

Enhancing ω -transaminase catalysis of bulky substrates guided by binding mechanism

Corresponding Author: Professor Gang Xu

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

This study systematically investigates the trade-off between channel bottlenecks, substrate binding stability, and stereoselectivity in ω -transaminases, addressing the limitation of catalytic efficiency toward macromolecular substrates. Combining free energy calculations, molecular dynamics simulations, and directed evolution experiments, the research holds significant theoretical value and engineering implications.

To facilitate publication, revisions are recommended for certain aspects. Specific suggestions are as follows:

1. Authors frequently designate specific channel segments as “rate-limiting regions” based on energy barriers within the free energy surface (FES). The FES reconstructed via PCV-metadynamics fundamentally reflects free energy changes during substrate entry and binding, not the true rate-determining steps of the entire catalytic cycle (e.g., chemical steps or product release). It is recommended to clarify whether “rate-limiting” is restricted to potential rate-limiting factors in the substrate entry/binding stage, rather than the macroscopic rate-determining step of the entire catalytic reaction.
2. The authors propose that channel expansion can alleviate substrate entry hindrance but may weaken binding stability or even induce stereoselective flipping. These phenomena are presented in parallel without distinguishing their scale or stage of occurrence. It is recommended to differentiate which types of channel expansion primarily affect substrate accessibility, which impact binding conformation stability, and which regions are highly coupled with stereoselectivity.
3. The paper suggests that DCCN analysis indicates distal residues can regulate channel and active site structures via conformational coupling. However, DCCN fundamentally reflects motion correlations rather than causality. We recommend revising this statement.
4. In analyzing multiple variants, the authors propose that the binding conformation with the lowest free energy is unfavorable for reaction geometry, leading to reduced catalytic efficiency. While conceptually plausible, the current evidence primarily relies on static conformation comparisons. Consider supplementing with statistics on reaction conformation distribution over time to demonstrate that this is not merely “the presence of an unfavorable conformation,” but rather one with significant temporal prevalence.
5. The authors primarily interpret the channel bottleneck structurally based on local minima conformations within the free energy surface. Strictly speaking, however, channel bottlenecks typically correspond to energy barrier regions rather than stable conformations themselves. It is recommended that the authors clarify in the text why local minima conformations were chosen for bottleneck analysis instead of conformations near the energy barrier.

Reviewer #3

(Remarks to the Author)

Teng et al. targeted the synthesis of chiral amine ABP from the sterically hindered substrate KBP, and successfully engineered an ω -transaminase from *Bacillus megaterium* (BmATA). The optimal mutant 13M exhibited a 10^6 -fold increase in catalytic activity compared with the wild-type (WT) enzyme, while maintaining an enantiomeric excess (e.e.) of >99%. The main innovation of this work does not lie in the proposed “computation–experiment integrated strategy” or the use of various simulation methods per se, but rather in the rational identification of potential mutation sites for this specific enzyme–substrate system through complementary simulation approaches, followed by systematic experimental validation. As a result, the authors obtained a highly active and highly selective transaminase and achieved the enzymatic synthesis of ABP for the first time. It is therefore recommended that the authors revise the manuscript to highlight this core innovation. In addition, the following points are provided for the authors’ reference:

1. The rationale for selecting the 8 key residues is insufficiently justified. The selection relied solely on “steric hindrance / channel bottleneck” considerations, while neglecting other core determinants of enzyme catalysis, including the chemical step, enzyme stability, evolutionary conservation, and so on.
2. The activity improvement from mutant 6M to 13M is mainly reflected in the increase in k_{cat} . However, quantum chemical or energetic calculations are strongly recommended to provide mechanistic validation, which would strengthen the mechanistic interpretation. At present, the k_{cat} values are obtained only from wet experiments; they are apparent activities and cannot be directly correlated with the outcomes of rational design.
3. Although mutant 13M shows a dramatic improvement in activity, simulations indicate that its Bürgi–Dunitz angle (60° – 80°) still deviates from the ideal value of 107° , suggesting that the substrate-binding mode has not yet reached the theoretically optimal catalytic geometry.
4. The authors applied a biased potential to overcome the energy barrier of the substrate entry channel, enabling precise localization of the rate-limiting step, this represents a key advantage of the FM+PCV method. However, its limitation is also evident: the sampling of conformational space is not fully comprehensive. Although the authors performed various molecular dynamics simulations totaling approximately 1000 ns, all simulations initiated from the same starting conformation, leading to relatively high stochasticity. Furthermore, convergence evidence for the free energy surfaces should be provided in the supplementary materials.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

