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Multiscale Modelling of Enzymatic Efficiency in Multienzyme Complex: Effect of Inter-enzyme Distance, Crowding Effect and Side Reactions

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Abstract

Molecular association is one of the most fundamental principles of chemistry and biology. Biomolecular complexes need to co-localize sequential catalytic and chemical processes such that the intermediates are precisely positioned to diffuse efficiently. The relative distance and orientation between the active sites within these complexes may affect the intermediate binding, which may also be different under different cell environments. In this work, we used Brownian dynamics simulations to model an intermediate substrate association in the tryptophan biosynthesis pathway in *Escherichia coli* (*E. coli*) for investigating potential advantages of using TrpCF bifunctional enzyme which fuses phosphoribosyl anthranilate isomerase (PRAI) and indoleglycerol phosphate synthase (IGPS). We focused on transporting a product of PRAI, 1-(o-carboxyphenylamino)-1-deoxyribulose 5-phosphate (CdRP), which is also the substrate of IGPS. We report the average association time and direct binding percentage when the substrate concentration is 10.0 μM as an estimated physiological concentration in cells. Our results suggest that final product synthesis efficiency can be achieved by optimizing the inter-enzyme distance and by properly aligning the orientation of the active sites. In addition, the intermolecular interactions between the substrate and macromolecule crowders largely affected the transportation of a substrate. The overall rate of product synthesis enhancement in a multienzyme complex depends on the distance between enzymes when the substrate leaking phenomenon exists. Our work highlights the importance of the enzyme spatial organization in regulating the production efficiency of a product in multi-enzyme complexes, broadens implications in synthesis processes under complex cell environments, and brings biophysical insights into the enzyme–substrate association and understanding of target ligand/substrate kinetics.

Introduction

In biological systems, multi-enzyme cascades have achieved enzymatic efficiency by controlling the relative catalytic sites and physical distance between different biomolecules¹⁻³. For a sequential chemical synthesis to produce a final product, nature has evolved multienzyme complexes that improve intermediate transportation, thereby increasing overall product synthesis rates. Notable examples include tryptophan (Trp) synthase (TRPS), polyketide synthase, 2-oxoacid dehydrogenase complexes such as pyruvate dehydrogenase complex (PDC), ketoglutarate dehydrogenase complex (OGDC), as well as cellulosomes⁴⁻¹⁰. Inspired by these natural systems, co-localizing multiple enzymes on a solid surface or forming multi-enzyme complexes are effective strategies in enzyme engineering, compound synthesis, and other commercial applications^{2,11-17}. In the cell environment, substrate-channeling in multi-enzyme complexes may further protect intermediate substrates from being used by other macromolecules¹⁸⁻²². Even in the absence of dedicated substrate channeling, in cells, enzyme proximity alone can enhance the enzyme-substrate association to accelerate final product synthesis.

In many bacteria and plants, the amino acid Trp is synthesized *de novo* with six catalytic steps in the Trp biosynthetic pathway (**Figure S1**). Because Trp production is essential for bacterial and plant survival, enzyme complexes and/or spatial arrangements have likely evolved to minimize intermediate loss and ensure the efficient synthesis of the final product. A well-studied example is TRPS, which uses substrate channeling to transport the intermediate indole, facilitating the final two enzymatic steps required for L-Trp synthesis¹⁰. In this pathway, the third and fourth steps are performed by phosphoribosyl anthranilate isomerase (PRAI) and indoleglycerol phosphate synthase (IGPS), respectively. In mesophilic *E. coli*, the two enzymes PRAI and IGPS are fused into a bifunctional enzyme complex, known as TrpCF²³, whereas in most other bacteria, PRAI and IGPS are separate^{18,24,25} (**Figure 1**). However, whether TrpCF confers an evolutionary advantage in enhancing IGP synthesis remains unclear. Previous studies showed that PRAI catalyzes the formation of CdRP at a rate approximately 10 times faster than IGPS converting CdRP into IGP^{26,27}. Consequently, unlike the unstable and slowly produced intermediate indole, the loss of some CdRP during the process might be less critical²⁸. Nevertheless, additional unknown factors may contribute to whether the fused enzyme has an evolutionary advantage, potentially enhancing overall Trp biosynthesis efficiency in the cellular context.

