quad (2)$$

X-ray Crystallography of KSI. Single-crystal diffraction data were collected at SSRL beamline BL9-1 using a wavelength of 0.98 Å.[46] The reflections were indexed and integrated with XDS;[47] the intensities were scaled, merged, and converted to amplitudes with SCALA and TRUNCATE.[48] The phases were derived from Protein Data Bank (PDB) entry 3CPO and

Scheme 2. Mechanism of KSI-Catalyzed Isomerization

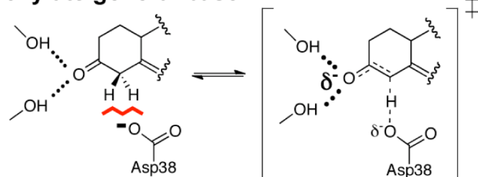


refined with REFMAC5.[49,50] Manual model building was conducted with COOT.[51]

■ RESULTS AND DISCUSSION

Bacterial KSI from *Comamonas testosteroni* (tKSI) and *Pseudomonas putida* (pKSI) catalyze a double-bond migration reaction in steroid substrates (Scheme 2).[40,41,52] In KSI, Asp38 (tKSI numbering is used throughout for the sake of simplicity) acts as a general base in the enolization step and as a general acid in the subsequent ketonization step (Scheme 2).

KSI's positioning of a charged general base abutting a hydrophobic steroid substrate in the Michaelis complex provides an additional possible contribution to catalysis in the E·S complex, beyond that from the presence of a general base that is positioned and stronger than water, through destabilization of this ground state. When compared to that of a reference state in water, the close positioning of the carboxylate and hydrophobic steroid in the active site would be expected to be unfavorable, relative to the close positioning of a neutral instead of a charged general base (Figure 1). This potentially destabilizing interaction would be expected to be largely relieved in the transition state as the proton transfer neutralizes the general base and the negative charge is translated to KSI's oxyanion hole (Figure 1).



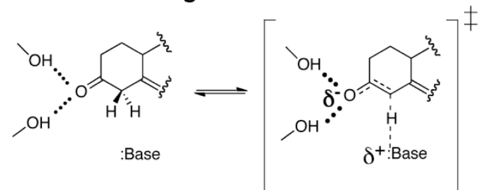
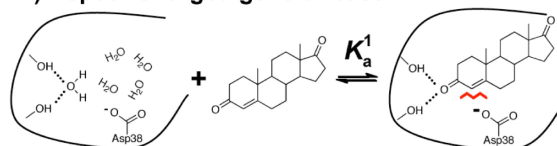


Figure 1. Model for ground state destabilization from an anionic Asp general base in KSI. (A) Close positioning of the charged Asp general base near the substrate could be unfavorable (red line) relative to (B) a hypothetical enzyme with a neutral general base. This destabilization would be relieved in the transition state as the charged general base is protonated and negative charge is localized in the oxyanion hole.

We first determined the binding affinity of the ground state analogue 4-androstene-3,17-dione (4-AND; see Scheme 1 for the structures of analogues used herein) for KSI constructs with the wild-type (WT) charged general base (Asp38) or with an uncharged general base analogue (Asp38Asn). This mutation mimics the reaction coordinate during the enolization step in which the proton is transferred to Asp38, thereby ablating its charge but maintaining its polarity. Unfavorable interactions between the ionized general base and ground state analogue would be expected to weaken binding to KSI with Asp38 relative to the Asp38Asn mutant; i.e., K_a^1 would be smaller than K_a^2 (Figure 2). An unperturbed binding affinity for charged and uncharged general base constructs ($K_a^1 = K_a^2$) would suggest the absence of an unfavorable interaction between the charged general base and hydrophobic steroid within the active site environment.



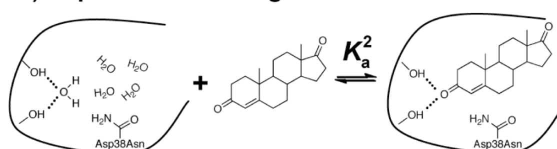


Figure 2. Comparison of binding affinity to provide a measure of ground state destabilization from close positioning of the charged general base and hydrophobic steroid substrate. Unfavorable interactions between the ionized general base and 4-AND, a ground state analogue (A, red line), are predicted to weaken binding to KSI relative to the Asp38Asn mutant (B).

