Published in final edited form as:

Biochemistry. 2018 June 12; 57(23): 3289–3298. doi:10.1021/acs.biochem.8b00185.

Directed Evolution as a Probe of Rate Promoting Vibrations Introduced via Mutational Change

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Abstract

In this article, we study with transition path sampling and reaction coordinate analysis how directed evolution in the Kemp eliminase family of artificial enzymes makes differential use of rapid rate promoting vibrations as a component of their chemical mechanism. Even though this family was initially created by placing the expected active site in a fixed protein matrix, we find a shift from largely static to more dynamic active sites that make use of donor–acceptor compression as the evolutionary process proceeds. We see that this introduction of dynamics significantly shifts the order of processes in the reaction. We also suggest that the lack of “design for dynamics” may help explain the relatively low proficiency of such designed enzymes.

Graphical abstract

Enzymes catalyze chemical reactions with incredibly high proficiency.¹ Significant effort has been directed to understanding the mechanism of enzymatic catalysis. Increasing evidence has shown that dynamic motions of enzymes play an important role in catalytic process.^{2–8} Recent theoretical and experimental studies have shown that sub-picosecond motions, which are on the same time scale as the chemical step of enzymatic reactions, can couple with the reaction coordinate of enzymes.^{2,4,5,9–20} These femtosecond motions are termed rate promoting vibrations (RPVs), since couplings between these motions and reaction coordinates can promote the rate of the chemical step in the reaction process. The RPV promotes the rate of the chemical step of an enzymatic reaction by dynamically modulating the height and width of the potential energy barrier.²¹ RPVs have been found in

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Notes

The authors declare no competing financial interest.

a variety of enzymes, such as in human heart lactate dehydrogenase,⁴ horse liver alcohol dehydrogenase,¹² and human purine nucleoside phosphorylase.^{10,11}

Protein structures are altered by evolutionary pressure.^{22–24} The existence of the RPV as a contributor to chemical mechanism gives rise to the question of how the RPV behaves during enzyme evolution? Is it introduced into an enzyme during evolution or does it exist from the very beginning and is left untouched during an evolutionary process? Past research comparing two members in the dihydrofolate dehydrogenase (DHFR) enzyme family^{6,9,25–27} shed some light on this question. It has been shown that in hsDHFR, a fast protein motion is coupled with the reaction coordinate,⁹ indicating the existence of a RPV; however, ecDHFR has been shown to not have an RPV involved in its catalytic process.^{6,9} Also, a recent study in two enzymes taken from the lactose dehydrogenase (LDH) enzyme family shows similar result.² While a RPV has been found in *Plasmodium falciparum* LDH, only modest signs of coupling between protein motions and the chemical step have been found for *Cryptosporidium parvum* LDH.² These two comparison results show that RPVs have been changed through the evolutionary process. The work described in this study further probes the importance of RPV introduction via mutational change induced by laboratory evolution. In many enzymes, chemistry is not the rate-determining step, and so it is expected that there will be minimal remaining evolutionary pressure on chemistry.^{28,29} In order to understand the effects of mutational changes on the chemical step, this study focuses on four enzymes in the directed evolution path of an artificially designed enzyme, KE59,³⁰ part of the Kemp eliminase family. Directed evolution is used to alter the properties of enzymes through in vitro mutagenesis screening.^{31–36} During the directed evolution process, mutants of a protein are generated by performing random mutagenesis.^{31,32,36} They are then screened for a desired property, an increase in catalytic efficiency or an increase in thermostability for instance. Only the best variant is kept as the intermediate, and another round of the mutation–screening procedure is performed starting from this intermediate. This procedure is repeated multiple times during a directed evolution study, until the property has been altered to a desired extent, or no further improvement is possible.^{31,32,36} The pathway of a directed evolution can serve as a mimic (imperfect though it may be) of a natural evolution process,³⁵ with obvious limitations: for example, no neutral mutations are kept. The lesson we glean from this mimic of true evolution, in some cases, may thus deviate from the reality, but the advantage of a directed evolution process lies in the ability to possess precise sequences and crystal structures for evolutionary intermediates, and a precise ranking of proficiency. We do admit the limitation of the directed evolution approach, and future work could be focused on the ancestral enzyme reconstruction approach.³⁷

