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Evaluation of the Catalytic Contribution from a Positioned General Base in Ketosteroid Isomerase

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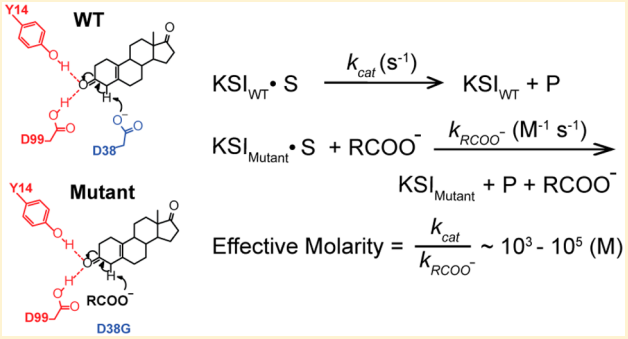
Supporting Information

ABSTRACT: Proton transfer reactions are ubiquitous in enzymes and utilize active site residues as general acids and bases. Crystal structures and site-directed mutagenesis are routinely used to identify these residues, but assessment of their catalytic contribution remains a major challenge. In principle, effective molarity measurements, in which exogenous acids/bases rescue the reaction in mutants lacking these residues, can estimate these catalytic contributions. However, these exogenous moieties can be restricted in reactivity by steric hindrance or enhanced by binding interactions with nearby residues, thereby resulting in over- or underestimation of the catalytic contribution, respectively. With these challenges in mind, we investigated the catalytic contribution of an aspartate general base in ketosteroid isomerase (KSI) by exogenous rescue. In addition to removing the general base, we systematically mutated nearby residues and probed each mutant with a series of carboxylate bases of similar pK_a but varying size. Our results underscore the need for extensive and multifaceted variation to assess and minimize steric and positioning effects and determine effective molarities that estimate catalytic contributions. We obtained consensus effective molarities of ~5×10^4 M for KSI from *Comamonas testosteroni* (tKSI) and ~10^3 M for KSI from *Pseudomonas putida* (pKSI). An X-ray crystal structure of a tKSI general base mutant showed no additional structural rearrangements, and double mutant cycles revealed similar contributions from an oxyanion hole mutation in the wild-type and base-rescued reactions, providing no indication of mutational effects extending beyond the general base site. Thus, the high effective molarities suggest a large catalytic contribution associated with the general base. A significant portion of this effect presumably arises from positioning of the base, but its large magnitude suggests the involvement of additional catalytic mechanisms as well.



INTRODUCTION

A central goal of biology is to understand how enzymes provide their tremendous rate enhancements compared to the corresponding solution reactions. Over the past decades, we have gained a substantial understanding of enzymatic mechanisms, with high-resolution X-ray structures in combination with site-directed mutagenesis implicating the residues directly involved in chemical steps.[1−6] Nevertheless, we are limited in our quantitative understanding of the mechanisms by which enzymes provide catalysis and our ability to dissect them.[7−9]

A common catalytic feature of enzymes is general acid/base catalysis, where positioned side chains facilitate proton transfers during the course of a reaction. A basic question is how much catalysis arises from these positioned general acids and bases in enzyme active sites. However, answering this question is challenging. Site-directed mutagenesis experiments give large deleterious effects upon removal of the side chains of these residues,[10−15] which is often interpreted as their catalytic contribution. However, the mutants lacking these residues may typically adopt a different mechanism, utilizing a solvent molecule or an alternative residue as a general acid or base, and the active site architecture of the mutants may facilitate or hinder these reactions. More generally, these experiments provide a difference between energetics of the mutant enzyme and the wild type, rather than an absolute contribution—i.e., a $\Delta\Delta G^{\ddagger}$ value for the two reactions is obtained and it is not possible to convert this free energy difference into an energy that represents an absolute catalytic contribution from a particular residue.[16,17]

Given that we can only obtain $\Delta\Delta G^{\ddagger}$ values, comparison states need to be clearly and explicitly defined to obtain quantitative understanding of enzymatic catalytic contributions. Perhaps the single most straightforward comparison would be to compare a wild-type enzymatic reaction to the analogous reaction in aqueous solution, and such comparisons are commonly made to obtain an overall rate enhancement afforded by enzymes.[18−21] On a deeper level, one would like, if possible, to obtain comparisons that isolate a contribution

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from a particular catalytic strategy. In this context, rescue reactions by exogenous acids/bases in mutants lacking these residues, can in principle provide an estimation of the contribution of an enzymatic general acid/base.

Toney and Kirsch pioneered exogenous rescue.[22,23] They mutated a catalytic lysine residue in aspartate aminotransferase (AAT) and rescued the reaction with exogenous amines. A comparison of the second-order rate constants of the rescued reactions with the first-order catalytic rate of wild-type AAT (k_{cat}) gave effective molarities for the catalytic lysine residue (Scheme 1) that were corrected empirically for steric effects.

Scheme 1. Measurement of Effective Molarity

$$E_{WT}\bullet S \xrightarrow{k_{cat}\,(s^{-1})} E_{WT} + P$$

$$E_{Mutant}\bullet S + X \xrightarrow{k_x\,(M^{-1}\,s^{-1})} E_{Mutant} + P + X$$

$$\text{Effective Molarity (EM)} = \frac{k_{cat}}{k_x}\ (M)$$

X = Exogenous Moiety

Subsequently, many examples have been reported where residues have been mutated and reactions rescued by exogenous functionalities.[24−36] While these studies are informative, they are limited. Even when multiple exogenous groups are used, these groups can experience complex positioning and steric effects from nearby residues that are difficult to assess empirically (vide infra), and the catalytic contributions may be under- or overestimated.