Binding affinities were determined from the quenching of intrinsic KSI fluorescence upon ligand addition (see Experimental Procedures for experimental methods). There is a 4–47-fold increase (depending on the bacterial KSI source) in binding affinity for an enzyme bearing an uncharged, polar Asn relative to an enzyme with the carboxylate of Asp (Table 1 and Figure 3). Binding and rate data as a function of pH strongly suggest that Asp38 remains anionic upon analogue and substrate binding above pH 5 (unpublished results and see below).[53,54]

To further test the effects described above, we conducted analogous experiments with 19-nortestosterone [19-NT (Scheme 1)], a reaction product analogue. The same effects, within 2-fold, were obtained for the substrate analogue 4-AND (Figure S1 of the Supporting Information). We also tested the simple prediction that a destabilizing interaction with the Asp38 anion would result in an increased pK_a of this residue with

Table 1. Effects of Asp38 Mutations on Association Constants of 4-AND for tKSI and pKSI

	tKSI		pKSI	
enzyme[a]	K_a (μM^{-1})	K_a ratio (mutant/WT)	K_a (μM^{-1})	K_a ratio (mutant/WT)
WT	$(1.6 \pm 0.07) \times 10^{-2}$	[1][b]	$(1.6 \pm 0.08) \times 10^{-2}$	[1][b]
D38N	$(6.3 \pm 0.6) \times 10^{-2}$	4	$(7.5 \pm 0.3) \times 10^{-1}$	47
D38A	1.1 ± 0.7	69	2.6 ± 0.8	163

[a]tKSI numbering used throughout. [b]Defined as unity for comparison.

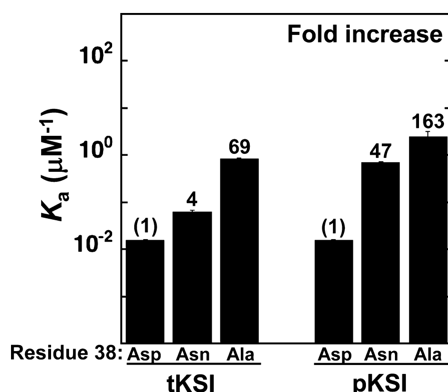


Figure 3. Effects of Asp38 mutations on 4-AND binding [K_a (Figure 2)]. Values are from Table 1.

bound substrate. We obtained pH dependencies for k_{cat}/K_M and k_{cat} for both pKSI and tKSI (Figure S2 of the Supporting Information). In all cases, there was an inactivating protonation at low pH. The derived pK_a values were higher for k_{cat} than for k_{cat}/K_M, for both enzymes. However, the effects were small, with perturbations of only 0.6 and 0.1 for tKSI and pKSI, respectively. These smaller-than-expected effects could arise from one effect or a combination of a number of effects, including poor solvation of protonated Asp within the E·S complex, the inactivating protonation occurring at a different residue or representing a more global unfolding event involving multiple protonations, or destabilizing interactions remaining with neutral Asp due to, for example, poor solvation of its hydroxyl group or oxygen lone pair electrons; the slope that was steeper than that predicted from a simple, single protonation event in the pH−rate dependencies for reactions of the free enzymes (Figure S2B,C of the Supporting Information; k_{cat}/K_M) is consistent with at least some complication from more global effects.

To further probe the interactions of Asp38, this residue was mutated to alanine (Asp38Ala), an uncharged and hydrophobic residue (Figure 3). Comparison of binding affinities between Asp38 and Asp38Ala KSI probes unfavorable interactions between a charged general base and steroid relative to potentially favorable interactions with a hydrophobic residue. The binding affinity increased by 69−163-fold for Asp38Ala relative to that with Asp38 (Table 1 and Figure 3). These results suggest that optimal binding interactions would be made with a hydrophobic group, as would be expected. However, enzymes are selected for catalysis, not optimal stability[55−58] or ground state binding. Our results provide a clear demonstration