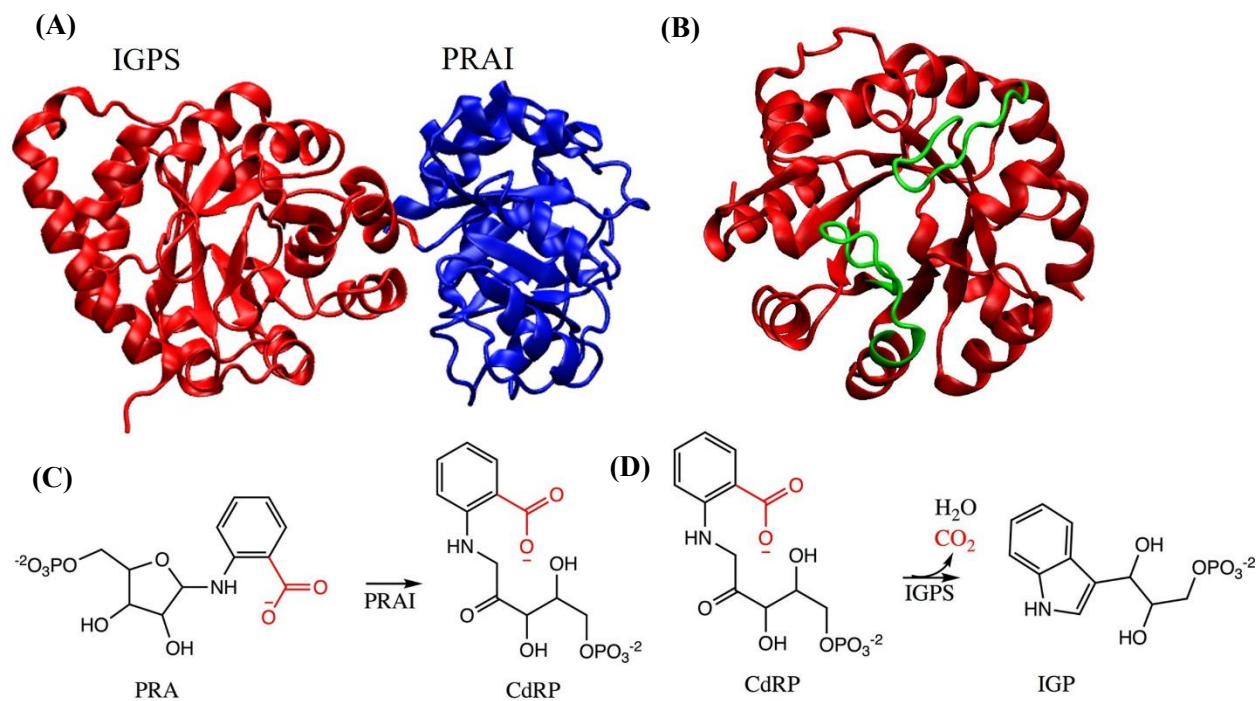


Figure 1. Structure and reaction steps of the bifunctional TrpCF protein in the tryptophan biosynthesis pathway. **(A)** *Escherichia coli* (*E. coli*), mesophile; bifunctional TrpCF fusion protein (PDB 1PII, *trpCF*), phosphoribosyl anthranilate isomerase (PRAI), blue; indoleglycerol phosphate synthase (IGPS), red. **(B)** $\beta\alpha$ -loop2 in IGPS shown in green (PDB ID: 1A53, obtained from PDB website²⁵). **(C)** 2D representation of the third step of chemical reaction. **(D)** Chemical reactions for the fourth step, in the tryptophan biosynthesis pathway.

Experimental techniques such as NMR spectroscopy and fluorescence assays have been used to investigate biomolecule diffusion in cell-like environments^{29–32}. These approaches can provide insights into large-scale molecular diffusion, and molecular modeling can further elucidate the underlying driving forces governing molecular encounters. Simulation techniques, such as atomistic molecular dynamics (MD) simulations, offer a cost-effective approach to study *in vivo* molecular diffusion while capturing the molecular recognition process and protein dynamics in atomic detail with explicit solvent models^{33–38}. However, large-scale and complex macromolecular crowding within the cellular environment poses a computational limitation for MD simulations. To overcome these challenges, Brownian dynamics (BD) simulations and numerical methods have been widely used for decades to study large-scale molecular encounters. BD simulations have modeled various ligand–receptor associations, rebinding kinetics, and gated ligand binding in various environments^{39–42}. For instance, the GeomBD3 program has been used to examine substrate diffusion between enzymes with specific surface and enzyme arrangements^{43–45}. This program has helped in exploring spatially organized macroscopic systems and quantifying the effects of increased overall product synthesis rates. Additionally, theoretical frameworks have been developed to numerically

model molecular encounters and compare kinetics in multi-enzyme complexes that catalyze multi-substrate reactions^{39,46,47}.

Molecular modeling studies have been conducted to understand why nature often co-localizes multi-enzymes involved in sequential catalytic steps within metabolic pathways. Using BD simulations, studies have modeled two enzyme spheres with oppositely charged enzymes and substrates, demonstrating that the reaction success strongly depends on the relative orientation and distance between the active sites⁴⁸. In addition, studies have highlighted the significant impact of viscosity on molecular diffusion. The product synthesis enhancement observed in multi-enzyme constructs on scaffolds is largely attributed to the “proximity effect”^{11,13,17,49–53}. Beyond proximity, several other factors, including pH near binding sites, protein dynamics within the multi-enzyme complexes, macromolecular crowding, and side reactions, may also contribute to the overall product synthesis efficiency^{54–57}. These effects are often system-dependent, and further studies across different systems will enhance our fundamental understanding of biosynthetic pathways, providing valuable insights for protein engineering and enzyme complex design.

In this study, we used multi-scale simulations, combining accelerated MD (aMD) and BD, to examine factors contributing to the substrate association of TrpCF, the PRAI:IGPS fused protein, and the separated PRAI and IGPS, to understand the advantage of the TrpCF complex and address diffusion and encounter mechanism in test-tube and cell-like environments. We began with modeling the substrate CdRP association with IGPS by using aMD and analyzed the dynamics of the protein in the fused complex PRAI:IGPS. To understand the effects of the fused enzymes on substrate association, we performed BD simulations with different inter-enzyme distances to investigate CdRP association time. We monitored the relative orientation of the catalytic sites of the two enzymes. We also included crowders in the system with different inter-enzyme distances. Additionally, we investigated the effects of competing side reactions in a cell-like environment, mimicking conditions in which many intermediates may be degraded during the transportation process. (**Figure S2**, schematic representation of the different BD simulation environments)

Methods

Structure preparation and MD simulations

The crystal structure of the TrpCF fusion enzyme of *E. coli* (PDB ID: 1PII) was obtained from the PDB website²³. Because the TrpCF fusion enzyme structure used in this study lacked a bound substrate, we aligned the CdRP-bound PRAI complex (from hyperthermophile *Thermotoga maritima*, *T. mari*, PDB ID:

1LBM)²⁴ with TrpCF to position the CdRP substrate within the PRAI catalytic site. The AM1-BCC charge model was used to calculate partial atomic charges of CdRP. Simulations involved using the ff14SB force field⁵⁸ for the enzymes and GAFF2⁵⁹ for the substrate in the molecular simulation package AMBER20⁶⁰.