MATERIALS AND METHODS

The directed evolution pathway we employ starts from KE59,^{30,38} an artificially designed enzyme catalyzing the Kemp elimination reaction. Kemp elimination is a ring opening–hydrogen transfer reaction on 5-nitrobenzisoxazole (5-nitro-BI),^{39,40} a schematic of which is shown in Figure 1. Directed evolution of KE59 led to a 2000-fold increase in its catalytic efficiency³⁰ and resulted in a $k_{\text{cat}}/K_{\text{M}}$ of $5.73 \times 10^5 \pm 1.9 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ ³⁸ for the end stage protein. KE59 and its directed evolution process is a useful system for study because of three reasons: First, KE59 and its variants are good candidates to possess RPVs in their catalytic

mechanism because of the hydrogen transfer nature of the mechanism. The potential energy barrier for reaction drops sharply for hydrogen transfer when the hydrogen donor and acceptor distance is compressed;^{28,29,41} thus these reactions are sensitive to motions that influence the donor–acceptor distance. In the Kemp elimination reaction, a hydrogen transfer step from the substrate to the catalytic base is involved in its reaction mechanism,^{38,42} as shown in Figure 1, making KE59 and its variants good candidates to have rate promoting vibrations coupled with the reaction coordinate. Second, crystal structures of several variants along the directed evolution process of KE59 were obtained,³⁰ which yield starting points for further theoretical studies. Third, the directed evolution process on KE59 increased its catalytic efficiency by 2000-fold.³⁰ Four variants along the directed evolution process have been studied in this research: R1-7/10H, R5-11/5F, R8-2/7A, and R13-3/11H. Crystal structures of these four variants are shown in Figure 2. Mutations from the prototype are highlighted in red, and it becomes immediately obvious that mutations are scattered in the protein, not restrained to the active site.

System Preparation

Transition path sampling (TPS) will be the method we use to generate an ensemble of reactive trajectories. From this ensemble, we will use our published methods to analyze the reaction coordinate. Preparation of crystal structures of the four enzymes we studied was performed using CHARMM39. For R1-7/10H and R13-3/11H, the Schrodinger/Maestro software was used to modify the cocrystallized 5,7-dichloro-BI substrate into the active substrate 5-nitro-BI and generate the coordinate file (CRD) and protein structure file (PSF). Hydrogen atoms that are missing in X-ray crystal structures were added by HBUILD command in CHARMM. Structures for evolutionary intermediates R5-11/5F and R8-2/7A require further manipulation, since they were crystallized without substrate. The crystal structure of the R13-3/11H–substrate complex was mutated in CHARMM39 according to the amino acid sequence of R5-11/5F to generate substrate binding structures for R5-11/5F and R8-2/7A. The quantum region of each enzyme for our QM/MM^{43,44} calculations is composed of the general base E231, a hydrogen bond donor T180 (S180 in R13), and the substrate molecule, as shown in Figure 3. The quantum region was treated quantum mechanically using the semiempirical AM1 potential.⁴⁵ Boundary atoms between the quantum region and molecular mechanic regions were treated using the generalized hybrid orbital (GHO) method⁴⁴.

The system was solvated explicitly in a spherical water box using a TIP3P water model.⁴⁶ Finally, potassium ions were added to the system to neutralize the negative charge carried by the enzyme. Each system was further treated with the following protocol prior to TPS. The system was minimized for 100 steps using the steepest descent method, then 1500 steps with gradually reduced harmonic constraint forces on all non-hydrogen atoms using adopted-basis Newton–Raphson algorithm (ABNR). Finally, the constraint forces were removed completely, and the system was minimized by the ABNR method for 1000 steps. The system was then heated from 0 to 300 K in 25000 steps. Previous molecular dynamics studies^{47–49} have shown that the active site for the Kemp eliminase enzyme family may take alternative nonreactive conformations during MD simulations. To maintain the active site geometry during system equilibration, we applied harmonic constraints to the active site during the

heating process. System equilibration was performed for the first 5000 steps under a gradually reduced constraint, then 25000 steps equilibration with all constraint forces removed.