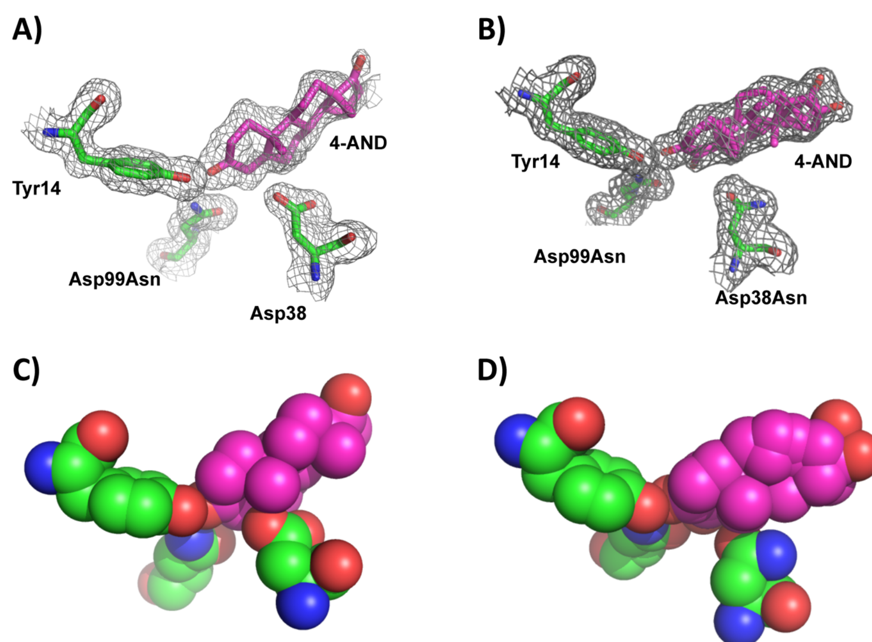


Figure 4. Crystal structures of 4-AND bound to D99N and D38N/D99N show the Asp and Asn side chains are 2.7 and 3.2 Å, respectively, from the steroid ligand. $2F_o - F_c$ electron density map (contoured at 0.5σ) for active site residues and the bound 4-AND ligand for tKSI D99N·4-AND (A) and tKSI D38N/D99N·4-AND (B) complexes (enzyme carbon residues are colored green, and 4-AND carbon residues are colored magenta). Space filling representations of tKSI D99N·4-AND (C) and tKSI D38N/D99N·4-AND (D) complexes show the residue 38 side chain abutting the steroid ligand. In the tKSI D38N/D99N·4-AND complex (D), the identities of the D38N side chain oxygen and nitrogen cannot be distinguished and are modeled arbitrarily. X-ray data and refinement statistics are listed in Table 2.

of this general and well-recognized concept: Asp38 is destabilizing in the ground state relative to a hydrophobic residue such as Ala, but Asp38 plays a critical role as a general base.

If the modestly increased binding affinity from mutation of Asp38 to Asn, to mimic protonated Asp38 subsequent to proton abstraction, represents a ground state destabilization that is relieved as the proton is transferred, then the position of Asp38 and Asn38 should be the same. Alternatively, a structural rearrangement upon mutation to Asn would suggest convolution with energetic effects that might be unrelated to ground state destabilization. We therefore determined the crystal structures of 4-AND bound to tKSI D99N and tKSI D38N/D99N (Figure 4A,B). X-ray data collection and model refinement statistics are listed in Table 2. The overall KSI structures for tKSI D99N and tKSI D38N/D99N bound to 4-AND obtained at 1.6 and 1.8 Å resolution, respectively, are the same as that observed previously for unliganded wild-type tKSI (Figure S4 of the Supporting Information). The Asp and Asn side chains are 2.7 and 3.2 Å from the steroid ligand, respectively, in the predominately hydrophobic active sites of tKSI D99N and tKSI D38N/D99N (Table S4 of the Supporting Information and Figure 4A,B).

Table 2. Crystallographic Data Collection and Refinement Statistics[a]

	tKSI D99N·4-AND	tKSI D38N·4-AND
PDB entry	3NHX	3NUV
resolution range (Å)	28.6−1.59 (1.63−1.59)	51.8−1.76 (1.80−1.76)
space group	$P6_522$	$P3_212$
a (Å)	61.2	59.8
b (Å)	61.2	59.8
c (Å)	142.9	144.5
α (deg)	90.0	90.0
β (deg)	90.0	90.0
γ (deg)	120.0	120.0
no. of unique reflections	305616 (44129)	195870 (22343)
completeness	100.0 (100.0)	99.0 (93.7)
multiplicity	13.7 (14.0)	6.6 (5.6)
R_{merge}[b] (%)	7.2 (7.4)	9.6 [104.7 (because of the multiplicity)]
I/σ	19.9 (3.8)	12.1 (1.7)
	Refinement	
no. of residues	125	125
no. of waters	143	54
R_{work}[c] (%)	18.5	19.2
R_{free}[d] (%)	21.9	24.1
root-mean-square deviation for bonds (Å)	0.032	0.028
root-mean-square deviation for angles (deg)	2.615	2.234

[a]Values for the outer shell are in parentheses. [b]$R_{merge} = \sum_{hkl}\sum_i |I(hkl)_i - \{I(hkl)\}| / \sum_{hkl}\sum_i I(hkl)_i$. [c]$R_{work} = \sum_{hkl}|F(hkl)_o - \{F(hkl)_c\}| / \sum_{hkl}F(hkl)_o$. [d]$R_{free}$ was calculated exactly as R_{work} was, where $F(hkl)_o$ were taken from 10% of the data not included in the refinement.

