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Kemp Eliminase Activity of Ketosteroid Isomerase

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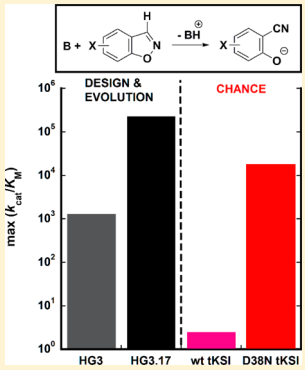
Supporting Information

ABSTRACT: Kemp eliminases represent the most successful class of computationally designed enzymes, with rate accelerations of up to 10^9-fold relative to the rate of the same reaction in aqueous solution. Nevertheless, several other systems such as micelles, catalytic antibodies, and cavitands are known to accelerate the Kemp elimination by several orders of magnitude. We found that the naturally occurring enzyme ketosteroid isomerase (KSI) also catalyzes the Kemp elimination. Surprisingly, mutations of D38, the residue that acts as a general base for its natural substrate, produced variants that catalyze the Kemp elimination up to 7000-fold better than wild-type KSI does, and some of these variants accelerate the Kemp elimination more than the computationally designed Kemp eliminases. Analysis of the D38N general base KSI variant suggests that a different active site carboxylate residue, D99, performs the proton abstraction. Docking simulations and analysis of inhibition by active site binders suggest that the Kemp elimination takes place in the active site of KSI and that KSI uses the same catalytic strategies of the computationally designed enzymes. In agreement with prior observations, our results strengthen the conclusion that significant rate accelerations of the Kemp elimination can be achieved with very few, nonspecific interactions with the substrate if a suitable catalytic base is present in a hydrophobic environment. Computational design can fulfill these requirements, and the design of more complex and precise environments represents the next level of challenges for protein design.



The Kemp elimination[1−3] involves abstraction of the proton bonded to the C3 atom of the aromatic benzisoxazole substrate (Figure 1A). Concurrently with proton transfer, bond cleavage of the weak O−N bond opens the five-membered ring and yields 2-cyanophenolate as the product. This reaction represents, at least in principle, an ideal candidate for the *de novo* computational design of enzymes because it is not present in any metabolic pathway, thereby eliminating biases that might arise from inclusions of already-known catalytic motifs in the computational design.

At least two distinct classes of artificial Kemp eliminase have been designed in the past decade. The first class, called KE, was developed using a quantum mechanical approach to position residues around the substrates.[4] This arrangement was then compared to known scaffolds in proteins using the RosettaMatch algorithm, and the scaffolds obtained were further optimized through computation. Most of the designed variants were inactive, but the most active variant (KE59), which was derived from a triosephosphate isomerase barrel scaffold, was found to use a catalytic glutamate in the active site and to perform the Kemp elimination of 5-nitrobenzisoxazole 6 orders of magnitude faster than acetate in water does.[4] The second class of Kemp eliminases, the HG eliminases, was designed using an iterative approach from a xylanase binding pocket and optimized using molecular dynamics.[5] The most active variant, HG3, uses a catalytic glutamate and accelerates the reaction 7 orders of magnitude relative to that of acetate in water. For both classes, subsequent directed evolution[4,6] resulted in an ∼100-fold rate increase, with k_{cat}/K_M for HG3.17 being equal to 2.3×10^5 M^{-1} s^{-1}, which is 9 orders of magnitude higher than the rate of the corresponding second-order reaction with acetate in water.[6] However, while the design led to considerable rate accelerations, these values fall at the lower end of rate enhancements observed for naturally occurring enzymes, which range up to $\sim 10^{27}$-fold.[7,8]

Although computational design yielded active enzymes, the Kemp elimination is also accelerated in very simple systems.[9−14] In particular, negatively charged species, such as acetate, perform the Kemp elimination 7−9 orders of magnitude faster in organic solvents than they do in water.[15] These significant rate accelerations have been ascribed to

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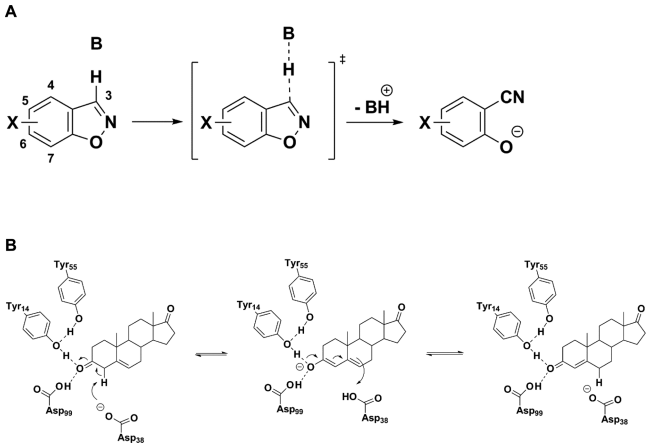

Figure 1. (A) Kemp elimination. X represents a generic substituent on the benzisoxazole ring, and B represents a base. (B) Reaction pathway for the KSI-catalyzed reaction, which involves a proton transfer reaction in the first step.[30]

electron delocalization in the reaction's transition state and poorer solvation of the anionic base in aprotic solvents, which destabilizes the base and makes it more available for proton abstraction.[15−17] Approaches such as evolution of catalytic antibodies[18−20] and rational redesign of cavitands[21−24] have also produced proteins that use an active site carboxylate, and these provide rate accelerations that rival those of computationally designed enzymes.