The authors have adequately addressed all my previous comments and revisions. The manuscript has been well revised with no remaining scientific and linguistic issues. I hereby recommend accepting this manuscript for publication.

Reviewer #3

(Remarks to the Author)

The authors have provided comprehensive, rigorous, and convincing responses. The revised manuscript addresses all the points I asked in the previous review round. In particular, the authors have supplemented a substantial amount of computational simulations and analyses, and have thoroughly rewritten the Abstract, Introduction, and Discussion sections. I believe the authors have fully addressed all my concerns, and the manuscript quality has been significantly elevated to meet the publication standard. I recommend publication in present form.

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Reviewer #2:

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To facilitate publication, revisions are recommended for certain aspects. Specific suggestions are as follows:

Comment 1: Authors frequently designate specific channel segments as “rate-limiting regions” based on energy barriers within the free energy surface (FES). The FES reconstructed via PCV-metadynamics fundamentally reflects free energy changes during substrate entry and binding, not the true rate-determining steps of the entire catalytic cycle (e.g., chemical steps or product release). It is recommended to clarify whether “rate-limiting” is restricted to potential rate-limiting factors in the substrate entry/binding stage, rather than the macroscopic rate-determining step of the entire catalytic reaction.

Response: We appreciate the reviewer’s valuable comments. This is an important distinction that we had not sufficiently clarified in the original manuscript. To avoid any potential confusion with the macroscopic rate-determining step of the entire catalytic reaction, we have replaced “rate-limiting” with “high-energy barrier” and explicitly added “binding process” as a qualifier where ambiguity might arise. These revisions have been implemented in the corresponding sections (line 13, 105, 113, 123, 125, 131, 191, 194, 509, 549).

Comment 2: The authors propose that channel expansion can alleviate substrate entry hindrance but may weaken binding stability or even induce stereoselective flipping. These phenomena are presented in parallel without distinguishing their scale or stage of occurrence. It is recommended to differentiate which types of channel expansion primarily affect substrate accessibility, which impact binding conformation stability, and which regions are highly coupled with stereoselectivity.

Response: We appreciate the reviewer's valuable comments. This is an important point that we had not adequately addressed in the original manuscript. In the revised version, to enable a clearer distinction between the effects of channel expansion on substrate accessibility, binding conformation stability, and stereoselectivity, as suggested by the reviewer, we have systematically categorized the identified residues into five functional groups based on the scale and stage at which they exert their primary effects (Supporting Information, Fig. S3): (1) sites that widen the substrate tunnel without disrupting binding stability (magenta; residues 25, 88, 420, 442); (2) sites that optimize binding stability without severely compromising tunnel accessibility (magenta; residues 163, 327); (3) trade-off sites between tunnel widening and active-pocket binding stability (orange; residues 56, 59, 86, 423, 436); (4) sites that influence diastereoselectivity (blue; residues 25, 164); and (5) distal sites that modulate tunnel and pocket dynamics indirectly (red; residues 26, 90, 387, 440).

Comment 3: The paper suggests that DCCN analysis indicates distal residues can regulate channel and active site structures via conformational coupling. However, DCCN fundamentally reflects motion correlations rather than causality. We recommend revising this statement.

