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Kemp Eliminase Activity of Ketosteroid Isomerase

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Abstract

Kemp eliminases represent the most successful class of computationally designed enzymes, with rate accelerations up to 10^9 -fold relative to 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 7,000-fold better than wild-type KSI, 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, carries out 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, non-specific interactions with the substrate if a suitable catalytic base is present in a hydrophobic environment. Computational design is able to fulfill these requirements, and the design of more complex and precise environments represents the next level of challenges for protein design.

Graphical Abstract

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SUPPORTING INFORMATION

Supporting information available: Figure S1, which represents a schematic view of KSI active site; Figure S2, which shows the expected pH-rate profiles for wt tKSI in case D99 is equally or less efficient than it is in D38N tKSI; Figure S3, which shows equilenin inhibition of wt tKSI; Figure S4, which shows the effect of product inhibition on D38N tKSI and on HG3.17, and Figure S5, which shows binding of 5-NBI to D38N tKSI.

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 scaffold 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 triose phosphate isomerase barrel scaffold, was found to use a catalytic glutamate in the active site and to carry out the Kemp elimination of 5-nitrobenzisoxazole six orders of magnitude faster than acetate in water.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 seven orders of magnitude relative to acetate in water. For both classes, subsequent directed evolution^{4, 6} resulted in ~100-fold rate increase, with k_{cat}/K_M for HG3.17 equal to $2.3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, which is nine orders of magnitude higher than the corresponding second-order rate 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, carry out 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 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 $40 \text{ M}^{-1}\text{s}^{-1}$ 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 three 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 literature examples, 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 were the case, we turned to ketosteroid isomerase from *Comamonas testosteroni* (tKSI), an enzyme that binds hydrophobic steroids²⁹ and catalyzes proton migration from C4 to C6 of 3-oxo-⁵ ketosteroids using an active site aspartate (Asp38, Figure 1B).³⁰ This reaction is facilitated by two additional residues, Asp99 and Tyr 14, which constitute an oxyanion hole (Figure 1B).³⁰ We have asked whether and to what extent 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 one 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.

(a) 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 $\text{NH}_2\text{OH}\cdot\text{HCl}$ (242 mg, 3.48 mmol). The solution was refluxed at 65 °C under stirring and monitored by TLC (70:30 hexanes/ethyl acetate). After 18 hours the solution was cooled to room temperature and cold water was added. The yellow solid precipitate that crashed out of solution was collected by filtration and dried under high vacuum for 4 hours. Yield: 398 mg (74%). The ^1H NMR spectra in CD_3OD was consistent with the one reported in the literature.³¹

(b) 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 minutes. Solvent was removed via rotary evaporation, and the crude reaction mixture was purified using SiO_2 column chromatography with 85% hexane / 15% ethyl acetate as the eluent. Solvents were removed *in vacuo*, and the product was isolated as a white solid (100 mg, 55% yield). ^1H NMR in CDCl_3 was consistent with the literature.³²

Protein expression and purification of KSI WT and 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 media containing 50 Rg/ml carbenicillin at 37 °C to an O.D. of 0.6–0.8. After that, IPTG was added to a final concentration of 0.5 mM to induce the protein expression. Cells were grown for additional 5–6 hrs and harvested by centrifuging at $4042 \times g$ for 20 min and purification was carried out using previously reported protocols.^{33–35} To prevent contamination, the deoxycholate affinity column as well as FPLC loops and gel filtration column Superose 12 were washed 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.³⁵ Phosphate buffer was removed via a Zeba Spin Desalting column (Thermo Fisher). Identity of the mutant was confirmed by mass spectrometry and purity of > 95% was determined using polyacrylamide SDS gel electrophoresis.

Kinetic assays of KSI-catalyzed reactions

Reactions were carried out 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 5.4–6.4; sodium bis-tris propane (BTP), pH 6.0–9.5. Components were mixed in semi-micro plastic cuvettes (BrandTech Scientific) to a total volume of 500 μL and reactions were initiated by addition of the benzisoxazole substrate (2–5 μL). Appearance of product was monitored at the following wavelengths: 5-nitrobenzisoxazole, 380 nm ($\epsilon = 18,400 \text{ M}^{-1}\text{cm}^{-1}$); 5-