An elegant example of positioning of an exogenous moiety was observed for the D189S mutant of trypsin, where exogenous acetate binds in a well-ordered manner in the position of wild-type Asp189, as revealed by an X-ray structure, and rescues the reaction with a rate constant similar to that of wild-type trypsin.[37] On the other hand, chemical rescue by imidazole was about 3 orders of magnitude slower in alanine mutants of His166 of hexose-1-phosphate uridylyltransferase and of His122 of NDP kinase, relative to glycine mutants of these residues, likely due to steric interference between the alanine methyl group and the rescuing moiety.[38,39]

With an understanding of the strengths and potential limitations of exogenous rescues, we have obtained effective molarities for an extensive series of general base mutants of the enzyme ketosteroid isomerase (KSI) in conjugation with a series of rescuing carboxylate bases with varying steric properties. These measurements provide an independent assessment of steric and binding effects, thereby allowing more dependable estimation of the catalytic contribution from the general base.

MATERIALS AND METHODS

Materials. Buffers were prepared from reagent grade or better materials. 5(10)-Estrene-3,17-dione (5(10)-EST)) was purchased from Steraloids, Inc. Sodium formate (#3700) and sodium acetate (#3470) were purchased from J.T. Baker. Sodium propionate (#A17440) was purchased from Alfa Aesar. Sodium butyrate (#B5887), valeric acid (#240370), and pivalic acid (#T71803) were purchased from Sigma-Aldrich.

KSI Expression and Purification. KSI constructs were transformed into BL21 cells and a single colony was picked for protein expression and sequenced to confirm the identity of the mutant. Cells were grown at 37 °C to an O.D. of 0.5−0.6 in LB media containing 50 μg/mL carbenicillin and then induced with IPTG at a final concentration of 0.5 mM. After induction, cultures were grown for 6−10 h. Cells were harvested by centrifugation at 4042*g* for 20 min and lysed using an emulsiflex. Lysed cells were centrifuged at 48 297*g* for 20 min. Mutants were purified either from inclusion bodies or from the soluble fraction with the exception of D38G which was purified both ways (and shown to have activity within 20% from the two methods). Purifications were carried out using previously reported protocols.[40,41] For each protein preparation, either a new deoxycholate affinity column was used or a previously used column was washed with a 40 mM sodium phosphate (NaPi), 6 M guanidinium, pH 7.2 buffer followed by 40 mM NaPi, 1 mM sodium EDTA, pH 7.2 buffer. FPLC loops and the Superose 12 column were also washed with 6 M guanidinium buffer or 0.2 M NaOH prior to each use, followed by 40 mM NaPi, 1 mM EDTA, pH 7.2 buffer. Purity of the KSI mutants was >98% by SDS gel electrophoresis based on Coomassie blue staining. To test reproducibility, eight tKSI general base mutants were made twice; the measured activities were within 2-fold from the independent preparations.

KSI Mutant Reactions without Rescue. KSI Michaelis−Menten parameters were obtained by monitoring the 5(10)-EST (extinction coefficient 14 800 M^{-1} cm^{-1}) reaction at 248 nm in a PerkinElmer Lambda 25 spectrophotometer. Reactions were measured at 25 °C in 4 mM sodium phosphate (or sodium MOPS), pH 7.2 buffer with 2% DMSO added for substrate solubility. Low buffer concentrations were used to minimize the background reaction rate. Values of k_w and K_M for reactions without added exogenous base (defined in Supporting Information, Scheme S1) were determined by fitting the initial rates as a function of substrate concentration to the Michaelis−Menten equation. Typically, eight substrate concentrations, varying from 2 to 600 μM, were used for each mutant. The k_w (maximal rate constant for general base mutants) and K_M values were averaged from two or more independent experiments using different enzyme concentrations varied over 2−10 fold (for tKSI mutants: 0.5−6 μM except for D38G/P39G/D99N for which higher concentrations of 5−10 μM were used, for pKSI mutants: 1−5 μM). Averaged values and errors representing the standard deviations are given in Table 1. Michaelis−Menten parameters for KSI mutants with general base intact were also determined as described above. The averaged k_{cat} and their standard deviations for these mutants are given in Table 2.

KSI Base Rescue Reactions. Base rescue reactions were carried out in 20 mM sodium MOPS, 2% DMSO, pH 7.2 buffer at 25 °C by monitoring the 5(10)-EST reaction as above but with the ionic strength maintained at 1 M NaCl (except for D38G/F116A KSI mutant for which the ionic strength was 2 M). As a control to show that ionic strength does not affect the activity, we measured D38G and D40G Michaelis−Menten constants with buffers of different ionic strengths ranging from 1 mM to 1 M and observed no significant effects (Supporting Information, Figures S1 and S2). Typically, four concentrations of exogenous base were used, varying from 0 to 1.5 M. The substrate concentration was usually 300 μM in these experiments at which most of the KSI mutants were 90% or more saturated, with the exception of D38G/F116A, D38G/P39G/A114G and D38G/P39G/D99N and D40G/D103N for which the calculated saturation was 70% or above, based on the K_M values obtained for the nonrescued enzymatic reactions (Table 1). Substrate concentrations above 300 μM caused precipitations at the ionic strength of 1 M. Increasing the percentage of cosolvent (DMSO) improved substrate solubility but increased K_M as well. For tKSI D38G and pKSI D40G general base mutants, we observed an acetate rescue rate constant within 12% at 100 and 300 μM substrate, both above K_M but with different saturation levels (82% and 93% for D38G; 77% and 91% for D40G). Given that these values were indistinguishable (Figure 1), we reported the rescue rate constants directly from observed values at near saturation and did not correct to theoretical saturation.