Response: We appreciate the reviewer's valuable comments. We agree that DCCN analysis fundamentally reflects motion correlations rather than causality. In the revised manuscript, we have carefully revised the relevant descriptions to accurately convey the correlative nature of DCCN analysis. The specific revisions are as follows: (1) Through dynamic cross-correlation network (DCCN) analysis, distal sites exhibiting significant correlated motions with the aforementioned key residues were identified and engineered, thereby avoiding the "tunnel-active pocket" trade-off associated with direct modification of key residues in the active pocket (line 126-129); (2) As shown in Fig. 4-g, the mean correlation of residues 87 and 94 with residue 90 decreased to 0 in 5M, indicating that the mutation of residue 90 has a significant effect on these two residues (line 327-328); (3) As shown in Fig. 6-i, the mean correlation of residues 323 and 324 changed significantly, indicating that the mutation of residue 26 has a significant effect on these two residues (line 495-497).

Comment 4: In analyzing multiple variants, the authors propose that the binding conformation

with the lowest free energy is unfavorable for reaction geometry, leading to reduced catalytic efficiency. While conceptually plausible, the current evidence primarily relies on static conformation comparisons. Consider supplementing with statistics on reaction conformation distribution over time to demonstrate that this is not merely “the presence of an unfavorable conformation,” but rather one with significant temporal prevalence.

Response: We appreciate the reviewer’s valuable comments. We agree that static conformation comparisons alone are insufficient to demonstrate the temporal prevalence of unfavorable binding conformations. To address this concern, we have provided time-evolution profiles of both the distance $d(\text{C}_{\text{LIG}}\text{-N}_{\text{PMP}})$ and the angle $\angle(\text{N}_{\text{PMP}}\text{-C}_{\text{LIG}}\text{-O}_{\text{LIG}})$ over the simulation trajectories in the Fig. S4 (Supporting Information). Additionally, we have quantified the population of the dominant conformation A for each variant, along with the corresponding mean values and standard deviations of $d(\text{C}_{\text{LIG}}\text{-N}_{\text{PMP}})$ and $\angle(\text{N}_{\text{PMP}}\text{-C}_{\text{LIG}}\text{-O}_{\text{LIG}})$ (Fig. S2-d), enabling a more detailed comparison of reaction geometry prevalence across variants.

Comment 5: The authors primarily interpret the channel bottleneck structurally based on local minima conformations within the free energy surface. Strictly speaking, however, channel bottlenecks typically correspond to energy barrier regions rather than stable conformations themselves. It is recommended that the authors clarify in the text why local minima conformations were chosen for bottleneck analysis instead of conformations near the energy barrier.

Response: We appreciate the reviewer’s valuable comments. We agree that channel bottlenecks more rigorously correspond to energy barrier regions rather than stable conformations at local minima. In the revised manuscript, we have addressed this concern in two ways. First, we have included conformations near the high-energy barriers in the bottleneck analysis for both WT and 13M, in addition to the local minima conformations, thereby providing a more complete characterization of the channel bottleneck regions. Second, to enhance the reliability of the path metadynamics results, we performed simulations along three independent routes for each variant and identified the common high-energy barrier regions across these routes for analysis (Fig. 3, 5&7; line 207-242, 343-367, 510-528). This approach ensures that the identified bottlenecks reflect robust features of the free energy landscape rather than artifacts of a single simulation path.

Reviewer #3:

Comment 1: Teng et al. targeted the synthesis of chiral amine ABP from the sterically hindered substrate KBP, and successfully engineered an ω -transaminase from *Bacillus megaterium* (BmATA). The optimal mutant 13M exhibited a 106-fold increase in catalytic activity compared with the wild-type (WT) enzyme, while maintaining an enantiomeric excess (e.e.) of >99%. The main innovation of this work does not lie in the proposed “computation–experiment integrated strategy” or the use of various simulation methods per se, but rather in the rational identification of potential mutation sites for this specific enzyme–substrate system through complementary simulation approaches, followed by systematic experimental validation. As a result, the authors obtained a highly active and highly selective transaminase and achieved the enzymatic synthesis of ABP for the first time. It is therefore recommended that the authors revise the manuscript to highlight this core innovation.