Transition Path Sampling

In this research, TPS method^{50,51} has been used to acquire reactive simulation trajectories describing the chemical step of the enzymatic Kemp elimination reaction. TPS is a Monte Carlo sampling method that aims to capture rare events in complex systems. Two features make TPS suitable for this study. First, TPS does not require prior knowledge of the reaction coordinate of the system under study,⁵¹ and second, TPS requires a well-defined starting state and an ending state,⁵¹ both of which are easy to define for this enzymatic reaction. In this research, the two states are defined as the reactant state and product state of the Kemp elimination reaction. To perform TPS, a biased trajectory is initiated from the reactant state. Artificial forces are then applied, forcing the system to propagate into the product state. A slice is chosen randomly from this seed trajectory. The momentum of this slice is perturbed with a random perturbation chosen from a Boltzmann distribution, and then all momenta are rescaled to maintain a microcanonical sampling process. A new trajectory is generated starting with the coordinates from the previously selected slice and the perturbed momentum, propagating both forward and backward in time. The acceptance criterion under microcanonical sampling takes the following form:

$$P_{\text{acceptance}} = h_{\text{R}}(X_0)h_{\text{P}}(X_t)$$

where $P_{\text{acceptance}}$ is the acceptance ratio for the new trajectory and $h_{\text{R}}(X_0)$ and $h_{\text{P}}(X_t)$ are Heaviside functions that determine if a state is in reactant state (h_{R}) or product state (h_{P}). X_0 marks the start slice of the new trajectory, and X_t marks the end slice of the new trajectory. That is, the new trajectory is accepted (considered reactive) if it starts from the reactant region and ends in the product region and is rejected if fails to meet either criterion. This new trajectory is used to initiate the next round of TPS following the same procedure. Thus, TPS creates an ensemble of reactive trajectories connecting the reactant region and the product region, which can be utilized for further study. This sampling method has been used successfully to study the chemical step of enzymatic reactions in a variety of enzymes, for instance, LDH,^{2,4} PNP,¹¹ and DHFR.^{6,9}

In this study, the reactant state and the product state are defined by three parameters, referred to as the order parameters: hydrogen-donor (C9–H13) distance and hydrogen-acceptor distance (H13–OEX, OEX indicates that the hydrogen can be transferred to both oxygen atoms on E231) for the hydrogen transfer step in Kemp elimination and nitrogen–oxygen (N8–O12) distance for the ring-opening step in Kemp elimination. The system will be defined as in the reactant state if the C9–H13 distance is smaller than 1.35 Å, the H13–OEX distance is larger than 1.10 Å, and the N8–O12 distance is smaller than 1.75 Å. The system will be defined as in the product state if the C9–H13 distance is larger than 1.35 Å, the H13–OEX distance is smaller than 1.10 Å, and the N8–O12 distance is larger than 1.75 Å. The initial biased trajectory was generated by QM/MM simulation starting from the equilibrated

structure, with constraint force applied to maintain the C9–OE X distance to be 1.1 Å and the N8–O12 distance to be 2.5 Å.

Committor Analysis

The committor analysis^{50,51} method was used to determine transition states for reactive trajectories. A committor value, $P_A(x)$, is defined as the probability that a trajectory initiated from a specific slice, with a random velocity in all degrees of freedom, will end in the state A, while P_B can be defined in a similar manner. A committor value of $P_A \cong P_B = 0.5$ indicates that the corresponding slice has an equal possibility of going into either region A or B, and this slice is defined as the transition state of this particular trajectory. To calculate committor values for a specific slice, we first extracted its coordinate information, and then random momenta chosen from a Boltzmann distribution were assigned to all degrees of freedom in the system. A total of 50 trajectories were initiated, each 250 fs long and with a new set of random momenta. P_A and P_B were determined as counts in reactant or product states, among the 50 trajectories, respectively. The collection of all acquired transition states for each enzyme, referred to as the separatrix, is the transition state surface of the enzyme.