bromobenzisoxazole, 349 nm ($\epsilon = 8,500 \text{ M}^{-1}\text{cm}^{-1}$), 4,6-dichlorobenzisoxazole, 333 nm ($\epsilon = 28,290 \text{ M}^{-1}\text{cm}^{-1}$); 6-nitrobenzisoxazole, 398 nm ($\epsilon = 6,600 \text{ M}^{-1}\text{cm}^{-1}$); 6-bromobenzisoxazole, 327 nm ($\epsilon = 6,100 \text{ M}^{-1}\text{cm}^{-1}$); benzisoxazole, 323 nm ($\epsilon = 5,080 \text{ M}^{-1}\text{cm}^{-1}$). The extinction coefficients of the reaction products were determined by addition of concentrated sodium hydroxide to their parent substrates solutions. Kinetic parameters were determined by initial velocities using Kaleidagraph (Synergy Software). Reported errors are from fitting. Inhibition reactions by 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 determined by allowing 0.30 mM 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 $480 \text{ M}^{-1}\text{cm}^{-1}$). After 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 responsible for the measured inhibition.

Docking Simulations

Docking simulations of benzisoxazole binding 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 5 α -extran-3,17-dione (PDB ID 1OHP) was used as the template. Chains B, C, and 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 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, $k_{\text{cat}}/K_{\text{M}}$, of $2.5 \text{ M}^{-1}\text{s}^{-1}$ (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 non-optimized 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 the acetate-catalyzed reaction in water ($k_2 = 5.8 \times 10^{-5} \text{ M}^{-1}\text{s}^{-1}$, ref 6).

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 in the order of $2.5 \text{ M}^{-1}\text{s}^{-1}$. Fitting the data to a single deprotonation event, using Equation 1, gives an apparent $\text{p}K_{\text{a}}$ 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

$$(k_{\text{cat}}/K_{\text{M}}) = (k_{\text{cat}}/K_{\text{M}})_{\text{max}} \times \frac{10^{-\text{p}K_{\text{a}}}}{10^{-\text{p}H} + 10^{-\text{p}K_{\text{a}}}} \quad (1)$$

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 position.³⁸ In contrast, the carbon center of reaction in the Kemp elimination is hybridized sp^2 , which requires the base responsible for proton abstraction to lay 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 proton abstraction from the C3 of benzisoxazoles. To determine whether D38 is the general base in the tKSI-catalyzed Kemp elimination, we performed mutagenesis in the active site and we 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 at 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 about four orders of magnitude *more* reactive than wt tKSI in the plateau region, with a maximum value of $k_{\text{cat}}/K_{\text{M}} = 1.7 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ for reactions of 5NBI (Figure 4, filled circles). Similar results were obtained with variants with D38 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 $\text{p}K_{\text{a}}$ value of 7.8 ± 0.2 (Figure 4, filled circles). To determine whether this value corresponds to a real $\text{p}K_{\text{a}}$ or if it reflects a non-chemical 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, filled squares) shows the same trend as with 5NBI, with an apparent $\text{p}K_{\text{a}}$ of 7.9 ± 0.2 that is identical to the value measured for 5NBI. However, the maximum $k_{\text{cat}}/K_{\text{M}}$ value of $6.0 \times 10^2 \text{ M}^{-1}\text{s}^{-1}$ is about 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 about 7.8.

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 is D99 (see Figure S1 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 reduced 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 the double mutant D38N/D99N. In this case, we used the substrate 4,6-dichlorobenzisoxazole (4,6-Cl₂BI), which gives a larger rate acceleration relative to the background reaction than 5NBI. The pH-rate profile using this substrate with D99N tKSI shows a trend analogous to those of 5NBI and 5BrBI (Figure 5, empty circles), with similar pK_a , suggesting that the chemical step is also rate-limiting in this case and that the reaction mechanism is the same. Reactivity of D38N/D99N (Figure 5, empty squares) was ~200-fold slower than the single mutant (D38N) in the pH-linear region. Intriguingly, this mutant was not completely inactive, reacting ~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 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, the residual catalysis may arise from proton abstraction from a different residue and/or from placement of the substrate in non-aqueous environment with solvent abstracting the proton.