To obtain second-order rescue rate constants, initial rates of product formation were corrected for the background enzyme-independent reaction. These rates were divided by enzyme concentration and fit to a linear equation as a function of exogenous base concentration. Intercepts agreed with k_w values measured in the

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Table 1. Michaelis–Menten Parameters for Reactions of tKSI (A) and pKSI (B) General Base Mutants with 5(10)-EST in the Absence of Rescuing Base[a]

(A)			
mutants	k_w (10^{-4} s^{-1})	K_M (μM)	k_w/K_M (M^{-1} s^{-1})
D38G	15 ± 2	22 ± 4	60 ± 7
D38G/A114G	1.1 ± 0.2	38 ± 1	3.1 ± 0.2
D38G/P39G	7.5 ± 0.04	24 ± 4	32 ± 3
D38G/F54A	8.9 ± 0.2	19 ± 5	45 ± 8
D38G/F116A	9.5 ± 0.7	120 ± 10	6.9 ± 0.7
D38G/M112G	1.3 ± 0.1	36 ± 6	3.1 ± 0.4
D38G/P39G/A114G	2.2 ± 0.3	130 ± 10	1.5 ± 0.2
D38G/P39G/V40G	1.5 ± 0.7	16 ± 3	5.0 ± 1.6
D38G/P39G/V40G/M112G	1.2 ± 0.1	35 ± 1	2.8 ± 0.1
D38G/P39G/D99N	0.51 ± 0.08	130 ± 8	0.33 ± 0.04
(B)			
mutants	k_w (10^{-4} s^{-1})	K_M (μM)	k_w/K_M (M^{-1} s^{-1})
D40G	21 ± 1	30 ± 6	56 ± 8
D40G/P41G	4.5 ± 0.1	12 ± 3	27 ± 6
D40G/W120A	4.1 ± 0.2	27 ± 11	10 ± 4
D40G/P41G/W120A	1.7 ± 0.2	38 ± 5	3.7 ± 0.5
D40G/P41G/F56A	14 ± 0.6	19 ± 3	47 ± 7
D40G/D103N	1.0 ± 0.1	54 ± 21	1.7 ± 0.5

[a]Conditions: 4 mM sodium phosphate (or sodium MOPS), 2% DMSO, pH 7.2. The rate constant k_w is defined in Scheme S1. Errors represent standard deviations from at least two independent experiments with enzyme concentrations varied over 2–10 fold.

Table 2. KSI Catalytic Constants Used in Comparisons with Rescue Reactions[a]

enzyme	k_{cat} (s^{-1})
tKSI Wild type	36 ± 2[b]
pKSI Wild type	9.9 ± 0.9[c]
tKSI D99N	2.1 ± 0.6
pKSI D103N	0.78 ± 0.06

[a]Conditions: 4 mM sodium phosphate, 2% DMSO, pH 7.2. [b]From ref 54. [c]From ref 40.

absence of base. The rate constant values obtained from the slopes were averaged from 2 to 5 independent experiments using enzyme concentrations varied over 2–10 fold. The fits to the averaged data are shown in Figures S3–S6. The averaged values and errors calculated from standard deviations are given in Table 3. Limits were calculated for cases in which no rescue was observed, conservatively estimating uncertainty as a 50% increase in k_w at 0.25 M valerate or trimethyl acetate or 0.75 M for the other bases, which correspond to the highest base concentrations used in each case.

For pKSI, general base mutants D40A and D40N, exhibit a pK_a of ~7 due to ionization of Tyr57 near to the active site,[42−44] and an analogous deprotonation could occur for other pKSI general base mutants. To determine if this or other deprotonation events affected the activities measured herein, we determined Michaelis–Menten constants from pH 4.0 to 8.9 for the pKSI D40G mutant. The kinetic parameters for the reaction were independent of pH over this range (Supporting Information, Table S1A). Additionally, we observed the same formate rescue rate constant within 2-fold in the mutant from pH 5.2 to 8.2 (Supporting Information, Table S1B). Thus, there is no indication of an enzymatic change in protonation state over the investigated range that affects rescue.

We also investigated pH dependency of tKSI general base mutants to assess any potential complications arising from ionization of Asp99, a H-bond donor in the active site, at the working pH of 7. This residue

