The evolution of multiple active site configurations in a designed enzyme

Nan-Sook Hong, Dušan Petrović, Richmond Lee, Ganna Gryn’ova, Miha Purg, Jake Saunders, Paul Bauer, Paul D. Carr, Ching-Yeh Lin, Peter D. Mabbitt, William Zhang, Timothy Altamore, Chris Easton, Michelle L. Coote, Shina C. L. Kamerlin & Colin J. Jackson

Developments in computational chemistry, bioinformatics, and laboratory evolution have facilitated the de novo design and catalytic optimization of enzymes. Besides creating useful catalysts, the generation and iterative improvement of designed enzymes can provide valuable insight into the interplay between the many phenomena that have been suggested to contribute to catalysis. In this work, we follow changes in conformational sampling, electrostatic preorganization, and quantum tunneling along the evolutionary trajectory of a designed Kemp eliminase. We observe that in the Kemp Eliminase KE07, instability of the designed active site leads to the emergence of two additional active site configurations. Evolutionary conformational selection then gradually stabilizes the most efficient configuration, leading to an improved enzyme. This work exemplifies the link between conformational plasticity and evolvability and demonstrates that residues remote from the active sites of enzymes play crucial roles in controlling and shaping the active site for efficient catalysis.
Efficient de novo computational enzyme design has been a long-held goal of protein engineers and would allow the catalytic power of enzymes to be directed towards a range of industrially and medically important chemical reactions. Studies have demonstrated that although de novo design is possible, the imperfect designs often require optimization through laboratory evolution\(^1\).\(^2\). Our ability to design enzymes rests upon our fundamental understanding of enzyme catalysis, yet the biophysical and chemical basis for their catalytic efficiency remains a topic of debate\(^3\).\(^4\). There is evidence for contributions to catalysis from electrostatic transition state (TS) stabilization, conformational changes, and quantum tunneling\(^5\).\(^6\). Conformational sampling has been shown to allow enzymes to adopt specific configurations that are suited to different steps in their catalytic cycle and recent work has shown how remote mutations can alter the conformational landscape to increase sampling of certain conformational substates\(^7\).\(^8\). Vibrational motions have also been suggested to contribute to the chemical step in catalysis by altering the probability of transmission through the TS barrier in some enzymes by quantum mechanical hydrogen tunneling\(^8\).\(^9\).\(^10\).

Kemp elimination (proton elimination from 5-nitrobenzoxazole; Fig. 1) has been extensively used as a model system in enzyme design owing to the simplicity of the base-catalyzed ring opening reaction\(^1\) and the absence of natural Kemp eliminases\(^1\), although some enzymes have been shown to catalyze Kemp elimination promiscuously\(^1\).\(^2\).\(^3\).\(^11\).\(^12\).\(^13\). Computational design of KE07 involved construction of a theozyme to catalyze the chemical reaction, which was then grafted into the scaffold of imidazole glycerol phosphate synthase (HisF) from *Thermotoga maritima*\(^1\). Catalytically essential residues from the initial design include a base (Glu101) that facilitates C–H bond cleavage, an H-bond donor (Lys222) to stabilize the phenoxyde intermediate, and a stacking residue (Trp50), which was designed to stabilize the transition state and favor substrate binding through interactions with the aromatic ring of the substrate. This initial KE07 design (Round 1; R1) catalyzes the cleavage of 5-nitrobenzoxazole (1), with 10-fold rate acceleration over the noncatalyzed reaction and a turnover rate (k\(_{\text{cat}}\)) of 0.018 s\(^{-1}\). Seven generations of directed evolution then enhanced this turnover-rate over 100-fold\(^1\).

Although the improvements to KE07 have been partially rationalized through experimental and computational characterization of the mutant proteins\(^14\).\(^15\).\(^16\).\(^17\), accounting for the effects of remote mutations in later rounds has been challenging. KE07 is not the most efficient of the several Kemp eliminases now designed\(^18\).\(^19\).\(^20\), but in the context of understanding how enzyme activity can be gradually improved through stepwise mutations, its low efficiency makes it an ideal model system to study the mechanisms by which evolution or engineering can improve an inefficient starting point.

In this study we use a combination of protein crystallography, enzyme kinetics, and computational approaches to investigate the structure, function, and dynamics of a series of improved variants of the KE07 series. By soaking crystals of various KE07 variants with substrate, we capture the enzymes with a series of different active site configurations. Using molecular dynamics simulations to investigate the sampling of the different conformational substates, we show that the evolutionary improvement of KE07 involves conformational selection of an alternative, nondesigned, active site configuration.