Response: We appreciate the reviewer’s valuable comments. Following this suggestion, we have substantially revised the Abstract (lines 7-20), Introduction (lines 26-91), and Conclusion (lines 539-558) to shift the emphasis from the computational-experimental strategy toward the core innovation of this work: the rational identification of mutation sites specific to the BmATA-KBP system through complementary simulations and the successful engineering of a highly active and enantioselective ω -TA for the first efficient enzymatic synthesis of ABP.

Comment 2: The rationale for selecting the 8 key residues is insufficiently justified. The selection relied solely on “steric hindrance / channel bottleneck” considerations, while neglecting other core determinants of enzyme catalysis, including the chemical step, enzyme stability, evolutionary conservation, and so on.

Response: We sincerely appreciate the reviewer’s insightful comments, which prompted us to more thoroughly justify our residue selection strategy. We acknowledge that the original manuscript focused primarily on steric hindrance and tunnel bottleneck considerations without adequately addressing other potential determinants of enzyme catalysis. In the revised manuscript, we have incorporated MM/GBSA binding free energy calculations and comparisons with reported ω -TA mutants capable of catalyzing bulky substrates, demonstrating that BmATA WT already adopts a substrate-binding conformation favorable for catalysis. Given that its binding affinity and

catalytic distances are comparable to or better than those of experimentally validated ω -TA mutants, the chemical step alone is unlikely to account for the observed low activity; instead, the narrow access tunnel likely represents the more critical bottleneck. In addition, we provide here in this response letter a complementary analysis of evolutionary conservation and enzyme stability to offer the reviewer a more comprehensive picture of our rationale. While PSSM-based conservation analysis provided informative guidance, it was not sufficient to replace experimental validation. Several experimentally beneficial mutations occurred at positions that would have been excluded by stringent conservation-based pre-screening, underscoring the need for experimental validation. Regarding stability, current computational methods for predicting mutational effects on protein stability still suffer from limited accuracy, and imposing stability-based filters at the residue selection stage could prematurely exclude mutationally tolerant positions. We therefore adopted a sequential optimization strategy, prioritizing catalytic activity through tunnel engineering while deferring stability optimization to a subsequent, independent phase. Collectively, these analyses support our rationale that tunnel accessibility represents the primary bottleneck for BmATA WT catalysis of KBP. The detailed justification is elaborated from three perspectives below:

Regarding the chemical step: To evaluate the stability of KBP binding in the WT active pocket and whether its conformation is favorable for catalysis, we calculated MM/GBSA¹ and compared the results with the best mutants reported in two other studies on (R)- and (S)-selective ω -TAs catalyzing bulky substrates (Table S2)^{2,3}. These studies used MM/P(G)BSA binding energies and the nucleophilic distances between the substrate and PMP ($d(\text{C}_{\text{LIG}}-\text{N}_{\text{PMP}})$ or $d(\text{O}_{\text{LIG}}-\text{N}_{\text{PMP}})$) as descriptors of catalytic competence. The MM/GBSA binding free energy of KBP to BmATA wild-type (WT) was -50.69 ± 4.3 kcal/mol, significantly lower than that of the RbTA mutant Y125A (-33.64 kcal/mol, MM/GBSA) and the BPTA mutant M78F/W82A/I284F/T440Q (-36.33 kcal/mol, MM/PBSA), indicating a stronger binding affinity of BmATA WT toward KBP. Furthermore, in the BmATA WT complex, the catalytically relevant key distances were $d(\text{C}_{\text{LIG}}-\text{N}_{\text{PMP}}) = 3.8 \pm 0.27$ Å and $d(\text{O}_{\text{LIG}}-\text{N}_{\text{PMP}}) = 3.1 \pm 0.29$ Å, substantially shorter than those in the BPTA mutant ($d(\text{C}_{\text{LIG}}-\text{N}_{\text{PMP}}) = 4.86$ Å) and the RbTA mutant Y125A ($d(\text{O}_{\text{LIG}}-\text{N}_{\text{PMP}}) < 3.5$ Å). The comparison of these two parameters indicates that BmATA WT already adopts a substrate-binding conformation favorable

for catalysis. However, this is inconsistent with the experimentally observed low specific activity (0.0065 ± 0.0012 U/mg). Given that the BmATA WT exhibits binding affinity and catalytic distances comparable to or better than those of experimentally validated mutants of ω -TAs, the chemical step alone is unlikely to account for this low activity. Instead, the narrow access tunnel likely imposes steric hindrance on substrate entry, representing the more critical bottleneck for catalysis. (line 157-175)