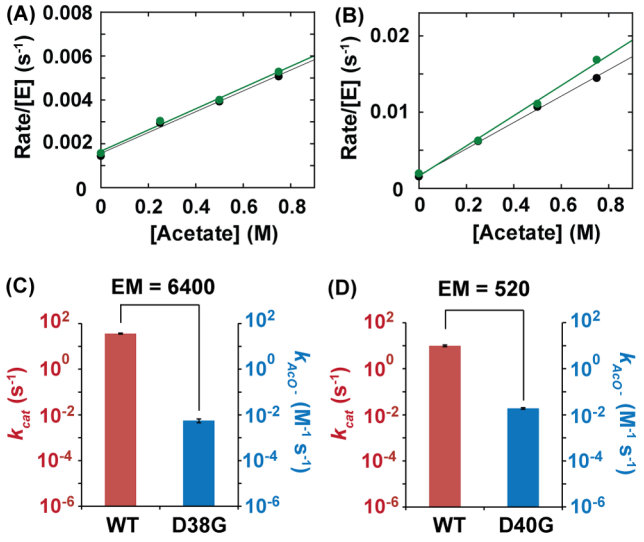

Figure 1. Effective molarity measurements. Acetate rescue for tKSI D38G (A) and pKSI D40G (B) mutants at 100 (black dot) and 300 (green dot) μM substrate 5(10)-EST. Both concentrations are above the K_M values for substrate (22 and 30 μM for tKSI and pKSI, respectively; Table 1A and 1B). (C, D) Comparison of WT and acetate-rescued rate constants, according to Scheme 1, to obtain the specific effective molarities for tKSI (C) and pKSI (D).

ionizes above pH 9 in wild type enzyme but its pK_a might be lowered upon mutation of nearby general base Asp38. The kinetic parameters of D38G reaction with or without base rescue were independent in pH range from 5.2 to 8.2 (Supporting Information, Table S2). We also investigated pH dependency of formate rescue rate constant in another general base mutant D38G/P39G from pH 6.2 to 8.2 and the observed rate constants were within 2-fold of one another (Supporting Information, Table S3).

Exogenous Base Solution Reactions. The second-order rate constant for exogenous base solution reactions were measured as described above but with no enzyme added. Briefly, the 5(10)-EST reaction was carried out in 20 mM sodium MOPS, 2% DMSO, pH 7.2 buffer at 25 °C with varying concentration of exogenous base ranging from 0 to 0.75 M, with a constant ionic strength constant of 1 M (NaCl). The initial rates of product formation were divided by substrate concentration and fitted to a linear equation as a function of base concentration. The second-order solution rate constant values obtained from the slopes were averaged from at least two independent experiments. The averaged fits for the data, rate constants, and standard deviations are given in Supporting Information, Figure S7.

Data were collected with a minimum absorbance change of 0.015 for the slowest reactions with no exogenous base added. These reactions gave linear and reproducible data. As an illustration, Figure S8 shows absorption traces for the acetate solution reaction along with a trace with no substrate added and with a substituted phenol instead of the substrate to serve as a control for an absence of an absorbance change.

X-ray Crystallography. Single-crystal diffraction data were collected at SSRL, beamline BL11−1, using a wavelength of 0.979 and at 100 K. Data indexing, integration, scaling, and merging were carried out using the XDS package.[45] Initial phases were derived by molecular replacement using PDB entry 3NXJ as a model in which residues 38 to 43 were removed. Model building was carried out with the program BUCCANEER from the CCP4 suite and manually in COOT.[46] The model was refined manually after visual inspection with Coot and using phenix.refine.[47,48] During phenix.refine cycles, torsion-angle simulated annealing was used and averaged kicked maps[49] (as implemented in phenix.refine) were used during manual refinement in combination with conventional maps. Model quality was checked by MolProbity[50] and gave an overall score of 1.92.

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Table 3. Rescue Rate Constants (k_{base}) for Exogenous Carboxylate Bases with D38G tKSI (A) and D40G pKSI (B) General Base Mutants[a]

(A)

	k_{base} (10^{-4} M^{-1} s^{-1})					
tKSI Mutants	Formate	Acetate	Propionate	Butyrate	Valerate	Trimethyl acetate
D38G	120 ± 25	56 ± 11	31 ± 9	<10	<30	<30
D38G/A114G	7.0 ± 0.3	8.4 ± 0.2	4.9 ± 0.3	5.9 ± 0.5	5.0 ± 0.1	5.8 ± 0.2
D38G/P39G	20 ± 2	20 ± 0.2	14 ± 1	9.6 ± 3.1	<20	<20
D38G/F54A	70 ± 8	700 ± 56	3600 ± 130	14000 ± 720	18000 ± 1600	110000 ± 9000
D38G/F116A	7.8 ± 1.6	6.6 ± 0.2	4.0 ± 1.3	4.1 ± 0.2	<20	<20
D38G/M112G	6.1 ± 0.2	5.0 ± 0.3	11 ± 0.3	100 ± 3	1300 ± 71	32 ± 0.7
D38G/P39G/A114G	5.3 ± 0.6	4.5 ± 0.2	4.5 ± 0.1	5.2 ± 0.2	<4	4.3 ± 0.4
D38G/P39G/V40G	13 ± 1	11 ± 2	14 ± 2	6.4 ± 0.9	7.4 ± 0.2	16 ± 3
D38G/P39G/V40G /M112G	2.5 ± 0.1	3.3 ± 0.2	7.3 ± 0.7	18 ± 1	250 ± 14	6.2 ± 0.7
D38G/P39G/D99N	-	0.95 ± 0.07	-	-	-	-

(B)