**Results**

**Computational design and initial catalytic improvement in rounds 1–4.** To investigate the progressive increase in catalytic activity, we determined Arrhenius parameters for several variants, including the activation energy (E\(_a\)) associated with the enthalpy of the reaction and pre-exponential factor (A; associated with frequency of collisions between molecules, or entropy) (Table 1). From R1 to R4 we observe a significant reduction in the E\(_a\) from 10.8 kcal mol\(^{-1}\) (R1) to 5.6 kcal mol\(^{-1}\) (R4), indicating that the Ile7Asp, Lys146Glu, Gly202Arg, and Asn224Asp mutations substantially improve the enzyme’s ability to catalyze the reaction, primarily through enthalpic effects such as an increase in basicity of the catalytic group or improved TS stabilization. However, the significant reduction in the activation energy from R1 to R4 was offset by a less favorable pre-exponential factor (containing the entropic component, or collision frequency)\(^21\) (Table 1).

Analysis of crystal structures of KE07 (Supplementary Data 1), with and without bound ligands, reveals how the mutations have increased the basicity of Glu101 and enhanced TS stabilization by changing the electrostatic character of the active site. The structure of KE07 R1 was soaked with substrate, allowing us to obtain a complex with the product of the Kemp elimination (through in crystallo substrate turnover). At higher pH values (8.50 vs. 7.25) we observed mixed occupancy between the product and the histidine tag of a neighboring protein molecule in the crystal lattice within the active site, presumably because deprotonation of the structurally analogous imidazole groups within the histidine tag at basic pH increases its affinity for the active site (Supplementary Fig. 1). At pH 7.25 we observe almost full occupancy of the product (Fig. 2a). Compared with the apo-enzyme, presence of the ligand induces Lys222 to move from the salt bridge it forms with Glu101 to coordinate the oxyanion of the product (2). It has previously been shown that once the trajectory had reached R4, a salt bridge between Lys222 and the Ile7Asp mutation replaces the Glu101 to Lys222 salt bridge\(^14\) (Supplementary Fig. 2), thereby increasing the basicity of Glu101. Indeed, PROPKA\(^22\) suggests the pK\(_a\) of Glu101 increases from 4.49 (R1) to 6.04 (average of six chains in R4; 3IIO (3IIO)).

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**Fig. 1** Reaction scheme for the Kemp elimination of 5-nitrobenzoxazole. The nucleophilic oxygen atom of the base (B) donates electrons to the electrophilic 3'-H of 5-nitrobenzoxazole and the electronegative oxygen atom of the isoxazole group forms a hydrogen bond with an acid (A) (1), forming a transition state in which the C–H and N–O bonds are weakened (2). The removal of the 3'-H from the substrate leads to an anionic phenoxyde intermediate, which is then protonated, forming the final product (3).
To test whether these in crystallo and in silico observations were consistent with the enzyme in vitro, we used fluorescence spectroscopy. Trp50 in the active site of KE07 is a convenient spectroscopic handle to interrogate the active site environment (a second tryptophan residue, Trp156, is invariant throughout evolution; Supplementary Fig. 3). A Trp50Ala mutation was made in KE07 R1 to assess the contribution of Trp50 to the fluorescence; the crystal structures showed no major changes to the active site nor overall fold (Supplementary Fig. 4). This showed that the overall fluorescence of the R1 Trp50Ala mutant was slightly lower than R1, which is consistent with some quenching of the fluorescence of Trp156 by Trp50, similar to what has been reported previously. It also revealed that the temperature dependence of KE07 R1 fluorescence is due to Trp50, with the fluorescence of the Trp50Ala variant showing almost no temperature dependence (Supplementary Fig. 3C). We observe that the Trp fluorescence of KE07 increases substantially from R1 to R4, which can reasonably be attributed to the changing environment of Trp50, given all of these mutations are in close vicinity (Fig. 3, Supplementary Fig. 2, Supplementary Table 1). The increase in fluorescence is consistent with increased negative charge on Glu101, as it has been shown that negative charge on Glu101 increases (Table 1). Thus, the mechanism by which the remote mutations that appear in R1 and R6 increase activity is qualitatively different. This is mirrored in their location within the protein; in contrast to the initial mutations that affect the active site directly, the mutations in R5 (Val12Met) and R6 (Lys146Thr) are remote from the active site (Supplementary Table 2). The mutations in R7 and R7-2 (Phe77Ile, Phe229Ser, Ile102Phe) result in further optimization of activation energy.