Sequential minimization steps were performed for the hydrogen atoms, side chains and the entire system complexes to remove any clashes, following a standard AMBER protocol⁶¹. The system was solvated in a TIP3P water box⁶² with a 12-Å padding from the edge of the box to the whole system. We added 14-Na⁺ ions to neutralize the system in the water box. Heating from 50 K to 300 K with an increment of 25 K was achieved in 200 ps (time step of 2 fs) for each temperature. The system was subsequently equilibrated at 300 K for 500 ps with all atoms relaxed. Long-range electrostatic (EEL) interactions were computed by using the particle mesh Ewald method⁶³ and constraint was applied to all atoms including hydrogens⁶⁴. A 10 Å cutoff was used for short range nonbonded and van der Waals interactions. Pressure was maintained at 1 atm using the Berendsen and the Langevin thermostat with a damping constant of 2 ps⁻¹ was used to maintain a temperature of 300 K. The NPT ensemble was used for MD production runs for 400 ns, and each frame was collected at a 1-ps time interval.

We conducted aMD simulations using different levels of alpha boost potential ($\alpha=0,1,2,3,4,6$; **see Table S1 for details**)⁶⁵ to investigate substrate dissociation pathways and to sample enzyme conformational arrangements, providing dissociated states to serve as starting configurations for our BD simulations. The simulation time step and trajectory frame interval were the same as the classical MD mentioned above.

BD simulations

The $\beta\alpha$ -loop2 in IGPS (**Figure 1B**) plays an important role in substrate binding within the active site. The closed conformation prevents the substrate binding according to previous studies⁶⁶⁻⁶⁸. The TrpCF crystal structure has a closed conformation, whereby the binding pocket in IGPS is not accessible by the substrate (see **Figure S3** for close and open conformation). Therefore, we selected a structure with an open loop conformation sampled from aMD simulations (aMD index 29 in **Table S1**) for BD simulations. As shown in **Figure 2A**, the centers of mass of the two enzymes were used for distance measurements. The separation of the two enzymes was defined along the x-axis by aligning their centers of mass. Initially, the enzymes were positioned 35 Å apart. Subsequently, IGPS was incrementally displaced relative to PRAI by 50 Å, 100 Å, and 150 Å along the same axis. The orientation of the enzyme catalytic sites was kept unchanged in this set up. To mimic a cell-like environment, 19 ribonucleases (PDB ID 4IBN)⁶⁹ were introduced as crowding agents since they are the most abundant macromolecules in bacteria⁷⁰. They possess active site

motifs and a concave binding groove that facilitates substrate binding⁶⁹, making them a suitable model for studying crowder effects. The crowders were held rigidly within a spherical volume, occupying 20% (4.53 g/L) of the total simulation space.

To simulate conditions in which substrates are consumed in a cell-like environment, such as competitive site reactions, we selected four crowder molecules with termination sites. Which means in the BD simulations, the run was terminated once the substrate interacted within 8 Å of the catalytic residue Asp 96 (**Figure S4A**), at which point the substrate was considered consumed. Two of these crowders were positioned approximately 100 Å from the PRAI starting point, and the other two were placed about 200 Å away (**Figure 2B**, indicated by orange circles).

To investigate the impact of structural orientation when two catalytic sites either face each other or are positioned further apart (**Figure 2C**), we adjusted the relative orientations of the two enzymes. Each enzyme's catalytic site was rotated in opposite directions at 90-degree intervals, creating six distinct simulated systems within a cell-like environment.

The GeomBD3 package was used to carry out BD simulations⁷¹. The proteins and substrate are treated as rigid bodies and the simulation temperature was 298.15 K. The simulation space, depicted in **Figure 3** (blue sphere), was spherical, with a radius of 341 Å, corresponding to an estimated concentration of 10.0 μM in cells. The ionic strength was set at 0.1 M NaCl, and the simulation time step was 0.05 ps. Substrate binding was deemed successful when the oxygen atom of CdRP was within 8.0 Å of residue K55 in IGPS (**Figure S4B**), at which point the simulation was terminated. K55 residue is one of the important residues in the catalytic site of the enzyme and it forms hydrogen bonds with the hydroxyl oxygens of the substrate⁷². Based on the experimental condition, pH ≈ 7.0, the total charges were as follows: CdRP carried a charge of -3.0, the fusion enzyme TrpCF a charge of -11.0, PRAI a charge of -7.0, and IGPS a charge of -4.0. The crowder enzyme ribonuclease carried a charge of +2.0.