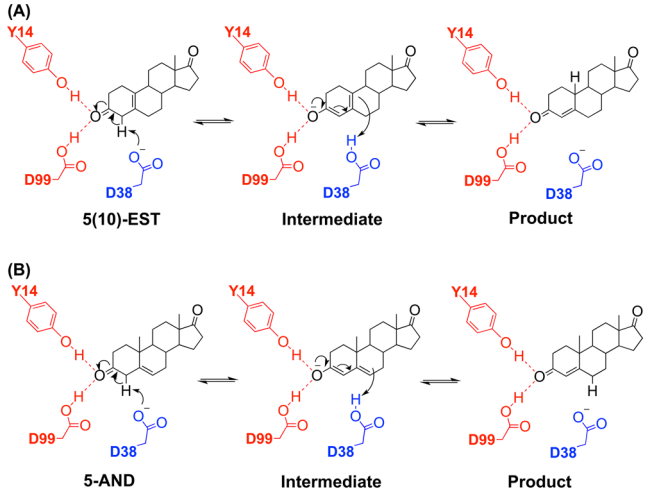
	k_{base} (10^{-3} M^{-1} s^{-1})					
pKSI Mutants	Formate	Acetate	Propionate	Butyrate	Valerate	Trimethyl acetate
D40G	9.1 ± 0.6	19 ± 1	8.2 ± 1.5	9.5 ± 1.0	<4	84 ± 14
D40G/P41G	19 ± 1	11 ± 3	7.8 ± 0.2	17 ± 1	10 ± 1	72 ± 6
D40G/W120A	15 ± 2	5.8 ± 1.1	6.5 ± 0.8	28 ± 2	32 ± 4	210 ± 21
D40G/P41G/W120A	6.9 ± 0.2	12 ± 0.3	4.7 ± 0.3	11 ± 1	8.0 ± 1	88 ± 5
D40G/P41G/F56A	1.5 ± 0.2	14 ± 3	190 ± 10	-	-	-
D40G/D99N	-	0.52 ± 0.01	-	-	-	-

[a]Conditions: 20 mM sodium MOPS, 2% DMSO, pH 7.2, ionic strength 1 M (NaCl), 25 °C. The average rate constant k_{base} is shown (defined in Scheme S1) along with standard deviations from 2 to 5 independent experiments. For tKSI, the k_{base} values shown in green and red in (A) were used to obtain the effective molarity (see text). For pKSI, the k_{base} values shown in green in (B) were used to obtain effective molarity (see main text).

RESULTS AND DISCUSSION

KSI catalyzes isomerization of a double bond in β,γ-unsaturated keto system to give an α,β-conjugated unsaturated ketone (Scheme 2). The substrate binds in a hydrophobic pocket, and the general base Asp38 abstracts an α-proton and generates a dienolate intermediate that is stabilized by hydrogen-bond donors Tyr14 and Asp99 that constitute an oxyanion hole. In the subsequent step, the intermediate is protonated by neutral Asp38 to generate the conjugated and more stable product.[51]

Scheme 2. KSI Reaction Mechanism[a]



[a]Residue numbering for the enzyme from *Comamonas testosteroni* (tKSI).

Effective molarities of the general base were determined for KSI from two different sources *Comamonas testosteroni* (tKSI) and *Pseudomonas putida* (pKSI). We began with tKSI D38G and pKSI D40G, in which only the general base side chain was truncated, and we determined whether the reactions could be rescued by exogenous acetate ion. We used 5(10)-EST (Scheme 2A) as the substrate as there is evidence that a chemical step that includes proton transfer is rate limiting.[52]

In the absence of added acetate ion, these mutants showed activities that were independent of buffer type and concentration as well as pH and ionic strength (Supporting Information, Figures S1 and S2). The averaged values of k_w (maximal rate constant without base) and K_M for these mutants are given in Table 1. A similar primary kinetic isotope effect for reactions of WT tKSI and D38G mutant, with no base added, suggests that a proton transfer step is rate limiting for both reactions and provides no indication of a change in mechanism upon general base mutation (Supporting Information, Table S4).

As shown in Figure 1A and 1B, each reaction rate increased linearly in the presence of acetate, giving second-order rate constants of 5.6×10^{-3} and 1.9×10^{-2} M^{-1} s^{-1} for D38G and D40G, respectively, with saturating substrate 5(10)-EST. The ratio of the first-order k_{cat} value (Table 2) with the second-order rescue rate constant (Scheme 1) gave effective molarities

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of 6400 and 520 M for the tKSI and pKSI mutant, respectively (Figure 1C and 1D).

To determine if these effective molarities are accurate measures or, due to steric or positioning effects, an over- or underestimation of the general base catalytic contribution, we mutated residues in proximity to the general base, and we did so in different combinations along with the D38G or D40G mutation.

In tKSI (Figure 2, left panel), Phe54 and Phe116 position the general base by anion-aromatic interactions;[53] Ala114 provides

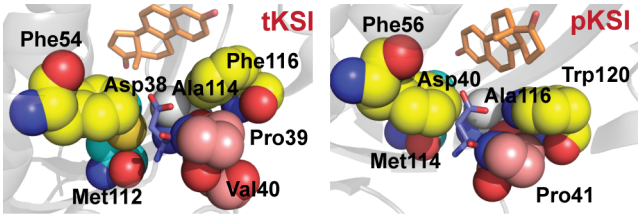

Figure 2. Residues near to the general base in tKSI (PDB: 3M8C) and pKSI (PDB: 1OH0). The general base and transition state analog are shown as sticks and the surrounding residues are shown as spheres.

hydrophobic packing against the methylene group of aspartate;[54] and Pro39 and Val40 are part of a loop that positions the general base.[54,55] We also mutated Met112, which is nearby but has not been established to have a role in general base positioning. We carried out analogous though less extensive mutagenesis in pKSI (Figure 2, right panel).