Thus, evolution, rational design, and computational design have provided researchers with active Kemp eliminases. In addition, it is known that some proteins possess promiscuous Kemp eliminase activity. Bovine serum albumin catalyzes the Kemp elimination of 5-nitrobenzisoxazole with a second-order rate constant of 40 M^{-1} s^{-1} at pH 8.0, but it does so using a catalytic lysine.[25,26] As amines are much better catalysts than carboxylates for the Kemp elimination in aqueous solution,[27] this value represents a rate acceleration of only 3 orders of magnitude. Other proteins known to accelerate the Kemp elimination are the putative oxidoreductase *ydbC* and the purine nucleoside phosphorylase *xapA*, although their mechanisms are unknown.[28]

Given the examples from the literature, it seemed likely that enzymes that possess a carboxylate residue in a relatively hydrophobic active site that can accommodate Kemp elimination substrates might be able to accelerate their elimination reaction. To determine if this is the case, we turned to ketosteroid isomerase from *Comamonas testosteroni* (tKSI), an enzyme that binds hydrophobic steroids[29] and catalyzes migration of a proton from C4 to C6 of 3-oxo-Δ^5 ketosteroids using an active site aspartate [Asp38 (Figure 1B)].[30] This reaction is facilitated by two additional residues, Asp99 and Tyr14, which constitute an oxyanion hole (Figure 1B).[30] We have asked whether and the extent to which the wild type and mutant KSI variants accelerate the Kemp elimination. We found that tKSI is a good Kemp eliminase. Unexpectedly, a single mutation in the active site of tKSI produced an even more active variant that accelerates the Kemp elimination more than the computationally designed Kemp eliminases and is only 1 order of magnitude less reactive than their evolved variants.

■ MATERIALS AND METHODS

Materials. 5-Nitrobenzisoxazole, 6-nitrobenzisoxazole, 5-bromobenzisoxazole, and 6-bromobenzisoxazole were from Ark Pharm (Libertyville, IL). Benzisoxazole, 4,6-dichlorosalicylaldehyde, and hydroxylamine were from Alfa Aesar. Triphenylphosphine and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) were from Sigma-Aldrich. Buffers and salts were from Alfa Aesar or Sigma-Aldrich. UV spectra and spectrophotometric kinetic assays were recorded using a Varian Cary 50 UV/visible spectrophotometer.

Synthesis of 4,6-Dichlorobenzisoxazole. 4,6-Dichlorobenzisoxazole was prepared according to a modified literature procedure, as described below.

Synthesis of 4,6-Dichloro-2-hydroxybenzaldoxime. 4,6-Dichlorosalicylaldehyde (0.500 g, 2.62 mmol) was dissolved in 7 mL of ethanol and mixed with 12 mL of ethanolic $NH_2OH{\cdot}HCl$ (242 mg, 3.48 mmol). The solution was refluxed at 65 °C while being stirred and monitored by TLC (70:30 hexanes/ethyl acetate). After 18 h, the solution was cooled to room temperature and cold water was added. The yellow solid precipitate that resulted was collected by filtration and dried under high vacuum for 4 h. The yield was 398 mg (74%). The ^1H NMR spectra in CD_3OD were consistent with that reported in the literature.[31]

Synthesis of 4,6-Dichlorobenzisoxazole. To a solution of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ, 332 mg, 1.46 mmol) and triphenylphosphine (Ph_3P, 383 mg, 1.46 mmol) in CH_2Cl_2 (5 mL) was added 4,6-dichloro-2-hydroxybenzaldoxime (200 mg, 0.971 mmol) at room temperature. The resulting solution was stirred for an additional 10 min. Solvent was removed via rotary evaporation, and the crude reaction mixture was purified using SiO_2 column chromatography with a 85% hexane/15% ethyl acetate eluent. Solvents were removed *in vacuo*, and the product was isolated as a white solid (100 mg, 55% yield). ^1H NMR data in $CDCl_3$ were consistent with the literature.[32]

Protein Expression and Purification of wt KSI and Its Mutants. BL21 cells were transformed with KSI constructs, and a single colony was picked up to grow an overnight culture. The colony was sequenced to confirm the identity of the mutant. From the overnight culture, cells were grown in Luria broth medium containing 50 μg/mL carbenicillin at 37 °C to an OD of 0.6−0.8. After that, isopropyl β-D-1-thiogalactopyranoside was added to a final concentration of 0.5 mM to induce protein expression. Cells were grown for an additional 5−6 h and harvested by centrifugation at 4042*g* for 20 min, and purification was performed using previously reported protocols.[33−35] To prevent contamination, the deoxycholate affinity column and FPLC loops and the gel filtration Superose 12 column were washed with 6 M guanidinium, 40 mM potassium phosphate, pH 7.2 buffer followed by 40 mM potassium phosphate, 1 mM EDTA, pH 7.2 buffer prior to each protein purification as described previously.[35] Phosphate buffer was removed via a Zeba Spin Desalting column (Thermo Fisher). The identity of the mutant was confirmed by mass spectrometry, and a purity of >95% was determined using sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

Kinetic Assays of KSI-Catalyzed Reactions. Reactions were performed at 20 °C in 25 mM buffer and with the appropriate variant and concentration of tKSI (0−400 μM). The following buffers were used: sodium acetate (pH 4.3−5.9), sodium 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH

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5.4–6.4), and sodium bis-tris propane (BTP) (pH 6.0–9.5). Components were mixed in semimicro plastic cuvettes (BrandTech Scientific) to a total volume of 500 μL, and reactions were initiated by addition of the benzisoxazole substrate (2–5 μL). The appearance of product was monitored at the following wavelengths: 380 nm for 5-nitrobenzisoxazole (ε = 18400 M^{-1} cm^{-1}), 349 nm for 5-bromobenzisoxazole (ε = 8500 M^{-1} cm^{-1}), 333 nm for 4,6-dichlorobenzisoxazole (ε = 28290 M^{-1} cm^{-1}), 398 nm for 6-nitrobenzisoxazole (ε = 6600 M^{-1} cm^{-1}), 327 nm for 6-bromobenzisoxazole (ε = 6100 M^{-1} cm^{-1}), and 323 nm for benzisoxazole (ε = 5080 M^{-1} cm^{-1}). The extinction coefficients of the reaction products were determined by addition of concentrated sodium hydroxide to their parent substrate solutions. Kinetic parameters were determined by initial velocities using Kaleidagraph (Synergy Software). Reported errors are from fitting. Inhibition reactions of 4-chlorophenol and equilenin were performed in the same way, except that the inhibitor was present in the reaction mixtures. Varying the incubation time with the inhibitor did not affect the initial velocities. Product inhibition in D38N tKSI was assessed by allowing 0.30 or 0.50 mM substrate to react to form the product (because of the high concentration of product, reactions were monitored at 460 nm using a measured ε value of 480 M^{-1} cm^{-1}). Once the reaction had reached completion, additional substrate was added and the reaction was monitored again to determine the decrease in rate. This process was repeated one more time. Controls were run with D38N tKSI left in buffer over the same period of time and showed no time-dependent decrease in reactivity, suggesting that product formation was the sole reason for the measured inhibition.