Regarding evolutionary conservation: In fact, we had already constructed a PSSM matrix for BmATA prior to experimental screening (score range: -6 to 6, with higher scores indicating stronger conservation). The PSSM scores for the eight selected channel residues are as follows: 25F (2), 56F (1), 59L (6), 86W (5), 164Y (1), 420Q (0), 436V (3), and 442R (2). Among these, residues 59L (6) and 86W (5) exhibit strong conservation, while 56F (1) is moderately conserved. Based solely on conservation analysis, these three positions would appear unsuitable for mutation. However, we contend that conservation-based predictions alone are insufficient to replace experimental validation. Without wet-lab data, it remains difficult to determine whether these conserved positions are truly immutable, or whether their functional roles can be compensated through indirect effects of distal mutations.

This reasoning has been validated by multiple studies. Mandecki et al. mutated the absolutely invariant active-site residue Asp101 in *Escherichia coli* alkaline phosphatase to serine, resulting in a 35-fold increase in specific activity, demonstrating that even completely conserved residues can yield substantial catalytic improvements upon mutation⁴. Similarly, Staar et al. systematically engineered three conserved residues in the halohydrin dehalogenase HheC and found that the conserved phenylalanine at position 12 tolerated multiple mutations; in particular, the F12Y variant exhibited up to a 5-fold increase in specific activity and enhanced enantioselectivity (E value increased from 22 for the wild type to 74)⁵. In the field of P450 engineering, mutation of the highly conserved active-site T268 in P450-BM3 significantly improved the yield and stereoselectivity of the cyclopropanation of styrene⁶. These examples collectively demonstrate that high conservation does not render a residue immutable. Conservation reflects the outcome of natural evolution optimized for the native function. When engineering objectives (such as

expanding the substrate scope or altering reaction types) diverge from the enzyme's native function, conserved positions can equally give rise to beneficial mutations. Accordingly, we chose to include all eight tunnel residues in experimental screening, allowing the experimental outcomes to determine the mutability of each site.

Notably, our experimental results retrospectively validated this decision. On the one hand, mutants at the strongly conserved positions 59L and 86W indeed failed to yield activity improvements, consistent with conservation-based predictions. On the other hand, the beneficial mutations identified at positions 25, 164, and 436 all shifted the PSSM scores toward less conserved values ($\Delta\text{PSSM} = \text{mutant} - \text{wild type}$: -6, -1, and -4, respectively), indicating that a stringent conservation-based pre-screening would have prematurely excluded these successful mutations. Taking these considerations together, we prioritized tunnel bottleneck analysis for identifying key residues and relied on experimental screening to determine their mutability in this study.

Regarding enzyme stability: We did not incorporate computational stability predictions as a pre-screening criterion during residue selection. This is because current computational methods for predicting mutational effects on protein stability — whether physics-based approaches (e.g., Rosetta) or data-driven methods (e.g., ThermoMPNN) — still suffer from limited accuracy and elevated false-positive rates. For instance, on the S669 benchmark dataset, the Pearson correlation between ThermoMPNN predictions and experimental $\Delta\Delta G$ values is only 0.43, while that of Rosetta is 0.39^{7,8}. Incorporating stability-based filtering criteria at the tunnel residue selection stage could prematurely exclude positions that are in practice mutationally tolerant, thereby causing potentially beneficial catalytic variants to be overlooked. Furthermore, given the limited accuracy of stability predictions, simultaneously optimizing both tunnel accessibility and protein stability would substantially expand the dimensionality of the design space, rendering rational prioritization impractical. We therefore adopted a sequential optimization strategy, prioritizing catalytic activity enhancement through tunnel engineering while deferring stability optimization to a subsequent, independent phase, thereby decoupling the two design objectives and circumventing the complexity associated with multi-property simultaneous optimization.