The variants of tKSI with Asp38 mutated to glycine in combination with mutations in one or more surrounding residues reacted with exogenous acetate ion with second-order rate constants, k_{base}, smaller than that with the D38G mutation alone by as much as ~20-fold, with the exception of D38G/F54A, which had a k_{base} value ~10-fold larger (Table 3A).

To explore the origin of these differences and to be able to obtain an effective molarity free from undesired steric and binding effects, we determined k_{base} values for a series of carboxylate bases varying in length and alkyl chain shape but with similar pK_a values (and similar solution reaction rates; Supporting Information, Figure S7) with each enzyme variant. We also obtained structural information to aid interpretation of these effects.

Seven of the eight tKSI variants investigated with mutations in addition to D38G gave very similar reaction rates with formate, acetate, and propionate. D38G/F54A KSI was again the outlier, giving faster rates with acetate, propionate and other longer-chain bases (Table 3A). These results observed saturation binding of valerate and trimethyl acetate (unpublished results) indicating that a hydrophobic binding pocket is formed upon mutation of F54, so we did not pursue additional F54 mutations herein. Variants with Met112 mutations also gave evidence for binding, in particular with butyrate and valerate as the rescuing bases, suggesting formation of a binding pocket, but one more distant than that formed in the F54A mutant as is consistent with the relative position of these residues within the active site (Figure 2).[56]

The other behavioral outlier was D38G tKSI, the only mutant that gave slower rates as the base chain length increased (Table 3A). This observation provides evidence for steric hindrance to reaction with the longer chain bases, and is consistent with slower reactions with D38 mutated to residues with side chains longer than glycine (Supporting Information, Figure S9). Conversely, the ~10-fold faster reactions of D38G than the other mutants with formate and acetate may reflect favorable interactions with these bases within a restricted pocket, and these effects may be offset by steric hindrance as the alkyl chain is lengthened. Overall, our results demonstrate favorable and unfavorable rescue effects with certain mutants and bases, underscoring the need for extensive variation in the mutant background and rescuing base.

To estimate the carboxylate reaction rates that best reflect reaction of a free base, unaided by binding interactions and unhindered by steric factors, we excluded the D38G variant, the only one with evidence for steric effects, and other variants with clear evidence for binding effects (D38G/F54A, D38G/M112G and D38G/P39G/V40G/M112G). The k_{base} values for the remaining mutants, D38G/A114, D38G/P39G, D38G/F116, D38G/P39G/A114G and D38G/P39G/V40G were remarkably constant, varying only ~5 fold from 0.4 to 2.0 ($\times 10^{-3}$ M^{-1} s^{-1}), with an average value and standard deviation of (0.86 ± 0.49) $\times 10^{-3}$ M^{-1} s^{-1} ($n = 25$; Table 3A, green). Further, the values for the shorter alkyl chain bases with the M112G mutants (Table 3A, red) were very similar to this average ($[0.59 \pm 0.31] \times 10^{-3}$ M^{-1} s^{-1}, $n = 6$). The average value of all 31 mutant/base combinations with no evidence of complications from steric or binding effects was 0.73×10^{-3} M^{-1} s^{-1}, which, combined with the value of k_{cat} of 36 s^{-1} for WT tKSI (Table 2), translates to an effective molarity of 5×10^4 M (Scheme 1).

To test whether this high effective molarity arises due to perturbation in the KSI structure that accompanies the mutations in the general base and its surroundings, and therefore overestimates the catalytic contribution that can be attributed to the positioned general base, we obtained a crystal structure for a tKSI general base mutant, D38G/P39G/D99N (Figure 3 and Supporting Information, Table S5). (The D99N mutation was included to avoid any potential ionization effects; the results described below (Figure 4) indicate that this mutation does not perturb base rescue.)

The oxyanion hole and nearby residues of the D38G/P39G/D99N mutant (light green) overlay well with D99N mutant (orange), suggesting that the general base mutation does not perturb the oxyanion hole (Figure 3A). The overall structure of the mutant is nearly identical to the previously reported D99N crystal structure with a Cα RMSD of ~0.5 Å (calculated with Superpose from the Phenix), excluding the general base loop residues 39–43.

This similarity suggests lack of any structural rearrangement beyond the general base site, within the coordinate error associated with the diffraction resolution of 2.15 Å. However, at a standard deviation of 1σ, the electron density is absent or greatly reduced for residues 40 to 42 (Figure 3B) that make a structured loop that leads to the general base and aids in its positioning (Figure 3C).[54] This observation suggests that the general base loop of tKSI mutant D38G/P39G/D99N is flexible, consistent with a prior crystal structure of a general base loop mutant (P39G/V40G/S42G) that also gave ill-defined electron density in this region (Supporting Information, Figure S10) as well as functional and molecular dynamic results.[54]

To further test for undesired effects on the oxyanion hole from mutation of the general base, we carried out double mutant cycle analysis. The simplest expectation is that an oxyanion hole mutation, D99N, should give the same fold decrease in wild type and general base mutated background if