Docking Simulations. Docking simulations of binding of benzisoxazole to tKSI were performed using the online server of Rosetta Ligand Docking (http://rosie.rosettacommons.org/ligand_docking). The chain A structure of D38N tKSI with bound 5α-estran-3,17-dione [Protein Data Bank (PDB) entry 1OHP] was used as the template. Chains B–D, the ligands, and all of the water molecules were manually removed before submitting the structure to the online server. The benzisoxazoles were drawn using ChemDraw 2015 (PerkinElmer) and converted into .sdf format with OpenBabel version 2.3.2 (http://openbabel.org/). Generated structures were visualized and analyzed using UCSF Chimera (http://www.rbvi.ucsf.edu/chimera/) and Pymol (Schrödinger Software).

■ RESULTS

tKSI Catalyzes the Kemp Elimination. We first tested whether wild-type tKSI (wt tKSI) catalyzes the Kemp elimination. At pH 7.0 in the presence of 5.8 μM wt tKSI, the Kemp elimination of 5-nitrobenzisoxazole (5NBI) was faster than the same reaction in the absence of the enzyme, with no apparent saturation observed up to 1.0 mM 5NBI (Figure 2). After correction for the rate of the reaction without enzyme, we calculated a second-order rate constant for the wt tKSI-catalyzed reaction, k_{cat}/K_M, of 2.5 M^{-1} s^{-1} (Figure 2, inset). Strikingly, this value is very similar to the second-order rate constants for the Kemp elimination of 5NBI catalyzed by some of the nonoptimized designed Kemp eliminases (Table 1). If wt tKSI uses a carboxylate residue as the general base, the measured second-order rate constant would correspond to an overall rate acceleration of 4.2 × 10^4-fold relative to that of the acetate-catalyzed reaction in water (k_2 = 5.8 × 10^{-5} M^{-1} s^{-1}, from ref 6).

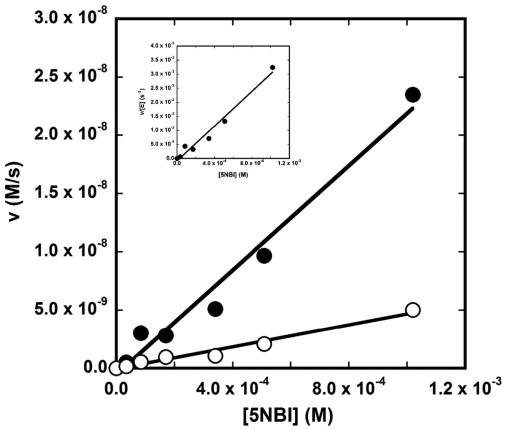

Figure 2. Initial velocities for the Kemp elimination in the presence (●) and absence (○) of wild-type tKSI as a function of 5-nitrobenzisoxazole concentration (25 mM sodium HEPES, pH 6.97, 22 °C, 5.7 μM tKSI). Points in the inset represent the difference between the rate of the reaction in the presence and absence of tKSI.

To determine whether a carboxylate, such as the general base for the normal reaction of tKSI [D38 (Figure 1B)], was the general base in the Kemp elimination, we first determined the pH–rate profile for the tKSI-catalyzed Kemp elimination of 5NBI. The value of the rate constant for this enzyme–substrate pair increases to reach a plateau on the order of 2.5 M^{-1} s^{-1}. Fitting the data to a single deprotonation event, using eq 1, gives an apparent pK_a of 5.2 ± 0.8 (Figure 3, circles). This value is within error of the value for the wt tKSI reaction of 4.6, which has been attributed to D38.[37]

$$k_{cat}/K_M = (k_{cat}/K_M)_{max} \times \frac{10^{-pK_a}}{10^{-pH} + 10^{-pK_a}} \quad (1)$$

The data presented in Figure 3 are consistent with a single catalytic deprotonation event, such as the deprotonation of D38, raising the possibility that D38 could be the general base. Nevertheless, simple geometrical considerations suggest that D38 might not be the general base for the tKSI-catalyzed Kemp elimination. The carbon center of reaction in the normal tKSI reaction is sp^3-hybridized (Figure 1B), which means that the general base is located out of plane with respect to the planar steroid ring. Indeed, crystal structures of tKSI show D38 in such a position.[38] In contrast, the carbon center of reaction in the Kemp elimination is sp^2-hybridized, which requires the base responsible for proton abstraction to lie in the same plane of the system formed by the two aromatic rings. If the benzisoxazole system arranges itself with the same geometry of the ketosteroid ring, then D38 would not be in a suitable position for abstraction of a proton from C3 of benzisoxazoles. To determine whether D38 is the general base in the tKSI-catalzed Kemp elimination, we performed mutagenesis in the active site and used docking simulations, as described below.

Mutation of D38 Leads to Faster Kemp Eliminase Activity. If D38 were the general base in the Kemp elimination, we expected the reaction to return to basal levels upon mutation of D38 to residues that cannot accept a proton. Contrary to this prediction, the tKSI variant with this residue mutated to neutral asparagine (D38N), a residue not effective in proton abstraction, is ~4 orders of magnitude more reactive than wt tKSI in the plateau region, with a maximal k_{cat}/K_M value of 1.7 × 10^4 M^{-1} s^{-1} for reactions of 5NBI [Figure 4 (●)]. Similar results were obtained with variants with D38