GeomBD3 utilized pre-computed grid representations for the enzymes' excluded volume potential, screened EEL potential, van der Waals (vdW) potential, and desolvation potential. The grids were extended to 15 Å for the vdW potential and 40 Å for the screened electrostatic potential to accommodate substrate diffusion. Substrate diffusion was modeled by using an implicit solvent approach with the overdamped Langevin equation which is coded in the package:

$$r_i(t + \Delta t) = r_i(t) - \frac{D_i}{k_b T} \frac{\partial E}{\partial r_i} \Delta t + \sqrt{2D_i \Delta t} R \quad (1)$$

Where D_i is the translation/rotational diffusion coefficient is calculated based on Stock-Einstein equation (equation (2)), K_b is Boltzmann's constant 1.380×10^{-23} J/k, and T is the simulation temperature which in our setups is 298.15 k. $\partial E / \partial r_i$ is the potential gradient, Δt is the time step, and R is a stationary Gaussian random number with a zero mean.

Diffusion coefficient

The diffusion coefficient was calculated using 500 ns of the entire trajectory as the substrate diffused in the solvent. In GeomBD3 package, Stock-Einstein equation is used to calculate translational/rotational diffusion coefficient of the substrate.

Transnational:

$$\frac{K_b T}{6\pi\eta} \left(\frac{1}{R_{CdRP}} \right) \quad (2)$$

Rotational:

$$\frac{K_b T}{8\pi\eta} \left(\frac{1}{R_{CdRP}^3} \right) \quad (3)$$

K_b is Boltzmann's constant 1.380×10^{-23} J/k, and T is the simulation temperature which in our setups is 298.15 k. η is the water viscosity, 8.9×10^{-4} pa.s, in the 298.15 k. R is the hydrodynamic radius of the substrate. If no hydrodynamic radius is provided, the hydrodynamic radius of ligand is approximated as the radius of gyration of the molecules.

In the BD simulations, the substrate diffusion coefficient was calculated internally by the GeomBD3 package using the Stokes–Einstein relation (Equations 2 and 3), and the resulting values for each setup are reported in **Table S2**.

Each simulation had 500 independent replicas to diffuse CdRP from the same initial position of the PRAI catalytic site. Once a replica satisfied the binding criteria, it was terminated, and a new independent replica would be generated from the same initial position with a different random number of seeds to move CdRP. Each simulation was terminated once the standard error of the mean (SEM) for association time was $\leq 5\%$ and with at least 500 successful bindings. In all BD simulations, a single substrate molecule was included, interacting with the enzyme and any crowders present; therefore, while substrate–substrate interactions were absent, the system does not correspond to strict infinite dilution.

BD simulations were conducted to generate trajectories for the IGPS:PRAI system at varying distances and orientations, aiming to elucidate the association events.

Simulation analysis

Relative interaction energy

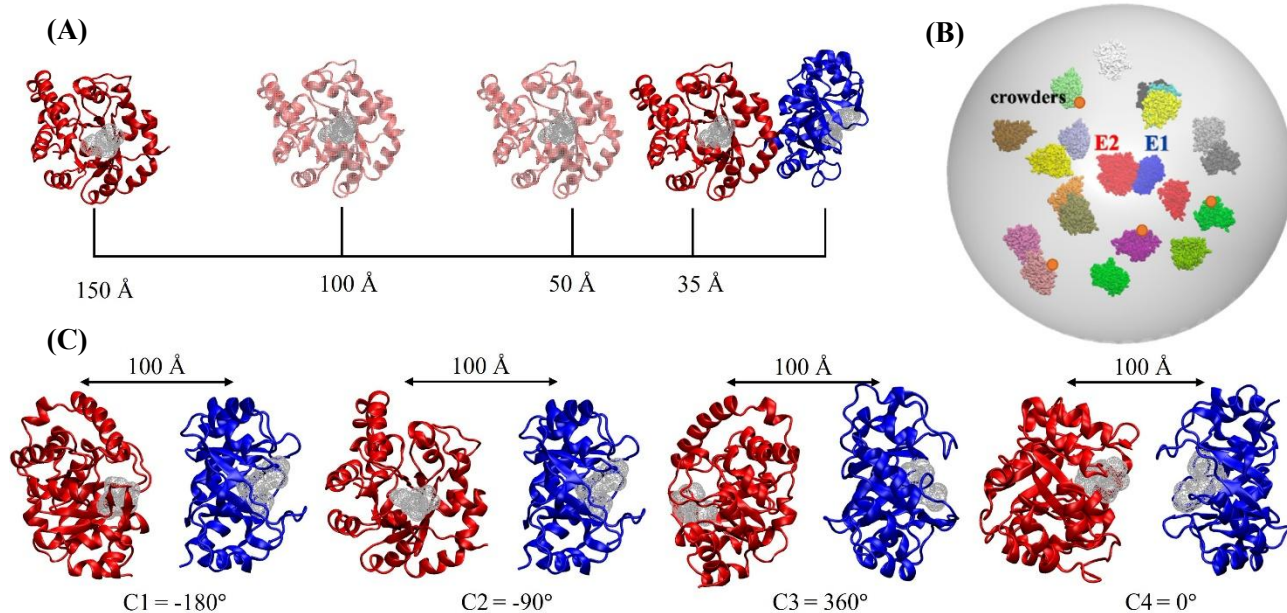
Molecular mechanics Poisson-Boltzmann surface area (MM/PBSA) ⁷³ were used to calculate the relative interaction energy between substrate and enzyme complex in successful and unsuccessful binding. The exterior dielectric constant was set to 15 to accommodate the polar protein residues at the system-water interface. Each energy component was calculated for the receptor (PRAI:IGPS), ligand, and the complex with interval 1 for 400 ns trajectory. The relative interaction energy (ΔE) was calculated manually by subtracting $E_{\text{Complex}} - E_{\text{Enzyme}} - E_{\text{Substrate}}$ for each frame.