To test whether D99 acts as the general base in wild type tKSI, we used the D99N mutant. This mutant is only marginally less reactive than wt tKSI in the flat pH region (k_{cat}/K_M value of $1.3 \pm 0.5 \text{ M}^{-1}\text{s}^{-1}$), 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 provides 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

In order 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 of the inhibition of the Kemp elimination by D38, we performed docking simulation using RosettaDocking. In these simulations, we used a crystal structure of D38N tKSI (PDB ID: 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, it 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 the C3 of 5NBI (Figure 6B). The angle between the nitrogen atom of N38, the proton attached to C3 of 5NBI, and C3 of 5NBI is 73°, which is significantly off from linearity (i.e., 180°). In contrast, both of the oxygen atoms of D99 are within 2.9 Å from the acidic proton of 5NBI, and the angles between 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 is > 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 non-specific 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 the C3 of the substrate and that 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 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 tight ($K_I < 1$ nM at pH values higher than 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-Cl₂BI with a K_I of (8 ± 1) μM (Figure 7), similar to the reported values of 8–20 μM for the cognate KSI

reaction 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 high concentration of inhibitor (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 a 5-nitro (5NBI), 5-bromo (5BrBI), and 4,6-dichloro (4,6-Cl₂BI) 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 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 speeding up 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 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 make any conclusion about the nature of the environment in D38N tKSI active site by simply comparing β_{LG} 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 $k_{cat}/K_M = 2,000 \text{ M}^{-1}\text{s}^{-1}$, while at pH 9.5

this value is $17,000 \text{ M}^{-1}\text{s}^{-1}$. By comparison (see Table 1), the two most successful designed Kemp eliminases catalyze the reaction of the same substrate with $k_{\text{cat}}/K_{\text{M}}$ values of $160 \text{ M}^{-1}\text{s}^{-1}$ (KE59)⁴ and $1,300 \text{ M}^{-1}\text{s}^{-1}$ (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 $(150 \pm 40) \mu\text{M}$ for reactions with D38N (see Figure S5). This observation implies that D38N binds the Kemp elimination substrate tighter than HG3 ($K_{\text{M}} = 2.4 \text{ mM}$) and KE59 ($K_{\text{M}} > 1 \text{ mM}$)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 5-NBI in the presence of the catalytic antibody 34E4 (0.66 s^{-1})²⁰ and the designed Kemp eliminase HG 3 ($1.7\text{--}3.0 \text{ s}^{-1}$)^{5, 6}, but about three orders of magnitude lower than the value measured for the evolutionally-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 ($k_{\text{non}} 1 \times 10^{-6} \text{ s}^{-1}$).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 ($10^5\text{--}10^6$), 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 ($k_{\text{non}} = 10^{-1} \text{ s}^{-1}$ and 10^{-2} s^{-1} , respectively) and hours for chorismate mutase and triosephosphate isomerase ($k_{\text{non}} = 10^{-5} \text{ s}^{-1}$ 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 be needed 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 complexity of this enzyme. However, because the tested functional groups at position 5 (NO_2 , CN , Cl , and Br) differ in shape and in charge distribution, this discrimination is likely to arise from non-specific 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 $39 \text{ M}^{-1}\text{s}^{-1}$ (Figure 8), which is about 100-fold faster than the value reported for HG3.17 ($0.34 \text{ M}^{-1}\text{s}^{-1}$).⁶ This difference is not dictated only by the difference in the β_{LG} values of the two enzymes, which predicts a ~ 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 non-specific interactions with the 5-position 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 either was designed or that arose through evolution, while D38N tKSI remains more promiscuous.

Nevertheless, the non-activated substrate BI would still react at least 3-fold intrinsically slower in HG3.17 than in D38N tKSI, suggesting that catalysis of non-activated 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 non-activated substrates, were observed in both the HG⁶ and KE³² Kemp eliminases (these β_{LG} values range from -1.2 to -1.8). These differences are somehow attenuated in D38N tKSI ($\beta_{LG} = -0.96$, 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 & 5) can be fit well to Equation 1, which describe a single deprotonation event. The simplest interpretation is that a single residue with $pK_a \sim 7.8$ needs to be deprotonated, probably to act as the catalytic base. Based on 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 6.0²⁰ and for the designed Kemp eliminases.^{5, 6, 49} (footnote 1) Because the Kemp elimination is very sensitive to base strength ($\beta \approx 0.7$),³ this difference in pK_a might contribute up to one 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 maximum value of k_{cat}/K_M 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}

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

footnote 1: 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 the pK_a value of 6.0 corresponds to the unknown deprotonation event and the 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 D 127 is 6.0 or 7.9 in the HG3 enzymes.

hydrophobic proteins with cavities are suited for 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 calmodulin²¹ 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 define 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, the evolved KE59.13 and HG3.17 are about two 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 S4, panel c, which is derived from Figure 2d of reference 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 maximum rate only one 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 to what extent this enzyme can be evolved *in vitro*.