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Table 1. Second-Order Rate Constants for the Kemp Elimination of 5-Nitrobenzisoxazole Catalyzed by Some Proteins at Their Respective pH Optima

protein	description	proposed catalytic amino acid	k_{cat}/K_M (M^{-1} s^{-1})	$(k_{cat}/K_M)/k_2$[a]	ref
tKSI	natural enzyme	Asp	2.5 ± 0.6	4.2×10^4	this work
L99A/M102H	engineered cavitand from T4 lysozyme	His	1.80 ± 0.06	7.2×10^4	23[b]
AlleyCat	engineered allosterically regulated protein	Glu	5.8 ± 0.3	9.9×10^4	21
1THF-2	computationally designed	Asp	7.9 ± 0.1	1.3×10^5	5
BSA[a]	natural lipid carrier	Lys	6500 ± 400	2.0×10^5	25, 36
KE07	computationally designed	Glu	12.2 ± 0.1	2.1×10^5	4
KE59	computationally designed	Glu	160 ± 20	2.7×10^6	4
AlleyCat7	evolved AlleyCat	Glu	1280 ± 10	2.2×10^7	24
HG3	computationally designed	Asp	1300 ± 200	2.2×10^7	5, 6
34E4	optimized catalytic antibody	Glu	5200 ± 200	5.5×10^7	20, 36
D38N tKSI	single mutation in KSI	Asp	17000 ± 1000	2.9×10^8	this work
KE59.13	evolved KE59	Glu	59000 ± 15000	1.0×10^9	32
HG3.17	evolved HG3	Asp	230000 ± 40000	3.9×10^9	6
ydbC	natural enzyme	unknown	430 ± 10	unknown	28
xapA	natural enzyme	unknown	1000 ± 30	unknown	28

[a]Rate accelerations were calculated using the values for the background reactions reported in the corresponding references, which match the nature of the enzymatic catalytic base (carboxylate, amine, or imidazole). [b]The rate constant for the background imidazole-catalyzed reaction (2.5×10^{-5} M^{-1} s^{-1}) was derived from the data reported in Figure S2 of this reference.

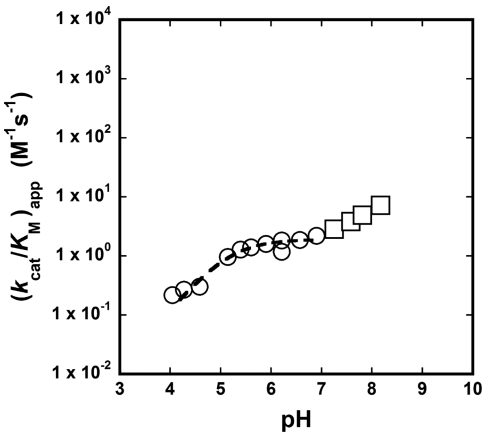

Figure 3. pH−rate profile for the reaction of wild-type tKSI in the presence of 1.0 mM 5NBI, 5.7 μM tKSI, and 25 mM buffer at 22 °C. Points represented by empty circles were fit to eq 1, which describes a single deprotonation event. Empty squares represent measurements for which the hydroxide-catalyzed reaction is faster than the KSI-catalyzed reaction, and they were excluded from the fit.

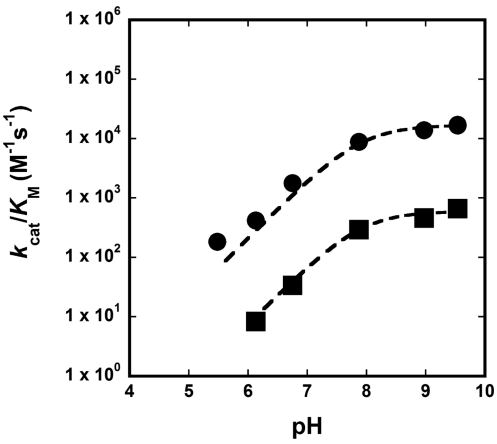

Figure 4. pH−rate profiles for the D38N tKSI-catalyzed Kemp eliminations of 5NBI (●) and 5BrBI (■) in 25 mM buffer at 22 °C. Fitting the data to eq 1 gives pK_a values of 7.8 ± 0.2 for 5NBI and 7.9 ± 0.2 for 5BrBI.

mutated to leucine, glycine, or lysine (Table 2). Thus, these results strongly imply that D38 is not the general base in these mutants and suggest that the negative charge on the oxygen atom of D38 might be inhibitory for the tKSI-catalyzed Kemp elimination, either directly or because it suppresses ionization of another group needed for reaction (see below).

The pH−rate profile for reactions of 5NBI with D38N tKSI revealed an apparent pK_a value of 7.8 ± 0.2 [Figure 4 (●)]. To determine whether this value corresponds to a real pK_a or if it reflects a nonchemical rate-limiting step, we used a slower substrate, 5-bromobenzisoxazole (5BrBI). As the bromo group is less electronegative than the nitro group, the negative charge developed in the transition state of the reaction (Figure 1A) will be less stabilized, and hence, a slower rate is expected with this substrate.[2,39] The pH−rate profile for 5BrBI [Figure 4 (■)] shows the same trend as with 5NBI, with an apparent pK_a of 7.9 ± 0.2 that is identical to the value measured for 5NBI. However, the maximal k_{cat}/K_M value of 6.0×10^2 M^{-1} s^{-1} is ∼30-fold lower than that for 5NBI (Figure 4, plateaus). These results strongly suggest that the saturation in the pH−rate profile corresponds to a deprotonation event of an enzyme residue with a pK_a of ∼7.8.

Table 2. Maximal k_{cat}/K_M Values for the Kemp Elimination of 5NBI by tKSI Variants with Different Residues at Position 38

residue 38	k_{cat}/K_M (M^{-1} s^{-1})	relative k_{cat}/K_M
D	2.5 ± 0.6	(1)
G	420 ± 30	170
K	2500 ± 300	1000
L	16000 ± 2000	6400
N	17000 ± 1000	6800

D99 Is Likely To Be the General Base in the D38N tKSI-Catalyzed Kemp Elimination. Because D38 cannot be the catalytic base in D38N tKSI, we looked at other residues in the active site of tKSI. The other residue in the active site of tKSI that can act as the general base base is D99 (see Figure S1

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for a diagram of the residues surrounding the steroid substrate in KSI), which normally stabilizes the oxyanion hole developing on the keto oxygen of the substrate by donating a hydrogen bond (Figure 1B). This residue is relatively in-plane with the ketosteroid ring, making it a better candidate for the general base role in the tKSI-catalyzed Kemp elimination. In wild-type tKSI, the pK_a of D99 is 9.5,[40] but it was proposed to drop to 8.5 in the D38H mutant.[41] The value of this pK_a in the D38N mutant is not known, but it is likely to be below 8.5 because of the weakened ability of asparagine (relative to aspartate and histidine) to destabilize the anionic form of D99 or stabilize protonated D99. Thus, the measured pK_a of 7.8 in the Kemp elimination is consistent with the expected pK_a of D99 in the D38N mutant.