Table 1 Arrenius parameters

| KE07 variant | k0 (s⁻¹) | k0 (H) (mM) | k0 (s⁻¹) | k0 (H) (s⁻¹·M⁻¹) | E_to (kcal/mol) | E_to (H) | E_to (H) | lnA0 | lnA0 | A0/A | k0/k0 |
|--------------|----------|-------------|----------|--------------------|----------------|----------|----------|------|------|-------|--------|
| R1           | 0.02 (0.0008) | 0.96 (0.06) | 245 (1.8) | 10.8 (1.1) | 15.1 (1.0) | 4.3 (1.5) | 14.0 (1.8) | 19.6 (1.7) | 0.004 (0.0006) | 4.9 (0.2) |
| R2           | 0.96 (0.09) | 1.10 (0.17) | 877 (59) | 5.6 (0.8) | 9.8 (0.8) | 4.2 (1.2) | 9.1 (1.3) | 14.4 (1.4) | 0.005 (0.0009) | 5.6 (0.2) |
| R3           | 1.43 (0.06) | 0.52 (0.05) | 2700 (257) | 7.7 (0.8) | 9.0 (0.7) | 1.4 (1.1) | 12.9 (1.3) | 13.0 (1.2) | 0.875 (0.121) | 8.6 (0.2) |
| R4           | 1.56 (0.07) | 0.50 (0.05) | 3000 (332) | 9.5 (0.5) | 11.7 (0.5) | 2.2 (0.7) | 16.1 (0.8) | 17.8 (0.6) | 0.181 (0.0126) | 6.5 (0.1) |
| R5           | 2.51 (0.14) | 0.58 (0.07) | 4310 (568) | 7.1 (0.7) | 11.0 (0.9) | 4.0 (1.1) | 12.5 (1.1) | 17.1 (1.5) | 0.009 (0.0011) | 7.0 (0.2) |
| R6           | 3.83 (0.17) | 0.55 (0.05) | 6970 (669) | 6.9 (0.4) | 11.3 (0.5) | 4.4 (0.6) | 12.7 (0.7) | 18.1 (0.8) | 0.005 (0.0003) | 7.3 (0.1) |

*Values, at 303 K, were from Michaelis–Menten saturation curves for the enzyme reaction using a substrate concentration range of 0.1–12 mM of 5-nitrobenzisoxazole from two independent experiments.

†E is in kcal mol⁻¹; lnA and k0/k0 values were calculated from the Arrenius equation from rate constants measured at a range of temperatures (283–323 K) at pH 7.25 from three independent experiments. Arrenius equations are shown in the Methods section and Arrenius plot is shown in Supplementary Fig. 11. Propagated standard errors in the fitted parameters are in parentheses.

‡ stands for the nonenzymatic reaction rate in buffer.

Catalytic improvement through conformational selection. In contrast to the improvement in turnover rate across the first four rounds of mutagenesis, which was unambiguously the result of reduced activation energy due to changes to the local electrostatics of the active site via mutations within and near the active site, the catalytic efficiency of R5 and R6 is improved by more favorable collision frequency (the activation energy actually increases) (Table 1). Thus, the mechanism by which the remote mutations that appear in R5 and R6 increase activity is qualitatively different. This is mirrored in their location within the protein; in contrast to the initial mutations that affect the active site directly, the mutations in R5 (Val12Met) and R6 (Lys146Thr) are remote from the active site (Supplementary Table 2). The mutations in R7 and R7-2 (Phe77Ile, Phe229Ser, Ile102Phe) result in further optimization of activation energy.

To understand the effect of these remote mutations on catalysis, crystals of KE07 R5 and R6 were soaked with substrate before flash-cooling to 100 K and data collection (Supplementary Data 1). Soaking of the genuine substrate was preferred to cocryostalization with an analog, to eliminate any possible artifacts that changes to the chemistry of the substrate could produce. We tested a variety of cryobuffers and crystallization conditions. When glycerol was used as the soaking buffer in R5, we captured the active site in the designed conformation, into which we were able to soak substrate to capture the product bound state (Fig. 2b). In contrast, when MPD was used as the cryobuffer, we captured the active site in a different configuration, with the tryptophan residue in the active site rotating ~100° (Fig. 2c). This configuration has been observed previously in R7, although it was thought to be an artifact due to the presence of the histidine tag from a neighboring protein molecule. We were able to capture the slow substrate monodeuterated (at the 3'-hydrogen) 5-nitrobenzisoxazole bound to this configuration (hereafter denoted configuration B) in a catalytically competent orientation, with C3 pointed towards the general base Glu101 (which undergoes rearrangement upon ligand binding to orient towards the substrate). These results demonstrate that substantial conformational change occurs from R5, with both configurations capable of binding substrate in a catalytically competent fashion. We also solved a higher resolution structure (1.61 Å) of R6, in which the active site of both monomers in the asymmetric unit were observed to be fully in configuration B, with the imidazole ring (an analog of the benzisoxazole substrate) of the histidine tag of a neighboring KE07 molecule in the crystal lattice bound to the active site (Supplementary Fig. 1). For R7 and R7-2, we were able to capture the active site in configuration A when glycerol/Bis-Tris was used as cryoprotectant (Fig. 2e), and configuration B with the neighboring hexahistidine tag bound and MPD used as cryoprotectant (Fig. 2g). However, the most striking result came from a crystal structure with 12 different KE07 molecules within the asymmetric unit, into which substrate was soaked. In this structure, we observe both configuration A (three subunits) and B (one subunit) (Supplementary Fig. 5), as well as two additional configurations (hereafter denoted configurations C and D). In configuration C, Trp50 is rotated back to a similar position as the designed conformation, except it is rotated such that the NH of the indole ring can hydrogen bond to the catalytic Glu101. The four subunits in configuration C all had product bound (Fig. 2f).
was observed. Four subunits were observed to adopt con
Supplementary Fig. 5), and were the only chains where product
configuration A with bound product after substrate soaking. c R5 in
configuration B with bound Bis-Tris molecule after cryoprotection. f R7 in
configuration C with bound product after substrate soaking. g R7-2 in
configuration B with bound hexahistidine tag from an adjacent protein chain
after cryoprotection. h R7-2 in configuration C with bound product after
substrate soaking, minor occupancy of configuration A is also observed.
Ligands and alternative conformations of residues are shown as pink sticks.
The mF_o-DF_c maps are shown as green meshes and contoured at 3.0σ.
The 2mF_o-DF_c maps are shown as blue meshes and contoured at 1.0σ. PDB ID of the structures: a (5D2W), b (6DKV), c (6C7M), d (6DNJ), e (6CAI), f (6DC1), g (5D38), h (6CT3)