Committor distribution analysis^{50,51} was performed to search for the reaction coordinate of each system. Needed inclusion of protein motion to obtain a valid reaction coordinate indicates the presence of an RPV. A trajectory of 250 fs long was initiated from a transition state, with constraints on all the degrees of freedom that are assumed to be in the reaction coordinate. Committor values for every 5 slices on this trajectory were calculated by shooting 50 times from each slice with random momenta. If all degrees of freedom that are involved in the reaction coordinate are under constraint in the initial trajectory, then the distribution of these committor values should remain peaked at 0.5. If not, further or different degrees of freedom are added, until a distribution peaked at 0.5 is observed. For each of the four enzymes, we use the sum of committor distributions starting from 7 transition states to search for reaction coordinate to eliminate random errors.

RESULTS AND DISCUSSION

Transition Path Ensembles

In this study, seven transition path ensembles, each containing 100 reactive trajectories, have been created. The first 6 transition path ensembles are created following the procedure described in the Materials and Methods section. In the equilibrated structures of R1-7/10H and R13-3/11H, two oxygen atoms on the acceptor glutamate E231 are roughly equally far from the hydrogen donor C9, and no previous work has reported either oxygen to be more favored than the other. Therefore, for each of these two enzymes, we have created two transition path ensembles, each only allowing the hydrogen to be transferred to one of the oxygen atoms. For both R5-11/5F and R8-2/7A, in the equilibrated structure, one of the two oxygen atoms is significantly further away than the other (OE1 in R5-11/5F, OE2 in R8-2/7A). Therefore, for each of these two enzymes, we have created only one transition path ensemble, only allowing the hydrogen to be transferred to the closer oxygen atom. When performing committor analysis on transition path ensembles of R1-7/10H, we discovered that when shooting from a slice chosen from a reactive trajectory, a significant

portion of these shooting moves end in another “product state” with the hydrogen atom being transferred to the other oxygen atom, showing that we should define the product state for R1 as only one state, which allows the hydrogen to be transferred to both oxygen atoms, instead of two product states each only allowing the hydrogen being transferred to one of the oxygen. A new transition path ensemble for R1-7/10H is then created with this new set of order parameters for product state and utilized for further calculation. We will refer to the five ensembles for distinct evolved artificial enzymes as R1, R5, R8, R13-OE1, and R13-OE2 in further discussion.

Through analyzing these transition path ensembles, we have found two important features of the Kemp elimination reaction catalyzed by KE59 enzyme family, as shown in Table 1. First, during the ring-open step of this reaction, the breaking N–O bond goes through an intermediate structure before it is fully broken. Intuitively, in the product of Kemp elimination reaction, the SP hybridized carbon of the cyano group will make N8 point in an opposite direction to that of the O12, making the product structure possibly possess a very large N8–O12 distance, as shown in Figure 1, while the N–O distance for all the intermediate states on average is 2.2 Å. Therefore, even though the distance between the two atoms at the intermediate state is already longer than a N–O single bond, in this research, we still define the ring-open step as being finished only after the intermediate state. Second, the two steps of the Kemp elimination reaction do not happen simultaneously, and the order of the two steps differs between different enzymes. Two general trends can be concluded from the change of these two features along the directed evolution process. First, along the directed evolution process, average time duration of the ring-open step becomes shorter, decaying from an average of 40 fs of R1 to an average of 23 fs of R13-OE1 and R13-OE2. Second, the order of the two steps has been reversed during the directed evolution process. While the hydrogen transfer step on average takes place 34 fs after the end of ring-open intermediate state for trajectories for R1 ensemble, the two steps take place almost simultaneously for both R5 and R8 ensembles. For one of the R13 ensembles, the hydrogen transfer step takes place even prior to the bond-breaking intermediate state. The reason for these changes will become apparent as we discuss variation of the reaction coordinate along the evolutionary path.