To test the role of D99 in the D38N mutant, we mutated this residue to asparagine to yield double mutant D38N/D99N. In this case, we used the substrate 4,6-dichlorobenzisoxazole (4,6-Cl_2BI), which gives a rate acceleration relative to the rate of the background reaction larger than that of 5NBI. The pH−rate profile using this substrate with D99N tKSI shows a trend analogous to those of 5NBI and 5BrBI [Figure 5 (○)], with a

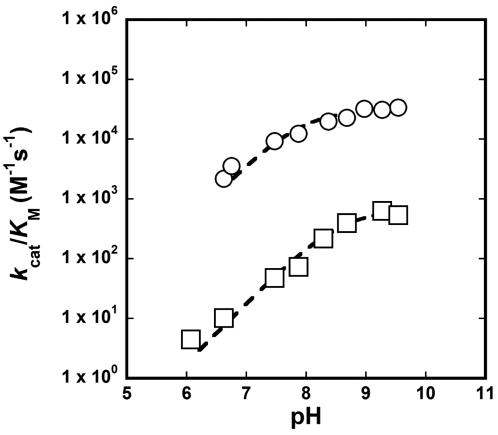

Figure 5. pH−rate profiles for the Kemp elimination of 4,6-Cl_2BI catalyzed by D38N (○) or D38N/D99N (□). Lines represent a fit to eq 1, which give pK_a values of 8.0 ± 0.2 for D38N and >9.0 for D38N/D99N.

similar pK_a, suggesting that the chemical step is also rate-limiting in this case and that the reaction mechanism is the same. The reactivity of D38N/D99N [Figure 5 (□)] was ∼200-fold slower than that of the single mutant (D38N) in the pH-linear region. Intriguingly, this mutant was not completely inactive, its reaction being ∼30-fold faster than the background, hydroxide-catalyzed reaction. It is not unusual that mutations of the general base in enzymes that catalyze the Kemp elimination produce variants with reduced but not abolished activity. For example, the E101A mutation in KE07 and the E100A mutation in KE61 resulted in only 4−8-fold decreases in the second-order rate constants of these enzymes,[4] despite mutation of the general base. The apparent pK_a for reactions of the double mutant was >9, suggesting that a different residue or solvent (OH^-) is responsible for proton abstraction. These results, combined with the analysis of the KSI active site and with docking simulations (see below), suggest that D99 acts as the general base in the D38N mutant; in the double mutant, residual catalysis may arise from abstraction of a proton from a different residue and/or from placement of the substrate in a nonaqueous environment with solvent abstracting the proton.

To test whether D99 acts as the general base in wt tKSI, we used the D99N mutant. This mutant is only marginally less reactive then wt tKSI in the flat pH region (k_{cat}/K_M value of 1.3 ± 0.5 M^{-1} s^{-1}), suggesting that D38, not D99, is the general base in wt tKSI. Further analysis suggests that anionic D99 is at least 100-fold less effective as a general base when anionic D38 is also present in the active site (Figure S2), a result consistent with suppression of D99 ionization by the presence of ionized D38.[41]

Docking Simulations and Inhibition Experiments Place Benzisoxazoles in the Active Site of tKSI. As mutation of active site residues decreases or enhances activity, the tKSI-catalyzed Kemp elimination likely occurs in the active site. Further, the results presented above suggest that D99 is the general base for this reaction in the D38N mutant and that anionic D38 has an inhibitory effect on D99 reactivity. A possible reason for this inhibitory effect is unfavorable interactions between the negatively charged oxygen of D38 and the substrate, as observed for the natural KSI substrate.[42]

To obtain more information about (i) the KSI region in which the Kemp elimination occurs, (ii) the nature of the general base, and (iii) the reason for the inhibition of the Kemp elimination by D38, we performed docking simulations using RosettaDocking. In these simulations, we used a crystal structure of D38N tKSI (PDB entry 1OHP) and docked various benzisoxazoles to it. In all of the simulations, the structures with the lowest energy contained benzisoxazoles in the active site of tKSI. Figure 6A shows the structure with the lowest energy for docking of 5NBI. In this structure, 5NBI is placed within the active site and, with the exception of D99, is surrounded by either neutral or hydrophobic residues. N38 sits above the benzisoxazole ring, and its nitrogen atom is 3.7 Å from the proton attached to C3 of 5NBI (Figure 6B). The angle among the nitrogen atom of N38, the proton attached to C3 of 5NBI, and C3 of 5NBI is 73°, which is significantly different from linearity (i.e., 180°). In contrast, both of the oxygen atoms of D99 are within 2.9 Å of the acidic proton of 5NBI, and the angles among these oxygen atoms, the proton attached to C3 of 5NBI, and C3 of 5NBI are 136° and 125°. In agreement with the relatively weak binding affinity of 5NBI (K_M values of >1 mM for wt tKSI and >200 μM for D38N tKSI), the only specific interaction between the benzisoxazole and the active site of tKSI is the potential hydrogen bond between D99 and the acidic proton of 5NBI.

Thus, docking simulations support the hypothesis that tKSI binds the Kemp elimination substrate through nonspecific interactions and that places it in the normal active site. Because of the different positions of residues 38 and 99, these simulations also support a model in which D99 abstracts the proton from C3 of the substrate and in which the negative charge on residue 38, when present, alters substrate positioning because of unfavorable interactions with the aromatic ring.