Comment 3: The activity improvement from mutant 6M to 13M is mainly reflected in the increase in k_{cat} . However, quantum chemical or energetic calculations are strongly recommended to provide mechanistic validation, which would strengthen the mechanistic interpretation. At present, the k_{cat} values are obtained only from wet experiments; they are apparent activities and cannot be directly correlated with the outcomes of rational design.

Response: We appreciate the reviewer's valuable comment. To provide mechanistic validation beyond apparent kinetic parameters, we performed additional computational analyses. Specifically, we performed an additional 300 ns MD simulation for 6M and calculated the MM/GBSA binding free energies for WT, 6M, and 13M (Supporting Information, Fig. S2). Furthermore, to compare the attack angle $\angle(\text{N}_{\text{PMP}}\text{-C}_{\text{LIG}}\text{-O}_{\text{LIG}})$ between 6M and 13M more rigorously, we carried out 400 ns metadynamics simulations for each variant using $d(\text{C}_{\text{LIG}}\text{-N}_{\text{PMP}})$ and $\angle(\text{N}_{\text{PMP}}\text{-C}_{\text{LIG}}\text{-O}_{\text{LIG}})$ as collective variables, enabling us to characterize the accessible range of attack angles at near-bonding distances ($d(\text{C}_{\text{LIG}}\text{-N}_{\text{PMP}}) \leq 2.8 \text{ \AA}$). By integrating the analysis of binding geometry, conformational population, and energetic stability, we provide a mechanistic rationale for the activity improvement from 6M to 13M that goes beyond k_{cat} values alone.

The detailed discussion is presented below: Analysis of Fig. S2-d shows that the dominant conformation A of 6M exhibits $d(\text{C}_{\text{LIG}}\text{-N}_{\text{PMP}}) = 3.90 \pm 0.16 \text{ \AA}$ and $\angle(\text{N}_{\text{PMP}}\text{-C}_{\text{LIG}}\text{-O}_{\text{LIG}}) = 41.8 \pm 3.48^\circ$ (proportion = 25.77%), whereas that of 13M displays a significantly shorter distance ($3.27 \pm 0.09 \text{ \AA}$) and a substantially larger angle ($81.98 \pm 3.79^\circ$), with its proportion increasing to 36.3%. MM/GBSA calculations corroborate this trend: the binding free energy decreases from $-29.25 \pm 6.3 \text{ kcal/mol}$ (6M) to $-32.03 \pm 2.56 \text{ kcal/mol}$ (13M). Thus, compared with 6M, 13M not only adopts a more catalytically competent binding geometry, but also populates this reactive conformation more frequently.

Although the $\angle(\text{N}_{\text{PMP}}\text{-C}_{\text{LIG}}\text{-O}_{\text{LIG}})$ of 13M ($81.98 \pm 3.79^\circ$) is considerably larger than that of 6M ($41.8 \pm 3.48^\circ$), it remains well below the ideal Bürgi-Dunitz angle (107°). However, this comparison is not entirely appropriate: the Bürgi-Dunitz angle is defined at incipient bond formation ($\sim 2.5 \text{ \AA}$), whereas the dominant conformation A of 13M still has $d(\text{C}_{\text{LIG}}\text{-N}_{\text{PMP}}) =$

3.27±0.09 Å, far from the bonding distance. To assess the attack angle more accurately at near-bonding distances, we performed 400 ns metadynamics simulations for both 6M and 13M using $d(\text{C}_{\text{LIG}}\text{-N}_{\text{PMP}})$ and $\angle(\text{N}_{\text{PMP}}\text{-C}_{\text{LIG}}\text{-O}_{\text{LIG}})$ as collective variables (Fig. S13-S15). Block analysis (Fig. S13-c & S14-c) indicates that neither simulation achieved full convergence. However, our key conclusion does not depend on precise free energy values but rather on the geometric characterization of sampled conformations at near-bonding distances ($d(\text{C}_{\text{LIG}}\text{-N}_{\text{PMP}}) \leq 2.8$ Å).