Transition States

We have succeeded in finding 7 transition states for each ensemble. Important distances between reaction-involved atoms at the transition state are listed in Table 2. Transition state structures, which indicate the point at which the system proceeds from reactant state to product, are distinct for different ensembles. In R1, all transition states lie in the ring-open step, as reflected by the longer average N8–O12 distance at transition states. The hydrogen transfer step, however, has not yet been initiated. In fact, in all other ensembles, transition states all lie in the ring-open intermediate state, that is, when the system is proceeding through the bond breaking step. To further support this finding, we matched the time period during which each system proceeds from its reactant state to the product state, characterized by its committor value, and it is not surprising that this time period overlaps to a great extent with the ring-open intermediate state. However, since the order of the two steps has been reversed along the directed evolution process, transition states have migrated to the other

side of the hydrogen transfer step. In R5, R8, and R13-OE2, the hydrogen transfer has been completed slightly before the transition state, reflected by their small hydrogen–acceptor distances in Table 2. Along with the earlier and earlier hydrogen transfer step, the average N–O bond length at the ring-open intermediate state has been shortened along the directed evolution process, from 2.17 Å in R1 ensemble to 1.68 Å of R13-OE2 ensemble. It is worth pointing out that the intermediate state is still well-defined for R13-OE2 ensemble even though its average intermediate bond length is just 1.68 Å.

Reaction Coordinate

Committer distribution analysis was performed under different constraint sets to search for the reaction coordinate in all four enzymes, each calculated for all seven available transition states in their respective ensembles. We started by applying constraints only to the quantum region (Figure 4, 1(a)–5(a)), which is essentially all atoms directly involved in the Kemp elimination reaction. Residues adjacent to the substrate were then incorporated into the constraint region, until an optimal committer distribution had been found (Figure 4, 1(b)–5(b)). The quantum region and all residues in the constraint region were fixed at their transition state configurations. Residues under constraint in Figure 4, 1(b)–5(b), together with the quantum region itself, are the reaction coordinate of each ensemble. These residues are listed in Table 3.

For the R1 ensemble, a reaction coordinate consisting of the quantum region itself is only marginally improved in quality by inclusion of protein residues in the reaction coordinate. For reasons that will become apparent as we analyze trajectories below, for the R5 and R8 ensembles, the quantum region itself yields a committer distribution reasonably peaked at 0.5. It is improved, however, in each ensemble by inclusion of active site residues into the constraint region. The R13-OE1 and R13-OE2 ensembles share the same chemical approach to reaction, despite the fact that their transition state structures are different. While their committer distributions are peaked at the reactant side with only the quantum region under constraint, the peaks are shifted to a significantly greater extent than in the previous cases to 0.5 when active site residues are incorporated into the constraint region. This means that for these most evolved members of this family, the motion of these residues is of maximal importance in the set. This in turn indicates that this mutational process selected for optimal activity “found” promoting vibrations as a method to optimize chemistry.

Despite the distinct performance of reactive trajectories from these five ensembles, all reaction coordinates share similarities, with I178 and V159 involved in the reaction coordinate of all 5 enzymes. Trajectory analysis below will show that no RPV exists for R1, whereas R5 possesses a RPV on the acceptor side, from V51 and V210, and R8, R13-OE1, and R13-OE2 each has a RPV coming from I178. I178, V159, and I133 together form a “platform” upon which substrate “sits”. The shape of the platform is not exactly the same in the 5 ensembles. While I133 and V159 are always in proximity to the substrate in all 5 ensembles, the distance between I178 and the substrate varies. While the I178 residue completely lost contact with the substrate in the R1 ensemble, it makes contact with the substrate through carbon $\gamma 2$ atom in R5 and R8 ensembles and through carbon $\delta 1$ atom in R13-OE1 and R13-OE2 ensembles. We have plotted the distance between I178 and the