Supplementary Fig. 5), and were the only chains where product
was observed. Four subunits were observed to adopt configuration D, in which Trp50 was disordered in-between the
well-defined conformations, supporting the idea that these
conformational substates exist in equilibrium (Supplementary
Fig. 5). Finally, crystals of R7-2 with the hexahistidine tag
crystallized in configuration B with the hexahistidine tag in the
active site (even when soaked with substrate) (Fig. 2g). However,
when we removed the hexahistidine tag, we captured a high-
resolution structure in configuration C with product bound
(configuration A was present in low occupancy; Fig. 2h).

Altogether, we have characterized the emergence of two
additional catalytically competent active site configurations in
KE07 that were not part of the original design, have used
substrate soaking and flash cooling to demonstrate that all three
can bind substrate in catalytically competent orientations, and
show that within the population of enzyme molecules within a
single crystal, all three (and an intermediate state) can be
sampled. However, in later generations (R7 and R7-2) evidence
for turnover was only observed with configuration C.
The structural basis for the dramatic reorganization of the active site of KE07 is coincident with the introduction of remote mutations and appears to involve differential stabilization of the three conformational substates. For example, Ile102Phe results in phenylalanine filling a hydrophobic cavity and a small adjustment of the main chain, which increases the distance between Glu101 and Trp50, allowing sampling of the Trp50 rotamer, which is then stabilized by an H-bond to Glu101, thereby stabilizing this alternative configuration (Supplementary Fig. 6). The Val12Met and Phe77Ile mutations, also in the second shell, cause changes to internal cavities that cause a slight rotation in the backbone at Trp50 that also favors the alternative configuration (Supplementary Fig. 6). From configuration B, the enzyme can more easily access configuration C as the indole ring has already rotated at this point.

The crystallography provides valuable snap-shots of different configurations that the active site can adopt, and using different buffers, we were able to selectively stabilize certain configurations, but the crystallography does not tell us much regarding their relative populations in solution (other than that they can all be sampled). To the solution sampling of these states, we used HREX-MD simulations, which are among the most comprehensive computational methods to investigate conformational sampling. These results were consistent with the crystallography and kinetic analysis: whereas in R1 (original design), configuration A was the dominant subtype that was sampled, by R5 we observed increased sampling of configurations B and C (Fig. 3; Supplementary Fig. 7). By R7 and R7-2, configuration C (which was the only state we saw associated with product in the crystal structures) becomes the dominant subtype sampled, with configurations A and B sampled only rarely. These simulations also reveal that the mobility of Glu101 was reduced as a result of the hydrogen bond formed to the indole nitrogen of Trp50 in R5; this could account for the improved pre-exponential factor from R5 onwards (Figs. 2, 3).

We again used tryptophan fluorescence to complement the crystallographic and computational analyses. In contrast to the increase in Trp50 fluorescence intensity over the first half of the evolutionary trajectory owing to the increased negative charge of Glu101, the fluorescence intensity decreases to below the level of R1 from R4 to R7-2 (Fig. 3; Supplementary Table 1). This marked reduction in fluorescence intensity is coincident with the introduction of remote mutations (generally conservative in terms of charge) that are unlikely to directly affect the local electrostatic environment of Trp50. The loss of fluorescence is consistent with the alternative active site configurations (B and C), in which Trp50 fluorescence is quenched: either via H-bonds with Glu101 in configuration B (it has been shown that H-bonding between the –NH atom of tryptophan and negatively charged amino acids leads to fluorescence quenching) or by solvent in the case of configuration C. It is notable that the reduction in fluorescence intensity is gradual, consistent with the progressive enrichment of the active site configuration C we observe in the MD simulations of structures between R5 and R7-2. A Trp50Ala mutant of R7-2 was made to compare against R1_Trp50Ala; no change in fluorescence intensity was observed other than ~15% lower intensity at 293 K in R7-2_Trp50Ala compared to R1_Trp50Ala due to the absence of two solvent accessible phenylalanine residues (Phe77Ile, Phe229Ser) (Supplementary Fig. 3, Supplementary Table 1). The Trp50Ala mutants in R1 and R7-2 result in significant (>95%) reductions in $k_{cat}$, although the $K_M$ of R7-2_Trp50Ala increased sevenfold, whereas the $K_M$ of R1_Trp50Ala was unchanged, suggesting more involvement of Trp50 in substrate binding in the evolved configuration C (Supplementary Table 3).