The X-ray data also raise a potential complication that was resolved by further experiment. The electron density map for the tKSI D38N/D99N·4-AND structure shows density for the 4-AND ligand bound both in a forward conformation with the steroid A-ring in the oxyanion hole and D-ring solvent exposed and in the other chain of the asymmetric unit, in a backward conformation with the D-ring in the oxyanion hole and A-ring solvent exposed (Figure 4). Multiple binding modes have been observed previously for KSI[59,60] and could complicate energetic comparisons. We therefore compared the binding, and relative binding, of a series of analogues with identical A-rings but different D-ring substituents (Figure S3A of the Supporting Information). The observation of the same binding affinity effects of mutations with independent extensive variation in the D-rings (Figure S3B of the Supporting Information) strongly suggests that the ligands bind in the forward orientation in solution so that the binding trends can be interpreted in terms of the reactive conformation with the A-ring in the catalytic pocket (also see the text of the Supporting Information).

We next conducted additional functional studies to further test the conclusions inferred from the structural and energetic analyses described above. Choi et al. reported that mutation of the proline adjacent to the general base (Pro39) to Ala or Gly decreased activity >20-fold, and a crystal structure of the Pro39Ala mutant complexed with the intermediate analogue equilenin showed Asp38 displaced by 1.5 Å relative to its position in wild-type KSI, thereby increasing the carboxylate oxygen−steroid distance from 3.5 Å in the wild type to 5.0 Å in the Pro39Ala mutant (Figure 5A).[54] Mutation of P39 appears to allow the enzyme backbone to access conformations that weaken unfavorable interactions between the charged Asp carboxylate and the hydrophobic steroid (ref 31 and unpublished results).

If the tighter binding of ground state analogues to KSI with an uncharged, polar, or nonpolar general base analogue relative to a carboxylate general base arises from an unfavorable interaction from close positioning between the charged general base and hydrophobic steroid, then mutations that relieve the close positioning would be predicted to reduce the differences in binding with Asp versus Asn or Ala at position 38. Indeed, the preferential steroid binding level with Asn or Ala at position 38 relative to Asp is reduced in the Pro39Ala mutant background and eliminated in the least restrictive Pro39Gly mutant (Figure 5B). Further, the steroid affinity in the presence of Asp38 is increased when Pro39 is mutated (Figure 5C), as expected for elimination of a destabilizing interaction. In contrast, steroid affinity is decreased for the Asp38Ala mutant upon substitution of Gly for Pro39 (Figure 5B), providing further evidence that residue 38 is no longer aligned for interaction with the steroid in the Pro39Gly mutant. These results provide evidence that the unfavorable energetic effect of Asp38 is linked to its proximity to the steroid ring, as predicted by the ground state destabilization model, and, thus, additional support for this model.

The results herein suggest that close positioning of the hydrophobic steroid substrate and charged general base in the KSI ground state complex facilitates the catalytic step via "ground state destabilization", with unfavorable ground state interactions relieved in the transition state as charge is transferred from the general base to the oxyanion hole. This effect is modest, with estimates of 4−47-fold from our binding studies for full ablation of charge and proportionately smaller effects expected in a transition states with partial proton transfer. On the other hand, Asn is an imperfect mimic of protonated Asp, and the true effects could be somewhat greater.