hydrogen donor, C9 for R8, R13-OE1, and R13-OE2, as shown by the orange line in Figure 5c,d,e. For all three representative trajectories, I178 moves close to C9 before the reaction, which pushes C9 closer to the hydrogen acceptor OEX, as shown by a compression of the donor–acceptor distance. Though in a real enzyme with full protein optimization, the magnitude of a RPV excursion is usually much larger, all three motions mentioned above are clearly coupled with the reaction coordinate and have resulted in a donor–acceptor compression, making them eligible to be defined as rate promoting vibrations. The R1 ensemble, however, shows no significant donor–acceptor compression before the reaction. I178 is not in contact with the substrate, as shown by the distance between I178 carbon γ_2 and C9, the orange line in Figure 5a. While the platform makes contact with the substrate through V159, no significant motion between them has been detected. Finally, for R5, the donor–acceptor is also compressed before the reaction, but this is caused by a different motion from a different direction. While no significant motion from the three platform residues have been observed, we do find that another residue that is in the reaction coordinate of R5 ensemble, V51, pushes the entire acceptor residue E231 toward the hydrogen donor C9 prior to the reaction. The orange line in Figure 5b, which is the distance between V51 carbon γ_2 and E231 carbon γ , describes this motion. To summarize, the directed evolution process for KE59 starts with a prototype possessing no RPV. While a RPV from the acceptor side has emerged in the evolutionary intermediate R5, it disappeared later as directed evolution proceeded. Later at stage R8, a RPV from the substrate side has been built in to the enzyme and is left unchanged until the final stage R13 of the directed evolution. The motions are small, but the barrier is exponentially dependent on donor–acceptor distance. As we will mention below, this fitting of an active site into a static framework not created for the specific evolution likely limits the range of motion that can be introduced.

The change of relative positions between the substrate and the hydrogen acceptor could be a possible explanation for the fact that R1 does not possess a rate promoting vibration. We can roughly define a plane from the atoms of the substrate and define the relatively position of the 3 platform residues to be underneath the substrate plane. In the R1 ensemble, both hydrogen acceptor atoms OE1 and OE2 are very close to the substrate plain before the reaction and remain in this relative position throughout the reaction process, as shown in Figure 6a, before the reaction and Figure 6b, at the hydrogen transfer reaction. Since the platform residues are all located underneath the substrate plane, a possible motion coming from the platform residues to the substrate would be in the “upward” direction, which has no effect on compressing the donor–acceptor distance. The story is completely different in the R13- OE2 ensemble. Acceptor atoms OE1 and OE2 now are “on top” of the substrate plane and remain in this structure throughout the reaction process, causing the donor–acceptor vector to point “upward”. A pushing motion comes from platform residues that can push part of the isoxazole ring upward, which then gives the hydrogen donor C9 an upward momentum toward the two acceptor atoms and compresses the donor–acceptor distance. This type of motion is exactly the motion that has been found for R13-OE2 ensemble. Figure 6c shows the pushing motion from I178 before the reaction, and Figure 6d shows the effect of this pushing when the hydrogen transfer reaction take place. As discussed above, the R8 and R13-OE1 ensembles also have this type of rate promoting vibration. The R5

ensemble does have the same acceptor position as R1, making the three platform residues not able to provide RPV. However, it possesses a RPV coming from the acceptor side.