**One enzyme: three active site configurations.** The structural, spectroscopic and computational results suggest that KE07 has evolved through maximizing the sampling of an active site configuration (C). To investigate whether the active site configurations that become enriched by R7-2 are catalytically competent, we performed empirical valence bond (EVB) simulations of the Kemp elimination, as catalyzed by variants R1 (configuration A), R5 (configurations A and B), R7 (configurations A and B) and R7-2 (configurations B and C). The EVB method describes chemical reactivity within a valence bond framework using classical force fields, and has been successfully applied to the investigation of the Kemp elimination reaction in enzymes. The reaction was modeled based on the valence bond states shown in Supplementary Fig. 8 and the resulting calculated activation-free energies, which are in excellent agreement with experiment, are shown in Supplementary Table 4. It is notable that configurations B and C are consistently better than the original, designed configuration (A).

Figure 4 shows the substrate positioning relative to key active site residues in the Michaelis complexes and transition states for the reactions catalyzed by the R7 or R7-2 A, B, and C configurations (Supplementary Fig. 9, Supplementary Tables 5-7). Firstly, and most importantly, these calculations confirm that both configurations B and C are similarly catalytically competent, i.e. the configurations are bona fide catalytic states. We observe, as in previous studies, that removal of the Glu101-Lys222 salt bridge appears to increase the pK$_A$/charge of the Glu101 side chain by ~2 pK$_A$ units (Supplementary Table 5), while the electrostatically unfavorable contribution of Lys222 to the calculated activation-free energy is substantially reduced by the removal of this salt bridge (Supplementary Fig. 10). In the most evolved versions of the three configurations we obtained from our structural studies (R7, A; R7-2, B; R7-2, C), configurations B and C displayed lower energy barriers than A (16.8 and 16.4 vs. 19.0 kcal mol$^{-1}$, respectively; Supplementary Table 4), which is consistent with the gradual conformational selection of state C along the trajectory. The reason for the increased efficiency of state C seems to be an accumulation of many small effects (we will focus on the A:C comparison since C was the state that was primarily selected). First, for all three Trp50 configurations, the substrate position is stabilized through π-stacking interactions with the Trp50 side chain. However, the Trp50:substrate alignment is on average slightly better in configuration C than configuration A (11.3 vs. 14.6°) consistent with the experimental observations: specifically, catalytically competent active site configurations emerged from disorder and the arrangement of the amino acids in these states (particularly configuration C) was superior to the original design, resulting in evolutionary conformational selection.

**The role of quantum tunneling.** Primary kinetic isotope effects (1$^o$ KIEs) occur when atoms that are directly involved in the reaction are replaced with heavier isotopes (e.g. hydrogen for deuterium) and can reveal much about the nature of the catalytic mechanism. Using hydrogenated and monodeuterated (at the 3’-
we investigated the magnitude of the KIE across quantum tunneling in enzyme catalysis and molecular quantum tunneling in solution, and recent work has implicated contribution to the reaction rate is likely to be relatively small. That although quantum tunneling is likely to occur in solution when starting from the isolated reactants. These results establish for the reaction from the pre-complex at 298 K, and 5.8 at 298 K for the lighter hydrogen isotope, with a tunneling-corrected KIE of 5.8 (Table 1, Supplementary Table 9). As expected, tunneling is greater for the deuterated molecules between 283 and 323 K are given in Supplementary Table 8. Given that the theoretical calculations show some level of agreement, we determined the activation energy ($E_a$) and pre-exponential factor ($A$) for the reaction in aqueous solution (Table 1). Given that hydrogen abstraction is the rate-limiting step in the Kemp elimination reaction, it was not surprising that ring opening in the deuterated analog occurred more slowly (Table 1, Supplementary Fig. 11). The relative rates of the hydrogenated and deuterated analogs allow calculation of the 1° KIEs for this reaction, yielding a value of 4.9 at 298 K. To investigate the contribution of quantum tunneling to this 1° KIE, the $H$ abstraction by a hydroxide ion-water cluster ($\cdot OH\cdot(H_2O)_4$) was studied from first principles (Supplementary Fig. 12), using quantum chemistry. The free energy activation barrier for H abstraction by the hydroxide ion-water cluster in the water continuum solvent model is consistent with previous work (17.4 vs. 19.8 kcal mol$^{-1}$). Gas-phase calculations reveal that the $H$ abstraction process is diffusion controlled without explicit H$_2$O molecules to stabilize the reactive $\cdot OH$ (Supplementary Table 8, Supplementary Fig. 13). QM/MM calculations were carried out with Polyrate in the H abstraction step of the intermediate complex (with the rate constant $k_{cat}$), and the corresponding values of the tunneling coefficients $\kappa$ for the hydrogenated and deuterated molecules between 283 and 323 K are given in Supplementary Table 9. As expected, tunneling is greater for the lighter hydrogen isotope, with a tunneling-corrected KIE of 5.8 for the reaction from the pre-complex at 298 K, and 5.8 at 298 K when starting from the isolated reactants. These results establish that although quantum tunneling is likely to occur in solution and contribute to the magnitude of the KIE, its overall contribution to the reaction rate is likely to be relatively small.