Active Site Binders Inhibit the Kemp Elimination. The mutational and computational analyses presented above strongly suggest that the Kemp elimination takes place in the active site of tKSI. Thus, we expected that inhibitors of the normal KSI reaction would inhibit the Kemp elimination. Equilenin, a strong inhibitor of the KSI reaction, inhibits the Kemp elimination of wt tKSI with an apparent K_I of (5 ± 1) μM (see Figure S3), which is similar to the literature value of 2−3 μM.[43] Equilenin also inhibits reactions of the D38 mutants (data not shown), but we could not determine its inhibition constant because it binds these mutants very tightly (K_I < 1 nM

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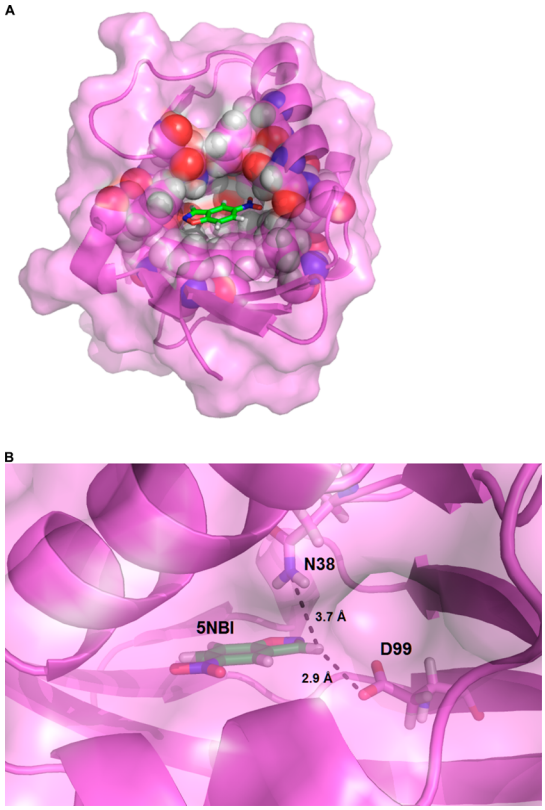

Figure 6. Docking simulation of binding of 5NBI to D38N tKSI (PDB entry 1OHP). Oxygen atoms are colored red, nitrogen atoms blue, hydrogen atoms white, and carbon atoms on the protein magenta and carbon atoms on 5NBI green. (A) Lowest-energy structure with residues within 4 Å of 5NBI shown as spheres. (B) Close-up view of the active site, with distances from the labile hydrogen of 5NBI to the nitrogen atom of D38 and to one of the oxygen atoms of D99.

at pH >7).[43] To determine an inhibition constant in D38 mutants, we used a much weaker inhibitor, 4-chlorophenol (4ClPh). This compound inhibits reactions of D38N KSI and 4,6-Cl_2BI with a K_I of (8 ± 1) μM (Figure 7), similar to the reported values of 8−20 μM for the cognate KSI reaction

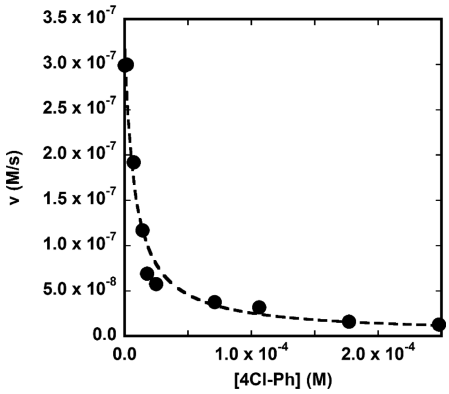

Figure 7. Inhibition of the D38N tKSI-catalyzed Kemp elimination of 4,6-Cl_2BI by 4-chlorophenol (4ClPh). Reactions were performed in the presence of 0.39 μM enzyme, 600 μM substrate (pH 7.5), and different concentrations of 4ClPh. Points were fit to the equation $v = v_{in} + v_{max} \times K_I/(K_I + [4ClPh])$, where v_{in} represents the velocity at full inhibition, v_{max} the velocity in the absence of the inhibitor, and K_I the inhibition constant.

catalyzed by D38N.[44] The very similar values for the inhibition constants of the normal KSI reaction and of the Kemp elimination, in conjunction with results from mutation of active site carboxylates and docking simulations, strongly suggest that the Kemp elimination takes place at the KSI active site. Additional experiments were consistent with the possibility of effects from binding of a second molecule at a high inhibitor concentration (data not shown).

The product of the Kemp elimination is a phenolate (Figure 1A), and phenolates are known KSI inhibitors.[34,44,45] In agreement with such observations, we measured a K_I value for the reaction product 2-nitro-5-hydroxybenzonitrile of 0.4−0.6 mM in D38N tKSI (Figure S4). Thus, the product weakly inhibits the reaction.

D38N tKSI Catalyzes the Kemp Elimination of Several Benzisoxazoles. The results reported above show that D38N tKSI catalyzes the Kemp elimination of benzisoxazoles with 5-nitro (5NBI), 5-bromo (5BrBI), and 4,6-dichloro (4,6-Cl_2BI) substituents. We included other benzisoxazoles to determine whether the trend in reactivity parallels those observed for other systems in which a linear free energy relationship (LFER) between the second-order rate constant of the reaction and the pK_a of the cyanophenol product was established.[2,6,27,46] As shown in Figure 8, the rate constant decreases as the pK_a

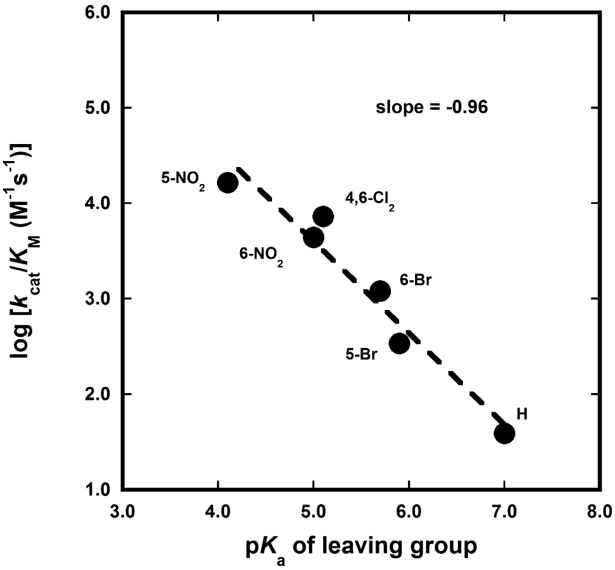

Figure 8. Relationship between the natural logarithm of the second-order rate constant for the D38N tKSI-catalyzed Kemp elimination and the pK_a of the product of the reaction. Reactions were performed at pH 9.5 using subsaturating concentrations of benzisoxazoles.