We also note that in R8, R13-OE1, and R13-OE2, the RPV helps orient the C–H bond to the acceptor direction. These effects are expected to lower the barrier of the hydrogen transfer step, making it take place prior to the ring-open step. However, since no RPV has been found in R1 ensemble, the hydrogen transfer barrier is likely large. We infer that for R1 ensemble, the completion of the ring-open step will aid the hydrogen transfer reaction in two ways. First, breaking the N–O bond can result in a redistribution of charges on the isoxazole ring. While O12 is negatively charged, the cyanide group must correspondingly carry a positive charge, decreasing the electron density on C9, and therefore weakening the hydrogen–C9 bond. Second, as shown in Figure 6e,f, breaking the N–O bond will cause a geometry change of the isoxazole ring, pointing the donor-hydrogen bond to the acceptor. The hydrogen transfer step can only happen with the aid of the ring-open completion. While it is hard to measure the influence of RPVs on the increase of catalytic efficiency along KE59 directed evolution process, we can see that its effect on the reaction mechanism of Kemp elimination is significant.

We have illustrated the role of I178 in the reaction coordinate. The roles that V159 and I133 play in the reaction coordinate can be explained in two ways. First, V159 and I133 may be supporting the I178 in its desired rotamer, preparing it for a possible pushing motion. Second, the three platform residues all play the role of fixing the substrate molecule in the transition state geometry during the reaction period. The fact that V159 and I178 are modestly involved in the reaction coordinate of the R1 ensemble without a significant motion supports this effect.

Discussion and Conclusions

The directed evolution process of KE59 has introduced 13 mutations into the prototype enzyme, and all five reaction coordinate residues are not part of these mutations.³⁰ The major effect of these mutations is to cause a change in active site geometry.^{30,42,48,49} It used to be believed that the change in active site geometry makes KE59 more frequently sample a reaction-favored substrate binding geometry⁴⁸ and also improves the base desolvation effect on the catalytic base E231.³⁰ We propose that aside from these known effects, this change of active site structure should be the cause of the change of relative positions between the substrate and E231 discussed above, which introduces the substrate side RPV into the enzyme family. We can see that the structural basis for a RPV, the three “platform” residues, exist even at the prototype of the KE59 directed evolution; it is just not positioned in a desired geometry that can result in a pushing motion along the donor–acceptor direction. In the KE59 directed evolution process, the introduction of RPV does not require mutations on residues involved in the reaction coordinate, nor does it require a *dramatic* change of the protein structure. As shown in this research, a subtle change of the active site structure is enough to put in a well-defined, if modest, RPV into the system. Therefore, this study shows that manipulating RPVs in an enzyme family could be done in a variety of ways, not just limited to manipulating the reaction coordinate residues, which has been the method to alter RPVs in several enzymes in previous work.⁵²

The rate promoting vibrations found in this study are small in magnitude compared to those found in natural enzymes. Also, all residues involved in the reaction coordinate of KE59 enzyme family are within the first shell of residues surrounding the active site. In this research, we have not found any amino acids further away from the active site to contribute to the reaction process. That is, the active site cannot feel influence from the outer sphere residues. RPVs found in other natural enzymes, in PNP and cpLDH, for instance, are usually composed of multiple residues along the donor–acceptor axis, some of which are far from the active site. Indeed, artificial enzymes like KE59 may be inherently lacking the power of coupling its motions with the catalytic process. The design philosophy of all Kemp eliminase enzyme families aims to put the designed active site into an existing protein scaffold. However, the design does not take into account if potential motions in the protein scaffold can contribute to the catalytic process. Instead, it is more of a “static” docking process. While this design procedure has succeeded in generating enzymes, the catalytic efficiency of the designed enzymes and their directed evolution families are still many orders of magnitude lower than natural enzymes. Not taking into account dynamic properties of the protein scaffold might be one reason for the deficiency of these artificial enzymes.

Acknowledgments

All computer simulations were performed at the University of Arizona High Performance Computing Center, on a Lenovo NeXTScale nx360 M5 supercomputer.

Funding: We acknowledge the support of the National Institutes of Health, Grant GM-068036.

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Figure 1.
Reaction mechanism of Kemp elimination.

Figure 2.
Crystal structures of the four mutations: R1-7/10H (a), R5-11/5F (b), R8-2/7A (c), R13-3/11H (d). Mutations are highlighted in red, reaction-involved residues and the substrate are shown explicitly.