Given that the theoretical calculations show some level of quantum tunneling in solution, and recent work has implicated quantum tunneling in enzyme catalysis and molecular evolution, we investigated the magnitude of the KIE across the trajectory; if enhanced quantum tunneling were selected for throughout evolution, as has been proposed, a substantial increase in the magnitude of the KIEs would be expected. Our results reveal that although there were transient changes to the KIEs in the middle of the evolutionary trajectory ($E_{a(D)}$ vs. $E_{a(H)}$ and $A_D/A_I$ values at R5 and R6), the KIEs from the start (R1) and end-point (R7-2) were very similar (Table 1, Fig. 5). Notably, R1 and R7/R7-2 are both relatively conformationally stable, as judged by the molecular dynamics simulations and tryptophan fluorescence spectroscopy. The anomalous KIEs observed in R5–R6 correlate with the increased sampling of the alternative active site configurations (Fig. 5). This is notable because QM/MM calculations have previously shown that, when the conformational coordinate is included in the catalytic model, the presence of distinct active site configurations with different catalytic efficiencies (as observed here) can account for anomalous KIEs without the need to invoke large changes in quantum effects. It therefore appears that evolution has not involved optimization of short-timescale (fs) vibrations that could enhance quantum tunneling in this example.

**Discussion**

In this work, we have followed the iterative improvement of a designed enzyme, dissecting the respective contributions of...
various effects on catalysis. In addition to traditionally considered effects, such as mutations increasing the basicity of a catalytic residue (e.g., Glu101), our results highlight the significance of catalytic tradeoffs: the same mutational changes that alter active site geometries with different catalytic efficiency. Conformational selection of the most efficient configuration then produced a superior enzyme. Conformational selection has been observed in the optimization of catalytic precision in other designed enzymes2,19,33, but the scale of the conformational reorganization observed here is particularly notable. Alongside our KIE data, our results suggest that to accurately model enzyme catalysis by populations of conformationally heterogeneous enzymes, catalytic models should incorporate multiple conformational substates comprising different active site geometries with different catalytic efficiencies3.

These results highlight the remarkable conformational plasticity of proteins and the degree to which the configuration of an active site can be modulated by the amino acid composition of the shell. It is notable that the selection of nondesigned active site conformations through directed evolution appears to be more common when working with computationally designed enzymes19. We suggest that this could be due to less focus on the optimization of outer-shell residues to stabilize the active site geometry, in comparison to naturally evolved enzymes in which the composition of the outer-shell has evolved over many generations to stabilize active sites via multiple mechanisms9.

This study adds to an emerging view in which the conformational plasticity of proteins underpins their remarkable evolutionary potential34 and establishes that, provided the active site contains plasticity of proteins underpins their remarkable evolutionary potential. In this study, we have determined the kinetic parameters for the reaction catalyzed by 2,3-Deuterio-5-nitrobenzoxazole and 2,3-Deuterio-5-nitrobenzoxazole, where $A \cdot R \cdot k$ and $T$ are pre-exponential factor, gas constant, reaction rate, and temperature in Kelvin, respectively. The activation energy $E_a$ was calculated from the slope of the Arrhenius plot. A is calculated from the y-intercept of the plot.

**Substrate synthesis.** 5-nitrobenzoxazole was prepared from 1,2-benzisoxazole (Sigma, >99%) following the published protocol19. 2 mL (2.3 g) of 1,2-benzisoxazole was dissolved in concentrated H$_2$SO$_4$ (20 mL) in a flask (placed on the salt–ice mixture), then 2 ml mixture of H$_2$SO$_4$ (1 ml) and HNO$_3$ (3 ml) was slowly added to the 1,2-benzisoxazole mixture. The solution was stirred for 30 min. Fifty milliliters of ice–water mixture was added to the stirred mixture and the filtered crude product was recrystallized by solvating it to heated anhydrous ethanol (70–80 °C) then slowly cooled and filtered. The yield of 2 days vacuum-dried product was 3.3 g. 3-Deuterio-5-nitrobenzoxazole was synthesized from 2-bromopropene following the published procedures9,30. The substrate synthesis scheme is shown in Supplementary Fig. 14 and consists of four steps:

1. 2-(2-Bromophenyl)tetrahydro-2H-pyran: Pyridinium p-toluenesulfonate (734 mg, 3.00 mmol) was added to a solution of 2-bromopropene (3.48 mL, 30.00 mmol) and 2,3-dihydropropyran (4.11 mL, 45.00 mmol) in dry CH$_2$Cl$_2$ (20 mL).