The FES (Fig. S13-a & S14-a) show that once $d(\text{C}_{\text{LIG}}\text{-N}_{\text{PMP}})$ falls below 2.8 Å, $\angle(\text{N}_{\text{PMP}}\text{-C}_{\text{LIG}}\text{-O}_{\text{LIG}})$ becomes confined to approximately 1-2 rad (57-115°). We therefore extracted all frames satisfying $d(\text{C}_{\text{LIG}}\text{-N}_{\text{PMP}}) \leq 2.8$ Å and plotted the corresponding angles as scatter plots (Fig. S15). The mean attack angles are nearly identical: 91.88±12.21° (6M) versus 91.87±12.17° (13M), indicating that the two variants share the same approach geometry at near-bonding distances. Moreover, when $d(\text{C}_{\text{LIG}}\text{-N}_{\text{PMP}}) \leq 2.6$ Å (dashed box in Fig. S15), the attack angle for both variants narrows to 85-110°, a range that encompasses the Bürgi-Dunitz angle (107°). This confirms that the approach trajectory of both variants is geometrically competent for bond formation. The critical difference lies in accessibility: out of 400,000 total frames, 13M reaches $d(\text{C}_{\text{LIG}}\text{-N}_{\text{PMP}}) \leq 2.8$ Å in 13,701 frames, compared to only 2,855 frames for 6M (about 4.8-fold increase). Taken together, these results demonstrate that while both variants can achieve a catalytically viable attack angle, 13M accesses the reactive near-attack conformation far more readily. (lines 429-459)

Comment 4: Although mutant 13M shows a dramatic improvement in activity, simulations indicate that its Bürgi-Dunitz angle (60°-80°) still deviates from the ideal value of 107°, suggesting that the substrate-binding mode has not yet reached the theoretically optimal catalytic geometry.

Response: We appreciate the reviewer's valuable comment. We acknowledge that the Bürgi-Dunitz angle measured from the dominant conformation of 13M in conventional MD (81.98±3.79°) does fall below the ideal 107° (Fig. S2-d). However, as we now discuss in the revised manuscript (lines 437-459), this comparison requires careful interpretation: the Bürgi-Dunitz angle is formally defined at incipient bond formation (~2.5 Å), whereas the dominant conformation of 13M maintains $d(\text{C}_{\text{LIG}}\text{-N}_{\text{PMP}}) = 3.27\pm0.09$ Å, well beyond the bonding

distance. To address this concern rigorously, we performed 400 ns metadynamics simulations for both 6M and 13M using $d(\text{C}_{\text{LIG}}\text{-N}_{\text{PMP}})$ and $\angle(\text{N}_{\text{PMP}}\text{-C}_{\text{LIG}}\text{-O}_{\text{LIG}})$ as collective variables (Fig. S13-S15). By extracting all frames where $d(\text{C}_{\text{LIG}}\text{-N}_{\text{PMP}}) \leq 2.8 \text{ \AA}$, we found that the mean attack angles of the two variants are essentially identical ($\sim 91.9^\circ$), and that when $d(\text{C}_{\text{LIG}}\text{-N}_{\text{PMP}})$ narrows further to $\leq 2.6 \text{ \AA}$, the attack angle converges to the $85\text{-}110^\circ$ range encompassing the ideal Bürgi-Dunitz value. This confirms that both variants are geometrically competent for bond formation at near-bonding distances.