Figure 3.
Selected quantum region in this study, important atoms involved in reactions are labeled.

Figure 4.

Committor distributions of all ensembles. 1(a)–5(a) are committor distributions for R1, R5, R8, R13-OE1, and R13-OE2, respectively, with only quantum region under constraint. 1(b)–5(b) are committor distributions for these five ensembles with the reaction coordinate residues under constraint.

Figure 5.

Distance-time series of representative trajectories from all five ensembles: R1 (a), R5 (b), R8 (c), R13-OE1 (d), and R13-OE2 (e). A total of five important atom-atom distances are shown on each figure: donor (C9)-hydrogen (H13) in black, acceptor (OEX)-hydrogen (H13) in red, ring-open (N8-O12) in green, donor (C9)-acceptor (OEX) in blue, distance between the substrate and residues possibly involved in a RPV (I178 carbon γ C9 for R1, R8; I178 carbon δ 1 C9 for R13-OE1 and R13-OE2; V51 carbon γ 2 E231 carbon γ distance for R5) in orange.

Figure 6. “Platform” residues involved in the reaction coordinate and the quantum region: I133 and V159 are in red, I178 is in orange, the substrate and E231 are in blue. Two representative structures are taken from R1 ensemble: the system at 200 fs before the hydrogen transfer reaction (a) and at the hydrogen transfer reaction (b). The next two are taken from R13-OE2 ensemble, 200 fs before the hydrogen transfer reaction (c) and at the hydrogen transfer reaction (d). Pushing motion from residue I178 to the substrate is represented by the solid black arrow in panel c). Effect of the pushing motion on the substrate is represented by the dashed black arrow in panel d). Reaction process in atomistic detail of R1 ensemble, before the reaction (e) and at the transition state (f).

Table 1
Average Time Duration of the Ring-Open Step and Time Lag between the Two Steps

system	R1	R5	R8	R13/OE1	R13/OE2
average time duration of bond breaking ^a (fs)	40 ± 19	27 ± 12	20 ± 8	25 ± 12	23 ± 18
average time lag ^b (fs)	34 ± 16	-18 ± 9	-15 ± 7	-2 ± 13	-31 ± 11

^aTime duration is counted as the time length during which the N–O distance is between 1.5 and 2.5 Å and the change of N–O distance is smaller than 0.1 Å/fs.

^bTime lag is counted as the time difference between the last slice of the previously defined intermediate state, and the slice when the hydrogen transfer takes place. A negative time lag indicates that the hydrogen transfer step takes place before the end of the bond-breaking intermediate state. Further, if the absolute value of a negative time lag is larger than that of the time duration, then in this trajectory the hydrogen transfer step takes place before the start of the bond-breaking intermediate state.

Table 2**Important Distances at the Transition State**

system	R1	R5	R8	R13/OE1	R13/OE2
hydrogen-acceptor (Å)	1.91 ± 0.17	1.23 ± 0.05	1.24 ± 0.05	1.52 ± 0.13	1.08 ± 0.06
hydrogen-donor (Å)	1.17 ± 0.05	1.39 ± 0.07	1.39 ± 0.06	1.25 ± 0.08	1.52 ± 0.12
donor-acceptor (Å)	2.97 ± 0.12	2.52 ± 0.03	2.60 ± 0.03	2.73 ± 0.08	2.55 ± 0.10
ring-open ^a (Å)	2.17 ± 0.02	1.80 ± 0.03	1.89 ± 0.05	1.92 ± 0.09	1.68 ± 0.03

^aSince the N8–O12 distance only fluctuates slightly during the intermediate state, the distance at the transition state can be used to represent the N8–O12 distance during the intermediate state.

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Table 3

Residues Involved in the Reaction Coordinate of Each Ensemble

system	R1	R5	R8	R13-OE1	R13-OE2
residues	I178, V159	I178, V159, I133, V51, V210	I178, V159, I133	I178, V159	I178, V159