Estimated travel time

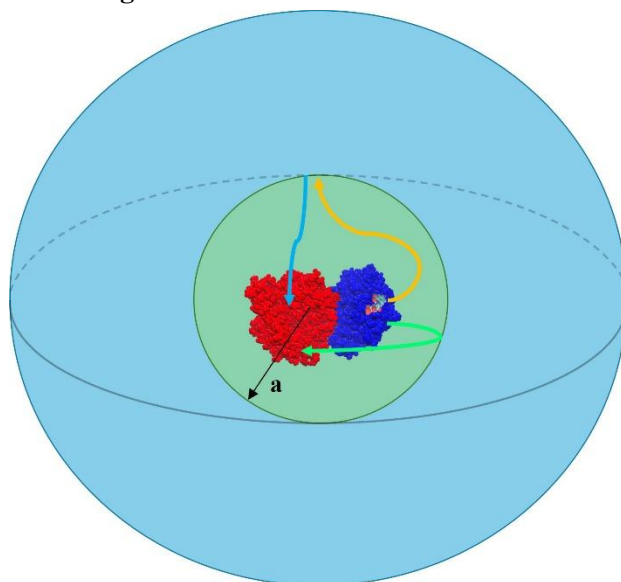
The calculation was based on the Einstein relation, $D = \langle r^2 \rangle / 2nt$, where r (inter-enzyme distance in each setup) is the displacement over time t , n is the dimensionality, which is 3 here, and $D_{\text{substrate}}$ is the diffusion coefficient calculated based on equation (2).

Direct versus indirect bindings

Direct bindings are cases in which the ligand originates from an enzyme and binds to the target enzyme directly without diffusing into the solvent. Because CdRP stays with the enzymes, direct bindings have a shorter travel distance than indirect bindings. To quantify the percentage of direct binding events, we analyzed all BD trajectories. First, we applied the Einstein equation to estimate the substrate's travel time based on the distance between the enzymes. This estimate served as the initial cutoff to identify potential direct bindings. Within this window, if the substrate dissociated and required > 5 ns to rebind, it was excluded—constituting a second cutoff. This filtering process was iterated to isolate consistently direct binding events. All simulation trajectories generated during this study are available from the corresponding author upon request.



265 **Figure 2.** Schematic representation of the orientation-dependent Brownian dynamics (BD) simulation
 266 setups. **(A)** The distance between the centers of mass of the two enzymes ranges from ~ 35 Å (fusion) to
 267 150 Å. **(B)** In the 20% crowding model, TrpCF (fusion) is positioned at the sphere's center. Crowder
 268 proteins are randomly distributed within the simulation sphere, with four crowder proteins featuring
 269 termination sites highlighted by orange circles. **(C)** Different relative orientations of the two active sites are
 270 depicted when they are separated by 100 Å. C1 represents the fused complex (PRAI:IGPS) orientation
 271 separated by 100 Å. (Refer to **Figure S5** for additional orientations C5 and C6).



272 **Figure 3.** Schematic representation of the binding dynamics between CdRP and two enzymes. Illustrates
 273 the distinction between direct and indirect binding in the BD setup. Any pathway within the green sphere
 274 represents direct binding, and the blue sphere shows where the substrate falls for indirect binding which
 275 corresponds to the simulation sphere. The black arrow labeled “a” indicates the direct binding sphere's
 276 radius (65 Å).

Results and Discussion

The results are organized as follows. To investigate whether the bifunctional TrpCF complex comprising the fused PRAI and IGPS domains exhibits inter-enzyme surface-channel or electrostatic advantages that facilitate efficient substrate transfer, we performed 34 independent aMD simulations. We analyzed the association pathways and examined conformational fluctuation during substrate diffusion to investigate the atomic-level behavior of the intermediate CdRP as it diffuses from the PRAI active site, where it is generated, to the IGPS binding pocket, where it acts as a substrate. Notably, substrate CdRP serves as both the product of PRAI and the substrate for IGPS. These simulations allowed us to probe whether a dedicated inter-domain channel or a favorable geometric configuration exists that might enhance IGP synthesis in the fused enzyme. The aMD trajectories were analyzed to characterize substrate dissociation/association pathways and to capture conformational fluctuations that might influence CdRP migration.

To further model substrate–IGPS encounters, we selected representative conformations from MD and set up BD simulations with different enzyme rearrangements and environmental conditions. We performed 500 BD runs for the fused PRAI:IGPS complex and three different inter-enzyme distances (50 Å, 100 Å and 150 Å, based on center-of-mass distance) on the CdRP association with IGPS. We then introduced environmental factors and selected a 100-Å inter-enzyme distance, rotating the enzymes to explore how different pocket orientations influence substrate transfer (**Figure 2**). In addition, we used the 100-Å configuration as a base to systematically rotate the IGPS around PRAI for examining the substrate association when the pair of enzymes have different spatial orientations. Finally, we explored how cell-like complexity influences CdRP binding by introducing crowding agents and competing side reactions. In these simulations (indices B1-B8), additional macromolecules were included that could bind or consume CdRP during its diffusion.