increases, as observed in all the other systems. The simplest interpretation is that the electron-withdrawing groups on the ring facilitate delocalization of charge in the transition state, thereby accelerating the reaction.[2]

The calculated global slope (β_{LG}) of −0.96 in reactions of D38N tKSI is different from that of HG3.17 (−1.36)[6] and more similar to the reported value for the reaction catalyzed by acetate in acetonitrile (−0.85).[46] However, the latter β_{LG} was calculated using the pK_a values of cyanophenols in water, and it has been pointed out that correction of these values for the different solvent (acetonitrile) resulted in a much shallower β_{LG} of −0.51,[47] which is instead similar to the β_{LG} of −0.60 for reactions catalyzed by acetate in water.[39] Although we cannot

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draw any conclusion about the nature of the environment in the D38N tKSI active site by simply comparing β_{LG} values measured in different systems and different solvents, our data show that D38N tKSI reacts with a wide range of benzisoxazoles, suggesting a hydrophobic active site that can accommodate all the tested substituents.

■ DISCUSSION

The Kemp elimination reaction has fascinated researchers for decades.[47] Because of its absence from any metabolic pathway, it has been used as a benchmark for the computational design of enzymes, with the assumption that high rate accelerations corresponded to a success in design. Nevertheless, much simpler systems such as organic solvents, micelles, and designed cavitands accelerate the Kemp elimination by several orders of magnitude.

The D38N variant of tKSI contains a single carboxylate in a relatively hydrophobic environment. This enzyme has been neither designed nor evolved to catalyze the Kemp elimination but nevertheless accelerates this reaction by several orders of magnitude. At pH 7.0, 5NBI is converted to products by D38N with a k_{cat}/K_M of 2000 M^{-1} s^{-1}, while at pH 9.5, this value is 17000 M^{-1} s^{-1}. By comparison (see Table 1), the two most successful designed Kemp eliminases catalyze the reaction of the same substrate with k_{cat}/K_M values of 160 M^{-1} s^{-1} (KE59)[4] and 1300 M^{-1} s^{-1} (HG3).[5] Importantly, all of these enzymes likely use a carboxylate to abstract the benzisoxazole proton, making comparison between the three reactions meaningful.

At pH 7.5, the value of K_M for 5NBI is equal to (150 ± 40) μM for reactions with D38N (see Figure S5). This observation implies that D38N binds the Kemp elimination substrate tighter than HG3 (K_M = 2.4 mM) and KE59 (K_M > 1 mM)[6] perhaps because of a higher extent of hydrophobic interactions. The value of k_{cat} for the same reaction of D38N KSI is 0.43 s^{-1} (see Figure S5). This value is not very different from those measured with the more active substrate 5NBI in the presence of the catalytic antibody 34E4 (0.66 s^{-1})[20] and the designed Kemp eliminase HG3 (1.7−3.0 s^{-1})[5,6] but ∼3 orders of magnitude lower than the value measured for the evolutionarily optimized HG3.17 (700 s^{-1}).[6] This large difference likely reflects a significantly better alignment between the base and the substrate achieved through the evolution process. It will be interesting to determine whether KSI, an enzyme specialized to catalyze a different reaction, possesses enough plasticity to be evolved to improve its turnover number in the Kemp elimination.

In terms of k_{cat}, the designed HG3 and HG3.17 provide rate enhancements of 3×10^6 and 6×10^8, respectively ($k_{non} = 1 \times 10^{-6}$ s^{-1}).[4] The HG3 rate enhancement is similar to those measured for enzymes such as chorismate mutase, carbonic anhydrase, and human cyclophilin,[48] which lie in the lower portion of rate enhancements measured in enzymes (10^5−10^6), whereas the rate enhancement provided by HG3.17 starts to approach those of more efficient enzymes such as triosephosphate isomerase (10^9). The natural enzymes mentioned above accelerate reactions that are intrinsically fast, with half-lives of seconds to minutes for carbonic anhydrase and cyclophilin (k_{non} values of 10^{-1} and 10^{-2} s^{-1}, respectively) and hours for chorismate mutase and triosephosphate isomerase (k_{non} values of 10^{-5} and 10^{-6} s^{-1}, respectively). As suggested for the Kemp eliminase, it is possible that these enzymes are less precise and complex than the enzymes that catalyze reactions with half-lives longer than weeks. Alternatively (and perhaps more likely), these enzymes may need to select against alternative substrates, so even enzymes with low rate enhancements may still be precise and complex. The evolved HG3.17 seems to be able to discriminate between substrates with and without an electron-rich group at position 5 (ref 6 and discussion below), suggesting that evolution might have somewhat increased the complexity of this enzyme. However, because the tested functional groups at position 5 (NO_2, CN, Cl, and Br) differ in shape and charge distribution, this discrimination is likely to arise from nonspecific medium effects rather than from specific interactions.

Intriguingly, D38N tKSI catalyzes the Kemp elimination of the unsubstituted benzisoxazole (BI) with a second-order rate constant of 39 M^{-1} s^{-1} (Figure 8), which is ∼100-fold faster than the value reported for HG3.17 (0.34 M^{-1} s^{-1}).[6] This difference is not dictated only by the difference in the β_{LG} values of the two enzymes, which predicts an ∼3-fold faster reaction for D38N tKSI. Instead, this difference is mainly due to the outlier position of BI (and other substrates) in the LFER of HG3.17. As mentioned above, it is possible that HG3.17 forms nonspecific interactions with position 5 of the substrate, which would exclude or differentially position substrates without an electron-rich group at position 5 in the active site of HG3.17.[6] Thus, it seems that HG3.17 possesses at least some binding pocket specificity that was designed or arose through evolution, while D38N tKSI remains more promiscuous.