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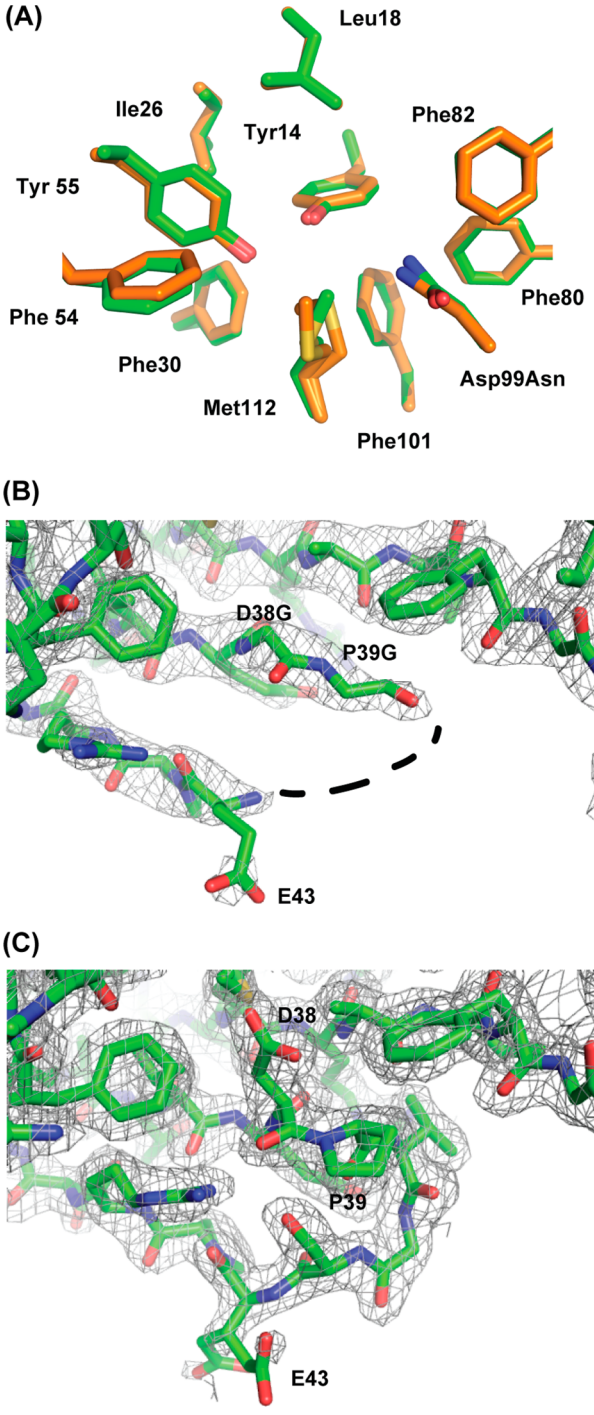

Figure 3. Crystal structure of tKSI D38G/P39G/D99N (PDB: 5DRE). (A) An overlay of the oxyanion hole and nearby residues of the mutant (light green) with D99N tKSI (PDB: 3M8C; orange). (B, C) Final $2F_o-F_c$ difference electron density maps of D38G/P39G/D99N (B) and tKSI mutant D99N (C) contoured at 1.0σ. Electron density is absent or greatly reduced for residues 40 to 42 in D38G/P39G/D99N that make a structured loop and is shown by dashed line. These residues were therefore not included in the final model. (Some electron density was observed at $\leq$0.5σ but attempts to model this portion of the loop with reasonable geometry were unsuccessful. See also Supporting Information, Figure S11.)

there is no perturbation of the oxyanion hole from the general base mutation. The 21 fold decrease observed in acetate rescue reaction upon D99N mutation in the D38G/P39G general base

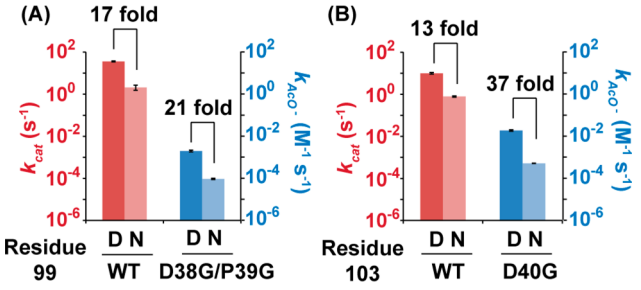

Figure 4. Double mutant cycle analysis of oxyanion hole and general base effects for tKSI (A) and pKSI (B). The value of k_{cat} with the wild type aspartic acid or an asparagine mutation in the oxyanion hole [D99 vs D99N (tKSI) and D103 vs D103N (pKSI)] in the WT background (with the general base intact, red), and the value of k_{rescue} for acetate ion for mutants lacking the general base (blue; D38G/P39G tKSI, panel (A); D40G pKSI, panel (B)). The rate constants are from Table 2 and 3. The numbers above the bars represent the fold effect from the mutation. Similar mutational effects were observed on the k_w values for the general base mutants (i.e., the rate constant in the absence of added general base; Supporting Information, Figure S12).

mutant matched, within error, the 17-fold effect of this mutation in wild type KSI (Figure 4A). This agreement suggests that oxyanion hole contribution is not affected by the general base and surrounding mutation.

The consistency of the observed k_{base} values with different mutants and different bases and the additional structural and functional analyses provide no indication of a possible change in mechanism for any of the mutants.

To further explore catalysis by the KSI general base we carried out analogous experiments with this enzyme from another source, *Pseudomonas putida* (pKSI). These enzymes have 34% sequence identity and have a similar constellation of oxyanion hole and general base active site residues.