Modeling CdRP diffusion from PRAI to IGPS with aMD simulations

We investigated the CdRP diffusion process from PRAI to IGPS in the Trp biosynthesis pathway using aMD (**Table S1** and **Figure S1**). Although enzymes typically arrange their conformations to facilitate product release from the catalytic pocket, protein conformational changes can take longer than several microseconds, so it is impractical to sample multiple product CdRP dissociation events. To address this challenge, we used the enhanced sampling method aMD with all-atom and explicit solvent models to enhance sampling. As shown in **Figure 4**, CdRP made attempts to exit from the catalytic site of PRAI, and we successfully modeled 12 dissociations among 34 aMD runs (**Table S1** and **Figure S6**). Although

intermolecular attractions retained CdRP on the TrpCF surface, diffusion across the surface did not always result in successful binding to the IGPS active site. Instead, CdRP could be trapped in local energy minima, preventing it from transferring to IGPS for next catalytic step (**Figure S1**, see catalytic steps in detail). During surface diffusion, although the global TrpCF structure remained stable, local side-chain rearrangements transiently stabilized CdRP at specific surface sites. In some cases, CdRP remained in local energy minima for > 400 ns. If it remained trapped for > 200 ns, we terminated the simulation to prevent excessive computation expense. The successful dissociation pathways for all 12 trajectories are shown in **Figure S7**, which indicates that substrate dissociation does not follow a guided or channeling pathway.

Figure 4A illustrates a representative conformation in which the product CdRP exits the catalytic site of PRAI (green sphere) and then binds to the active site of IGPS (grey sphere). **Figure 4B** depicts a successful binding event pathway in green arrows. At time zero (0 ns), CdRP is initially located within the binding pocket of PRAI. It remains in this pocket for approximately 100 ns, during which it exhibits dynamic fluctuations. These fluctuations are maintained primarily via a combination of hydrogen bonding and vdW interactions (see **Figure 4C–G**). During the initial ~100 ns, most of the hydrogen bonds are formed between CdRP and several key residues located within the PRAI pocket. These residues include SER287, ARG289, ARG312, SER328, ASP425, ASN427, and SER428. They collectively help maintain CdRP in the catalytic pocket via persistent hydrogen bonding, reflecting a relatively stable enzyme–product complex during this phase. Between 200 and 340 ns, CdRP gradually transitions from the PRAI binding site toward the IGPS interface. During this period, it simultaneously forms transient hydrogen bonds with residues from both enzymes, thus indicating a dual-enzyme interaction. Notably, around 200 ns, CdRP begins to interact more prominently with SER385 via hydrogen bonds, primarily involving the oxygen atoms on the OPO_3^{2-} moiety of the substrate. In parallel, ARG388 participates via hydrogen– π interactions and additional hydrogen bonds formed with the carbonyl and hydroxyl groups located in the central portion of CdRP. These interactions occur while CdRP is positioned near the bottom surface of PRAI, which suggests an intermediate anchoring point before full release. From 340 to 390 ns, CdRP fluctuates around the peripheral region of IGPS, during which it intermittently forms hydrogen bonds with key IGPS residues ARG199 and HIE228. These interactions help guide and stabilize the substrate near the entrance of the IGPS pocket. Finally, at approximately 390 ns, CdRP fully enters the IGPS active site and becomes stably positioned within the catalytic pocket. Once inside, it remains anchored primarily via hydrogen bonding with catalytic residues of IGPS, thus indicating successful inter-enzyme substrate channeling and recognition.