Nevertheless, the nonactivated substrate BI would still react at least 3-fold intrinsically slower in HG3.17 than in D38N tKSI, suggesting that catalysis of nonactivated substrates is still a problem for the designed enzymes. Indeed, large and negative β_{LG} values, which are indicative of dramatic differences in reactivity between activated and nonactivated substrates, were observed in both the HG[6] and KE[32] Kemp eliminases (these β_{LG} values range from −1.2 to −1.8). These differences are somehow attenuated in D38N tKSI [β_{LG} = −0.96 (Figure 8)], perhaps because of some general acid catalysis by an unknown residue or because of the differences in the active site environment.

The pH−rate profiles for reactions of D38N tKSI (Figures 4 and 5) can be fit well to eq 1, which describes a single deprotonation event. The simplest interpretation is that a single residue with a pK_a of ∼7.8 needs to be deprotonated, probably to act as the catalytic base. On the basis of mutational analysis and the simulations presented herein, we propose that this residue is D99. Interestingly, the pK_a of this carboxylate is ∼1−2 units higher than the pK_a values measured for the catalytic antibody 34E4 of 6.0[20] and for the designed Kemp eliminases.[5,6,49,a] Because the Kemp elimination is very sensitive to base strength ($\beta \approx 0.7$),[3] this difference in pK_a might contribute up to 1 order of magnitude to the relative efficiency of D38N tKSI.

The pH−rate profile for wt tKSI (Figure 3) is also consistent with a single deprotonation event, such as deprotonation of D38. In agreement with the model of suboptimal positioning of this residue derived from docking simulations, the maximal k_{cat}/K_M value of is ∼7000-fold smaller than that of D38N tKSI (Table 2). In addition, the negative charge present on D38 might interfere with binding of electron-rich substrates such as benzisoxazoles, as suggested by comparing the binding data in Figure 2 (wt tKSI) and Figure S5 (D38N tKSI). A direct interaction between D38 and D99 seems less likely, as these residues are more than 5 Å apart in crystal structures.[51,52]

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Regardless of the mechanism(s) used to catalyze the Kemp elimination, the observation that many proteins possess Kemp eliminase activity (Table 1) implies that this reaction is easier to accelerate than many reactions catalyzed by enzymes. The high second-order rate constant for the Kemp elimination using D38N tKSI further indicates that design is not necessary to obtain efficient Kemp eliminases. We suspect that detection of Kemp eliminase activity of many proteins is limited by the high rates of the background hydroxide- and buffer-catalyzed reactions, which can obscure detection when protein concentrations are in the commonly used nanomolar and low micromolar ranges. Nevertheless, not all hydrophobic proteins with cavities are suited to the Kemp elimination. For example, the xylanase from *Thermoascus aurantiacus,* which was used as the initial scaffold for the computationally designed HG Kemp eliminases,[5] the T4 lysozyme,[23] and calmodulin[21] have no detectable Kemp eliminase activity. Thus, a combination of the right base in the right environment is needed; nevertheless, these requirements still lack the specificity requirement that defines catalysis for most naturally occurring enzymes.

In vitro evolution of designed Kemp eliminases has produced significantly better variants, with higher rates and diminution of product inhibition. For example, evolved KE59.13 and HG3.17 are ∼2 orders of magnitude more efficient than their parent variants (Table 1), and product inhibition is at least qualitatively minimal in HG3.17.[6] Although no quantitation of this product inhibition was provided, we estimate the K_i to be ∼1.9 mM (see Figure S4c, which is derived from Figure 2d of ref 6), a value similar to the measured K_M of 5NBI in this enzyme.[6] Remarkably, we have shown that a single mutation in the active site of tKSI produced a variant that catalyzes the Kemp elimination with a maximal rate only 1 order of magnitude lower than that of the best computationally designed enzyme, although product inhibition is somewhat higher and the value of k_{cat} is significantly lower than that of HG3.17. As mentioned above, it will be intriguing to determine the extent to which this enzyme can be evolved *in vitro*.

In summary, we have shown that ketosteroid isomerase is a remarkable Kemp eliminase, in spite of a lack of design. Our results, in conjunction with many examples in the literature,[53] showed that the Kemp elimination is a simple reaction that needs minimal catalytic features of a hydrophobic cleft with a positioned general base. Although some specificity arose during the course of the *in vitro* evolution of the Kemp eliminases, this specificity seems to be related to nonspecific effects rather than to precise interactions. In addition, the reactions of non-activated substrates remain problematic. For future challenges, computational design should seek more sophisticated benchmark systems that increase specificity and catalytic complexity or use reactions which are intrinsically slow and difficult to catalyze.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.6b00762.

A schematic view of the KSI active site (Figure S1), the expected pH−rate profiles for wt tKSI in which D99 is equally or less efficient than it is in D38N tKSI (Figure S2), equilenin inhibition of wt tKSI (Figure S3), the effect of product inhibition on D38N tKSI and HG3.17 (Figure S4), and binding of 5NBI to D38N tKSI (Figure S5) (PDF)

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Notes

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■ ABBREVIATIONS

KSI, ketosteroid isomerase; tKSI, *Comamonas testosteroni* ketosteroid isomerase; wt, wild type; 5NBI, 5-nitrobenzisoxazole; 5BrBI, 5-bromobenzisoxazole; 4,6-Cl_2BI, 4,6-dichlorobenzisoxazole; 4ClPh, 4-chlorophenol.

■ ADDITIONAL NOTE

[a]The pH−rate profiles for the HG3 and HG3.17 enzymes are bell-shaped, with inflections at pK_a values of ∼6.0 and 7.9.[5,6] It has been assumed that the first pK_a value (6.0) corresponds to the deprotonation of the catalytic aspartate (D127) while the second pK_a value (7.9) corresponds to an unknown anticatalytic deprotonation event. Nevertheless, a kinetically equivalent model in which a pK_a value of 6.0 corresponds to the unknown deprotonation event and a pK_a value of 7.9 corresponds to the deprotonation of D127 would give exactly the same profile.[50] This alternative model has not been tested or ruled out. Thus, it is uncertain whether the pK_a value of D127 is 6.0 or 7.9 in the HG3 enzymes.

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