The detailed discussion is presented below: Although the $\angle(\text{N}_{\text{PMP}}\text{-C}_{\text{LIG}}\text{-O}_{\text{LIG}})$ of 13M ($81.98 \pm 3.79^\circ$) is considerably larger than that of 6M ($41.8 \pm 3.48^\circ$), it remains well below the ideal Bürgi-Dunitz angle (107°). However, this comparison is not entirely appropriate: the Bürgi-Dunitz angle is defined at incipient bond formation ($\sim 2.5 \text{ \AA}$), whereas the dominant conformation A of 13M still has $d(\text{C}_{\text{LIG}}\text{-N}_{\text{PMP}}) = 3.27 \pm 0.09 \text{ \AA}$, far from the bonding distance. To assess the attack angle more accurately at near-bonding distances, we performed 400 ns metadynamics simulations for both 6M and 13M using $d(\text{C}_{\text{LIG}}\text{-N}_{\text{PMP}})$ and $\angle(\text{N}_{\text{PMP}}\text{-C}_{\text{LIG}}\text{-O}_{\text{LIG}})$ as collective variables (Fig. S13-S15). Block analysis (Fig. S13-c & S14-c) indicates that neither simulation achieved full convergence. However, our key conclusion does not depend on precise free energy values but rather on the geometric characterization of sampled conformations at near-bonding distances ($d(\text{C}_{\text{LIG}}\text{-N}_{\text{PMP}}) \leq 2.8 \text{ \AA}$).

The FES (Fig. S13-a & S14-a) show that once $d(\text{C}_{\text{LIG}}\text{-N}_{\text{PMP}})$ falls below $2.8 \text{ \AA}$, $\angle(\text{N}_{\text{PMP}}\text{-C}_{\text{LIG}}\text{-O}_{\text{LIG}})$ becomes confined to approximately 1-2 rad ($57\text{-}115^\circ$). We therefore extracted all frames satisfying $d(\text{C}_{\text{LIG}}\text{-N}_{\text{PMP}}) \leq 2.8 \text{ \AA}$ and plotted the corresponding angles as scatter plots (Fig. S15). The mean attack angles are nearly identical: $91.88 \pm 12.21^\circ$ (6M) versus $91.87 \pm 12.17^\circ$ (13M), indicating that the two variants share the same approach geometry at near-bonding distances. Moreover, when $d(\text{C}_{\text{LIG}}\text{-N}_{\text{PMP}}) \leq 2.6 \text{ \AA}$ (dashed box in Fig. S15), the attack angle for both variants narrows to $85\text{-}110^\circ$, a range that encompasses the Bürgi-Dunitz angle (107°). This confirms that the approach trajectory of both variants is geometrically competent for bond formation. The critical difference lies in accessibility: out of 400,000 total frames, 13M reaches $d(\text{C}_{\text{LIG}}\text{-N}_{\text{PMP}}) \leq 2.8 \text{ \AA}$ in 13,701 frames, compared to only 2,855 frames for 6M (about 4.8-fold increase). Taken together, these

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Comment 5: The authors applied a biased potential to overcome the energy barrier of the substrate entry channel, enabling precise localization of the rate-limiting step, this represents a key advantage of the FM+PCV method. However, its limitation is also evident: the sampling of conformational space is not fully comprehensive. Although the authors performed various molecular dynamics simulations totaling approximately 1000 ns, all simulations initiated from the same starting conformation, leading to relatively high stochasticity. Furthermore, convergence evidence for the free energy surfaces should be provided in the supplementary materials.

Response: We appreciate the reviewer's valuable comments. To mitigate the stochasticity inherent in initiating simulations from a single starting conformation, we performed path metadynamics simulations along three independent routes for each variant and identified the common high-energy barrier regions across these routes for analysis (Fig. 3, 5&7; line 207-242, 343-367, 510-528). By cross-validating the barrier locations across independent pathways, we ensure that the identified rate-limiting regions are robust rather than artifacts of a particular initial trajectory. Furthermore, we conducted block analysis for all path metadynamics simulations (Supporting Information, Fig. S11). All nine simulations (three independent routes per variant) exhibit clear plateau behavior at large block sizes, indicating sufficient sampling and convergence of the free energy estimates.

Please find the re-submitted manuscript. We hope that our revisions will be suitable, and we are looking forward to hear a positive response from you. Thank you again for providing us an opportunity to resubmit our manuscript. If you have any queries, please feel free to contact us.

Sincerely Yours

Prof. Gang Xu

References

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