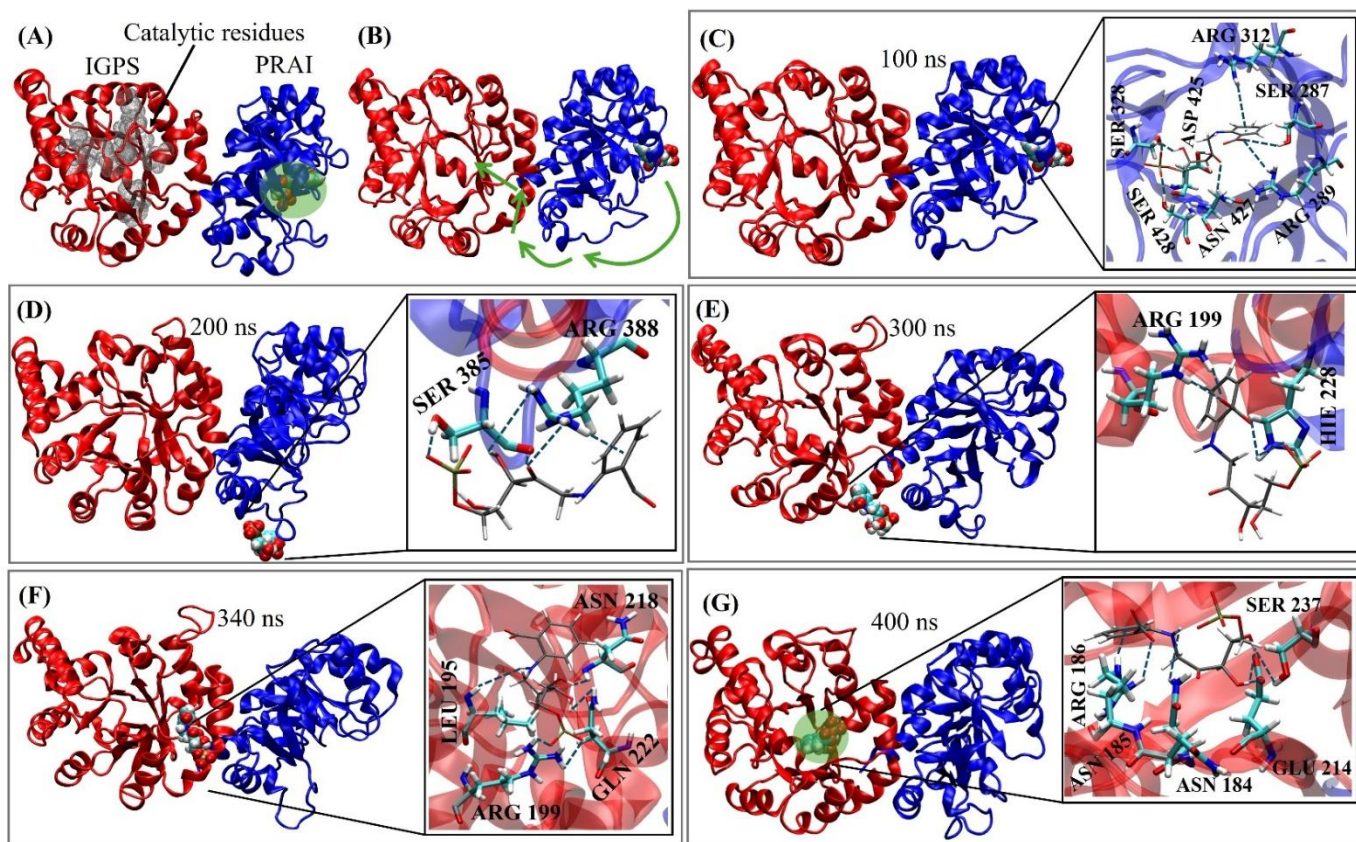
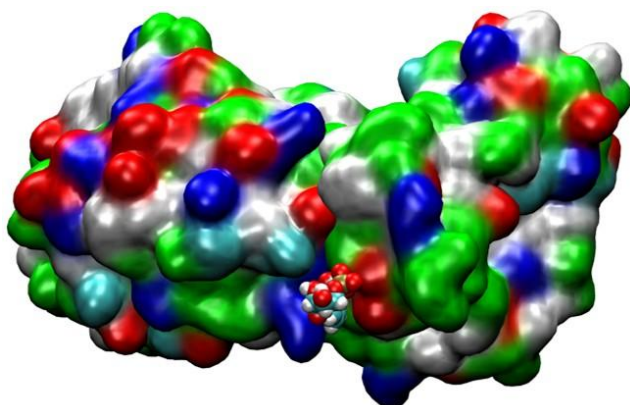


Figure 4. Representative binding events of CdRP in the fused PRAI:IGPS during a 400-ns accelerated molecular dynamics (aMD) simulation in the fused system (TrpCF). **(A)** CdRP is depicted in a van der Waals (vdW) representation. CdRP is highlighted for enhanced clarity, and the catalytic residues of IGPS are shown as gray beads. PRAI and IGPS are shown in blue and red, respectively. Green arrows show the next move of CdRP. **(B)** Successful binding pathway of CdRP in fused TrpCF is shown in green arrows. **(C-G)** Each step of the successful binding pathway and the close-up snapshots of hydrogen bonds at different times between CdRP and different parts of PRAI:IGPS in successful binding. **(C)** At ~100 ns, CdRP fluctuates within the pocket before moving toward the PRAI surface, primarily via hydrogen bonding and non-specific vdW interactions. Hydrogen bonds are formed within the first 100 ns with residues SER 287, ARG 289, ARG 312, SER 328, ASP 425, ASN 427, and SER 428, anchoring CdRP in the PRAI pocket. **(D)** At 200 ns, CdRP is moved toward the bottom of the PRAI and forms hydrogen bonds with residues SER 385 and ARG 388, stabilizing the ligand for about 100 ns in the area. **(E)** At 300 ns, CdRP fluctuates between the bottom of PRAI and IGPS for about 40 ns via hydrogen bonds between the ligand and the residues ARG 199 and HIE. **(F)** From 340 to 390 ns, it fluctuates near the IGPS edge before entering and stabilizing in the IGPS pocket at 390 ns. Hydrogen bonds at the PRAI:IGPS interface (IGPS side) (~340 ns) involving LEU 195, ARG 199, ASN 218, and GLN 222, holding CdRP near IGPS. **(G)** Represents the ligand binding in the IGPS pocket at the end of the simulation, which is stabilized via hydrogen bonds between the residues ASN 184, ASN 185, ARG 186, GLU 214, and SER 237.