Similar to tKSI, some of the pKSI mutants showed evidence for binding effects—i.e., increased k_{base} values with longer or more branched alkyl chains (Table 3B). As observed for the analogous mutation in tKSI, the F56A mutation gave increasing k_{base} values with increasing alkyl chain length of the carboxylate exogenous base, suggesting the creation of hydrophobic binding pocket (Table 3B, D40G/P41G vs D40G/P41G/F56A and Supporting Information, Figure S13). Therefore, pKSI variants with F56 mutations were not explored further. Nevertheless, there was a remarkable coherence of the other 17 k_{base} values for the four other mutants studied, and these gave a mean and standard deviation of $(11 \pm 4.5) \times 10^{-3}$ M^{-1} s^{-1} (Table 3B, green). This rescue rate constant, combined with k_{cat} of 9.9 s^{-1} for WT pKSI (Table 2), gives an effective molarity of 900 M for pKSI.

The oxyanion hole mutation, D103N, gave a decrease in activity within 3-fold in WT pKSI and in a general base mutated background, suggesting that the general base mutation does not substantially perturb oxyanion hole contribution (Figure 4B).

CONCLUSIONS AND IMPLICATIONS

Our best estimates of the effective molarities for tKSI and pKSI, unencumbered by steric or binding effects, are 50 000 and 900 M, respectively. In obtaining these measures of effective molarity, we observed a range of k_{base} values, underscoring a general inability to obtain a reliable determination of the effectiveness of a native general base from any single base rescue measurement. Stated another way, the observation that

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effects can differ, in direction and magnitude, for different mutants and different bases indicates the importance of carrying out rescue reactions across a range of mutants and with a spectrum of rescuing bases.

With this body of data (Table 3), we were able to identify binding and steric effects that increase or decrease reactivity of the rescuing base, presumably resulting from idiosyncratic interactions with protein pockets and surfaces. Notably, increases in k_{base} values did not follow a simple or simply predictable relationship, suggesting that empirical corrections to account for the steric effects should be implemented with caution, especially when the data set is limited. The approach of removing additional residues to create a solution-like environment for rescue reactions may be a generally useful strategy for obtaining effective molarities and deciphering catalytic contributions in enzymes, although controls for additional, undesired conformational and catalytic effects are important.

The high effective molarities of 10^3−10^5 M for the two KSI enzymes suggest a substantial catalytic contribution arising from the enzymatic general base, given the structural and double mutant cycle controls that appear to localize this effect to the general base region (Figures 3 and 4). Perhaps the most fundamental property of enzymes is to bind and position groups, and there is likely a large rate advantage from positioning the aspartate general base, via its covalent connection, anion-aromatic interactions, and packing interactions, with respect to its substrate, which is positioned within its binding site.[53,54] Nevertheless, we cannot yet assign a specific catalytic contribution to general base positioning per se, and our results raise several interesting questions.

KSI has the difficult task of abstracting a proton from one carbon and donating it to a position two carbons away (Scheme 2A). In addition, KSI is even more efficient at catalyzing the reaction of a substrate that requires protonation at a different position (Scheme 2B).[57,58] Thus, it is hard to imagine that the large KSI effective molarities accompanied by an ability to efficiently catalyze proton transfer at multiple positions arises solely from a conformational entropic effect.[59]

In model studies, lower effective molarities of up to ∼10 M have been typically observed for general acids and bases in solution reactions, from comparisons of intramolecular and corresponding intermolecular reactions.[60] However, these values derive from proton transfer from and to heteroatoms and it has been suggested that these effective molarities are suppressed because of hydrogen bonding in the intermolecular reaction that increases the reaction probability of the reference reaction.[61] As these hydrogen bonds would be absent or less favorable for carbon acids, it was further suggested that higher effective molarities are expected for proton abstraction from and to carbon acids.[61,62]

In addition, some model studies have yielded higher effective molarities, even greater than 10^4 M, for intramolecular proton transfers in highly constrained systems.[60] These studies and the results obtained herein for KSI underscore the need to further dissect molecular features such as positioning, steric strain and relief, hydrogen bonding, and solvation differences that may contribute to effective molarities in both nonenzymatic and enzymatic systems.[63−66]

Indeed, prior KSI results provide evidence for substrate destabilization of ∼5−10 fold arising from an interaction with the aspartate general base, presumably from placing the anionic base in contact with the hydrocarbon substrate.[67] It is also possible that the positioning of the general base and substrate within the relatively hydrophobic KSI environment has additional effects on local solvation in a manner that enhances reactivity and that such effects are removed in the mutants used herein to obtain the observed effective molarities.

It was surprising that the effective molarities obtained for tKSI and pKSI differed by nearly 2 orders of magnitude. The lower effective molarity of the pKSI general base could potentially result from faster rescue reactions due to an inability to remove interactions that facilitate reaction of exogenous bases in its mutants. However, the rescue rate constants were very consistent for pKSI general base mutants, other than for the mutant containing F56A mutation that presumably formed a binding pocket (Table 3B), and therefore provide no indication for such an effect. Alternatively and intriguingly, the two KSI variants may have different catalytic contributions from the general base, despite their close evolutionary relationship and the presence of the same oxyanion hole residues and general base.[68] For tKSI, the additional mutations made around the ablated general base might eliminate solvation effects, and the smaller effective molarity and the absence of additional mutational effects on pKSI effective molarities could reflect solvation differences within these active site, or a differential local response to mutation. Regardless, the common literature assumption of considering effects and properties of tKSI and pKSI to be interchangeable may not be warranted, and new mechanistic insights may emerge from additional careful comparisons between KSI variants from different species.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b04796.

Figures S1−S13, Tables S1−S5, and Scheme S1 (PDF)
Crystal data (CIF)
Crystal data (CIF)

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Notes

The authors declare no competing financial interest.

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