The observed ~10^2-fold stronger binding with Ala replacing Asp38 provides a clear demonstration of the oft-noted principle

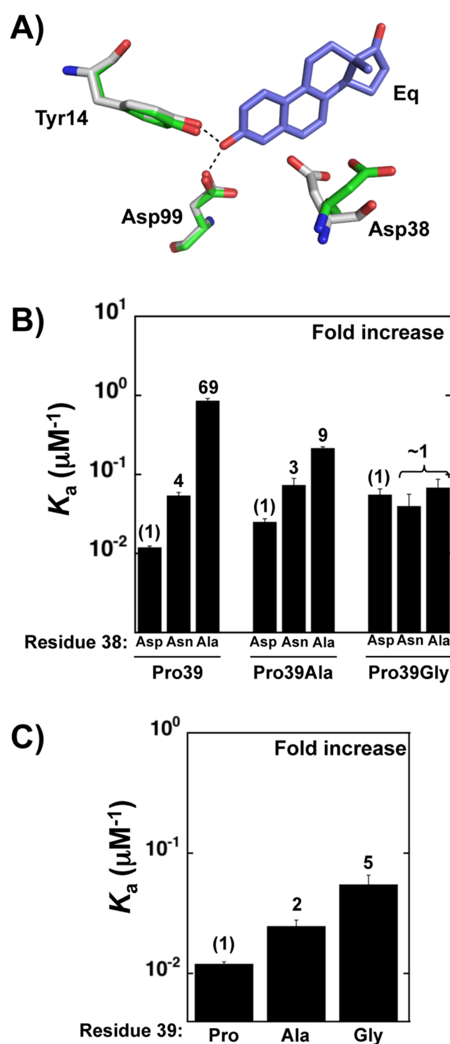


Figure 5. Effect of displacing the general base on differential binding with Asp, Asn, or Ala at position 38. (A) Superposition of the previously determined structures of wild-type tKSI (PDB entry 8CHO, carbon atoms colored gray) and tKSI Pro39Ala bound to equilenin, a transition state analogue (PDB entry 1OGZ, carbon atoms colored green, equilenin colored violet). (B) Effects of Asp38 mutations on 4-AND binding (K_a) with Pro39 (WT), Pro39Ala, or Pro39Gly. (C) Effects of Pro39 mutations on 4-AND binding in the presence of Asp38. Values are from Tables S5 and S6 of the Supporting Information.

that enzymes optimize catalysis in lieu of making the strongest ground state binding interactions. Indeed, overly strong ground state interactions can interfere with catalysis by causing saturation at lower-than-desired substrate concentrations.[9,10,61,62] In addition, in cases where it is possible to weaken ground state binding without causing corresponding weakening of transition state interactions, a catalytic advantage in "k_{cat}" can ensue. Ground state destabilization, via placement of an anionic general base abutting the hydrophobic steroid substrate, appears to provide a modest advantage for KSI. Enzymes generally appear to utilize multiple catalytic strategies to together yield the enormous overall catalysis achieved by enzymes.[5,42,45]

The destabilization in the KSI ground state complex is relative to a reference state in water and requires desolvation of the interacting groups. It has been suggested that the stronger binding of neutral versus charged substrates of phenylalanyl- and valyl-tRNA synthetase arises from the greater energetic price for desolvating the charged substrate than the neutral substrate analogue.[10,63−65] As desolvation presumably plays a role in the KSI destabilization described herein, any Asp or Glu residue involved in forming a carbanion would be expected to be subject to the same desolvation penalty and thus provide analogous ground state destabilization.

It is possible that such advantages do not require a formal negative charge but also accrue when the lone pair of a neutral base is desolvated and positioned up against a CH group from which a proton must be abstracted. Such effects could account for the observed limited pK_a perturbation of Asp38 upon substrate binding. It will be of interest to further probe the energetics in these situations, to determine if this ground state destabilization mechanism is used by other enzymes that deprotonate carbon acids, and to determine whether similar or larger effects are observed in other active sites.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

19-NT association constants for tKSI and pKSI (Figure S1); pH dependencies of k_{cat}/K_M and k_{cat} for tKSI and pKSI Asp99Asn with 5(10)-EST (Figure S2); association constants for 19-NT ligands bearing varied D-ring substituents (Figure S3); superposition of the X-ray structures of tKSI D99N and tKSI D38N/D99N bound to 4-AND with with-type tKSI (Figure S4); effects of Asp38 mutations on 19-NT association constants (Table S1); effects of Asp38 mutations on 19-NT, 19-NT-sulfate, and 19-NT-hemisuccinate association constants for tKSI (Table S2); effects of Asp38 mutations on 19-NT, 19-NT-sulfate, and 19-NT-hemisuccinate association constants for pKSI (Table S3); residue 38 side chain−ligand distances in tKSI D38N·4-AND and tKSI D38N/D99N·4-AND crystal structures (Table S4); effects of Asp38 mutations on 4-AND association constants for tKSI in the P39A background (Table S5); and effects of Asp38 mutations on 4-AND association constants for tKSI in the P39G background (Table S6). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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