The resulting mixture was stirred for 21 h under an atmosphere of nitrogen at room temperature. Thereafter, the reaction mixture was quenched with sat. aq. NaHCO$_3$ (20 mL) and the resulting phases were separated. The aqueous phase was then dried over Na$_2$SO$_4$, and the organic phases were combined, dried over Na$_2$SO$_4$, and the filtrate concentrated in vacuo. The residue was dissolved in and flushed by flash chromatography (SiO$_2$, EtOAc:Hex (1:10)) to give 1 (6.61 g, 86%) as a colorless oil.

2. 3-Deuterio-5-nitrobenzoxazole: A mixture of HNO$_3$ (400 MHz, CDCl$_3$) $\delta_{7.8, 7.8, 1.6}$ Hz, 1 H), 7.24 (dd, $J = 7.8, 4.7, 4.6, 1$ H), 7.16 (dd, $J = 8.6, 1.2, 1$ Hz, 1 H), 6.87 (dd, $J = 7.8, 7.4, 1.6$ Hz, 1 H), 5.53 (t, $J = 2.7$ Hz, 1 H), 3.92 (dd, $J = 11.0, 3.1$ Hz, 1 H), 3.67–3.57 (m, 1 H), 2.19–2.05 (m, 1 H), 2.04–1.95 (m, 1 H), 1.94–1.83 (m, 1 H), 1.80–1.59 (m, 3 H).

13C NMR (100 MHz, CDCl$_3$) $\delta_{153.4, 133.2, 128.3, 122.7, 121.7, 113.6, 93.6, 91.6, 63.8, 30.2, 25.3, 18.3$. MS (EI) $^+$ m/z 258 (100%, [M$^+$]$^+$), 256 (10%, [M$^+$]$^+$)

3. 2,3-Deuterio-5-nitrobenzoxazole: A solution of 2,3-Deuterio-5-nitrobenzoxazole was added to a solution of 2,3-Deuterio-5-nitrobenzoxazole and 2,3-Deuterio-5-nitrobenzoxazole, where $A \cdot R \cdot k$ and $T$ are pre-exponential factor, gas constant, reaction rate, and temperature in Kelvin, respectively. The activation energy $E_a$ was calculated from the slope of the Arrhenius plot. A is calculated from the y-intercept of the plot.

**Methods**

**Mutagenesis and protein purification.** KE07 variants, cloned into the PET-29b (+) vector (Invitrogen), were expressed with C-terminal His$_6$-tags in Escherichia coli BL21(DE3) cells (Invitrogen). The cell pellet was resuspended in buffer A (50 mM Tris-Cl pH 8.0, 100 mM NaCl, 20 mM imidazole) and lysed with a French pressure cell press (Thermo Fisher). The soluble fraction was loaded onto a Ni-NTA column (Qiagen) and elution was achieved with buffer B (50 mM Tris-Cl pH 8.0, 100 mM NaCl, 250 mM imidazole). After extensive dialysis against elution buffer (25 mM HEPES-NaOH pH 7.25, 100 mM NaCl), protein concentrations were determined using a NanoDrop spectrophotometer (Thermo Fisher) at 280 nm.

**Kemp elimination kinetics.** Kemp elimination of 5-nitrobenzoxazole (PubChem CID: 142383) results in the formation of 2-cyano-4-nitrophenol (PubChem CID: 111777) in 95% yield. The reaction rate was determined using an Agilent Technologies spectrophotometer at 242 nm (1 cm path length, 1 M solution in 1% (v/v) CH$_3$OH:CH$_2$Cl$_2$).

1. **Kemp elimination kinetics.** Kemp elimination of 5-nitrobenzoxazole (PubChem CID: 142383) results in the formation of 2-cyano-4-nitrophenol (PubChem CID: 111777), which is a colorless compound. The reaction rate was determined using an Agilent Technologies spectrophotometer at 242 nm (1 cm path length, 1 M solution in 1% (v/v) CH$_3$OH:CH$_2$Cl$_2$).

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mixing of ice and water (50 mL, 1:1). The resulting precipitate was collected and recrystallized from dry EtOH to give the final product (273 mg, 28% over two steps) as a colorless solid. \(^{1}H\) NMR (400 MHz, CDCl\(_3\), \(\delta\) 8.73 (d, J = 2.0 Hz, 1 H), 8.52 (d, J = 9.2, 2.0 Hz, 1 H), 7.77 (d, J = 9.2 Hz, 1 H). \(^{13}C\) NMR (100 MHz, CDCl\(_3\), \(\delta\) Ms (Eu\(^{3+}\)).