In summary, we have shown that ketosteroid isomerase is a remarkable Kemp eliminase, in spite of 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 in course of the in-vitro evolution of the Kemp eliminases, this specificity seems to be related to non-specific effects rather than to precise interactions. Further, 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 slow in intrinsic nature and difficult to catalyze.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

KSI	ketosteroid isomerase
tKSI	<i>Comamonas testoteroni</i> ketosteroid isomerase
wt	wild type
5NBI	5-nitrobenzoxazole
5BrBI	5-bromobenzoxazole
4,6-Cl₂BI	4,6-dichlorobenzoxazole
4CIPh	4-chlorophenol

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FIGURE 1A

FIGURE 1B

Figure 1.

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

Figure 2.

Initial velocities for the Kemp elimination in the presence (filled dots) and absence (empty dots) of wild type tKSI as a function of the concentration of 5-nitrobenzisoxazole (25 mM sodium HEPES, pH 6.97, 22 °C, [tKSI] = 5.7 μ M). Points in the inset represent the difference between the rate of the reaction in the presence and in the absence of tKSI.

Figure 3.

pH-rate profile for the reaction of wild type tKSI in the presence of 1.0 mM 5NBI, 5.7 μ M tKSI, 25 mM buffer, 22 $^{\circ}$ C. Points represented by empty circles were fit to Equation 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 were excluded from the fit.

Figure 4. pH-rate profiles for the D38N tKSI-catalyzed Kemp eliminations of 5NBI (filled circles) and 5BrBI (filled squares) in 25 mM buffer, 22 °C. Fitting the data to Equation 1 gives pK_a values of 7.8 ± 0.2 for 5NBI and 7.9 ± 0.2 for 5BrBI.

Figure 5. pH-rate profiles for the Kemp elimination of 4,6-Cl₂BI catalyzed by D38N (empty circles) or D38N/D99N (empty squares). Lines represent a fit to Equation 1, which give pK_a values of 8.0 ± 0.2 for D38N and > 9.0 for D38N/D99N.

FIGURE 6A

FIGURE 6B

Figure 6. Docking simulation of 5NBI binding to D38N tKSI (PDB ID 1OHP). Oxygen atoms are in red, nitrogen in blue, hydrogen in white, and carbon on the protein in magenta and carbon on 5NBI in 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.

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Figure 7.

Inhibition of the D38N tKSI-catalyzed Kemp elimination of 4,6-Cl₂BI by 4-chlorophenol (4Cl-Ph). Reactions were carried out in the presence of 0.39 μM enzyme, 600 μM substrate, pH 7.5, and different concentrations of 4-ClPh. Points were fit to the equation

$v = v_{in} + v_{max} \times \frac{K_I}{K_I + [4-ClPh]}$ where v_{in} represents the velocity at full inhibition, v_{max} the velocity in absence of the inhibitor, and K_I the inhibition constant.

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 carried out at pH 9.5 using subsaturating concentrations of benzisoxazoles.

Table 1

Second order rate constants for the Kemp elimination of 5-nitrobenzoxazole catalyzed by some proteins at their respective pH optima.

Protein	Description	Proposed Catalytic a.a.	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 cavitant 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	$6,500 \pm 400$	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	$1,280 \pm 10$	2.2×10^7	24
HG3	Computationally-designed	Asp	$1,300 \pm 200$	2.2×10^7	5, 6
34E4	Optimized catalytic antibody	Glu	$5,200 \pm 200$	5.5×10^7	20, 36
D38N tKSI	Single mutation in KSI	Asp	$17,000 \pm 1,000$	2.9×10^8	this work
KE59.13	Evolved KE59	Glu	$59,000 \pm 15,000$	1.0×10^9	32
HG3.17	Evolved HG3	Asp	$230,000 \pm 40,000$	3.9×10^9	6
ydbC	Natural enzyme	Unknown	430 ± 10	Unknown	28
xapA	Natural enzyme	Unknown	$1,000 \pm 30$	Unknown	28

^aRate 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).

^bThe rate constant for the background imidazole-catalyzed reaction ($2.5 \times 10^{-5} M^{-1} s^{-1}$) was derived from the data reported in Figure S2 of this reference.

Table 2**Maximum values of k_{cat}/K_M**

for the Kemp elimination of 5NBI by tKSI variants with different residues at position 38.

Residue 38	k_{cat}/K_M ($\text{M}^{-1}\text{s}^{-1}$)	Relative k_{cat}/K_M
D	2.5 ± 0.6	(1)
G	420 ± 30	170
K	$2,500 \pm 300$	1,000
L	$16,000 \pm 2,000$	6,400
N	$17,000 \pm 1,000$	6,800