X-ray crystallography. Crystals of all KeO7 variants except one (R7-2 with product) were grown at 4 °C by hanging-drop vapor diffusion. Equally volume reservoir solution (12-25% PEG 3350, 0.1 mM Tris-Propanehydrochloride 8.5) or (25 mM HEPES-NaOH pH 7.25, 100 mM NaCl) were mixed with protein (5-30 mg mL\(^{-1}\)) and crystals reached maximum size after 7-60 days of incubation. One of the R7-2 crystals was grown mixing 2 μL of reservoir solution (0.5 M (NH\(_4\))\(_2\)SO\(_4\), 0.8 M LiSO\(_4\), 0.1 M Nacitrate pH 5.6) and 1 μL of protein (11 mg mL\(^{-1}\)) the crystals reached maximum size after 1–2 months. \(^{1}H\) NMR (400 MHz, CDCl\(_3\)) of the re

200 ns simulations, giving the average exchange acceptance ratio of 35

0.723, and 0.667. The exchange between replicas was attempted every 4 ps during

101, 128, 201, and 222). The Hamiltonian scaling factors for the nonbonded

interactions from the protein. The system was solvated with TIP3P water molecules\(^49\) and the

system, and 367 ns simulation time over all systems (in addition to the

reservoir solution (12

7.25, 100 mM NaCl) and the spectra were measured at 283

Cary Eclipse Fluorescence Spectrophotometer with a Single Cell Peltier Accessory

for our EVB simulations of each system. Our EVB calculations were performed using a simple two-state model, using the valence bond states described in Supplementary Figs. 8 and 16 (see also refs.\(^{15,28}\)). The EVB free energy perturbation/umbrella sampling (EVB-UES) calculations were performed in 51 individual mapping frames of 100 ps simulation length per frame, leading to a total of 5.1 ns simulation time per individual EVB trajectory, 45.9 ns simulation time per system, and 367 ns simulation time over all systems (in addition to the equilibration time leading to a total simulation time of 1.09 μs). The EVB parameters were calculated using the uncatalyzed background reaction in aqueous solution as a baseline, which was modeled using the 4-

nitrobenzoxazole substrate and propionate as a model for Glu101 (again, see ref.\(^{20}\)). All simulations of this reference state were performed using the same protocol as for the corresponding enzymatic reaction, with the exception that a larger harmonic restraint of 1.0 kcal mol\(^{-1}\) Å\(^{-2}\) was placed on the reacting atoms to stop them from drifting out of the simulation sphere. The EVB off-diagonal element and gas-phase shift, which are described in detail in e.g. ref.\(^{27}\), were adjusted to reproduce an activation-free energy of 21.2 kcal mol\(^{-1}\) in aqueous solution based on the calibration provided in refs.\(^{15,28}\) and the same EVB parameters were then used unchanged to model the reaction in all enzyme variants. This then provides a common reference point to compare the relative energies of all enzyme variants to each other. All EVB parameters used in this work are provided in Supplementary Tables 10 to 21 (Supplementary Fig. 16). Finally, all analyses were performed using the Q simulation package and tools \(^{50,61}\). the RMSD and clustering analyses were performed using GROMACS\(^{45}\) (the clustering algorithm described by Daura et al.\(^{64}\), with the cutoff of 0.5 Å for the protein atoms), and the geometry analysis was performed using the MDTraj library\(^{64}\).

Computational tunneling evaluation in the Kemp elimination. All molecular orbital theory and density functional theory calculations were carried out with Gaussian 09. Geometries and frequencies of all species were calculated using the M06-2X functional. All species were optimized in vacuo or in a field of solvent using SMD/M06-2X/6-31 + G(d,p) method\(^{46}\) with water. All transition state structures were characterized with a single imaginary frequency and minimum with zero imaginary frequencies. The tunneling probability was evalu-

ated using the reaction-path and transition state theory (RP-VTST/MT) and multi-

dimensional tunneling (RP-VTST/MT) method\(^67\). These calculations were performed with Polyrate 2010-A via the Gaussian 2009-A interface to Gaussian

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Data availability

The crystal structures of KE07 variants, with and without ligand, have been deposited in the Protein Data Bank under accession codes 6C7H, 4Z08, SD2T, SD2V, SD2W, 6C7V, 6DNI, 6C7T, 6C8B, SD30, SD32, SD33, 6CAL, 6D31, 6D38, 6CT3. PDB validation reports are all available at www.rcsb.org. All relevant data are available from the corresponding author upon request.

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**Author contributions**

N.-S.H., S.C.L.K., D.P., M.P., P.B., R.L., G.G., J.S., C.-Y.L., W.Z. and T.A. performed experiments/simulations and analyzed the results. P.D.C., P.D.M., W.Z. and C.E. supervised research and analyzed the results. M.L.C., S.C.L.K. and C.J.J. supervised research, analyzed results and wrote the manuscript with input from all authors.

**Additional information**

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