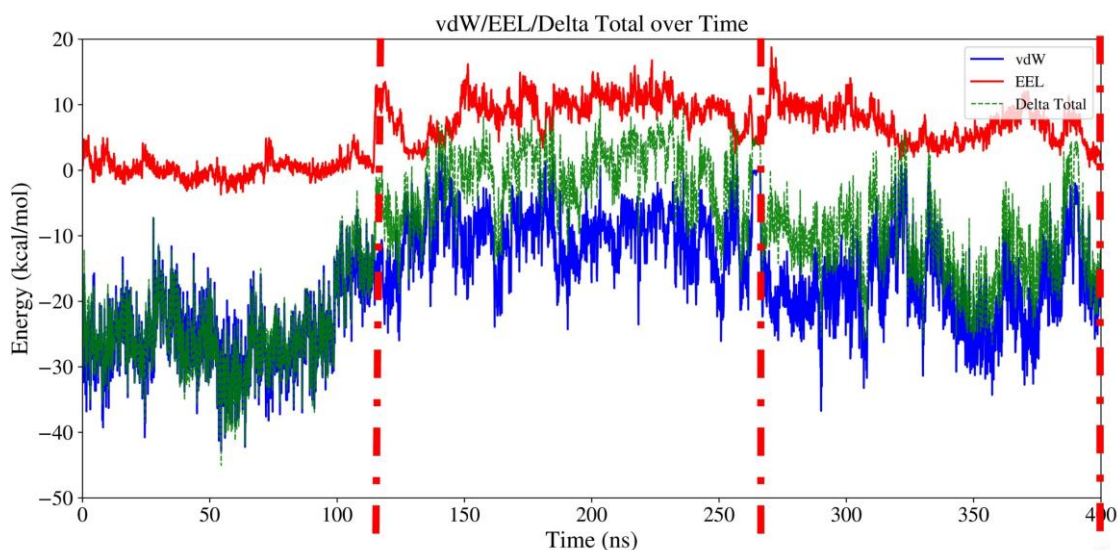
To further characterize the molecular basis of CdRP binding during the successful binding trajectory, we performed energy calculation and structural analyses over 400-ns aMD simulation. The relative binding energies between CdRP and the bifunctional PRAI:IGPS enzyme were computed using the molecular mechanics Poisson-Boltzmann surface area (MM/PBSA) method. As shown in **Figure 5B**, the binding process is energetically dynamic and can be divided into three distinct phases. During the first third of the simulation, favorable vdW interactions and electrostatic contributions stabilize the ligand within the PRAI binding pocket. These interactions are primarily non-specific in nature and are complemented by an extensive hydrogen-bonding network, which collectively holds CdRP in close association with PRAI. In the second third of the trajectory, as CdRP migrates toward the IGPS interface, charge–charge repulsion arises because of the proximity of the OPO_3^{2-} moiety of CdRP to negatively charged residues on the TrpCF surface (see **Figure 5A**; negatively charged residues are highlighted in red, and CdRP is shown in vdW representation). When overlaid with the vdW representation of CdRP, it becomes evident 19% via hydrogen bonds and vdW contacts. Although CdRP does not fully dissociate from the enzyme complex, the local arrangement and persistent hydrogen bonding in the interfacial region appear to mitigate repulsive forces and maintain ligand proximity. The temporal profile of hydrogen bonding further supports this conclusion. As shown in **Figure 5C**, the number of hydrogen bonds between CdRP and PRAI:IGPS is highest during the early phase of binding, consistent with the strong interaction energies observed. Although the number of hydrogen bonds decreases during the repulsive middle phase, a sufficient number remains to prevent complete disengagement. This situation highlights the critical role of hydrogen bonding not just in initial substrate recognition but also in guiding inter-domain transfer. Together, these findings underscore the complex energetic interplay that governs substrate retention and transfer within the fused PRAI:IGPS system. Hydrogen bonding and these non-specific vdW interaction provide the necessary specificity and persistence required for successful interdomain binding. These results reveal a coordinated mechanism in which both long-range and local interactions function in concert to facilitate efficient substrate handoff between active sites.

To contrast the successful CdRP binding trajectory described above, we examined a representative unsuccessful binding event captured in a parallel 400-ns aMD simulation of the fused PRAI:IGPS (TrpCF) system. As shown in **Figure 6**, this trajectory illustrates key deviations in substrate behavior and interaction dynamics that ultimately prevent productive binding. Initially, from 0 to ~200 ns, CdRP remains near the PRAI domain, fluctuating between its binding pocket and adjacent surface regions. Although transient

(A)



(B)



(C)

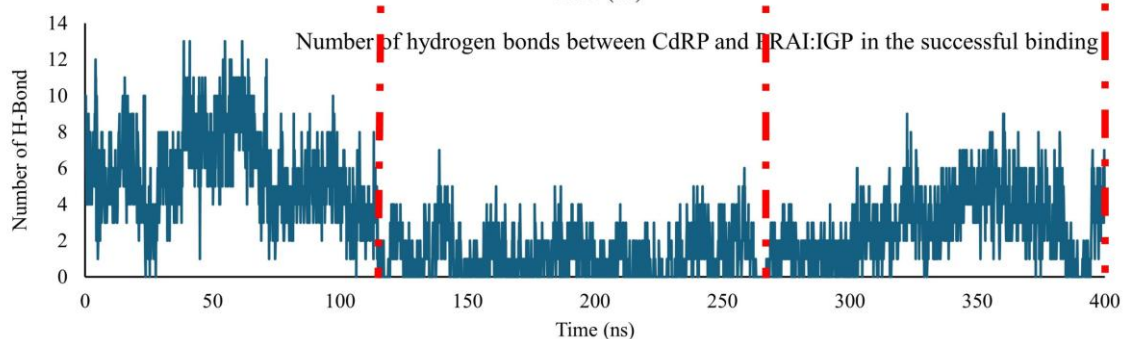


Figure 5. Quantifying the interactions between CdRP and PRAI:IGPS (TrpCF) in the successful dissociation that moreover results in successful binding to IGPS pocket. **(A)** Quicksurf representation of PRAI:IGPS and vdW representation of CdRP. This visualization highlights the negatively charged surface regions, in red, of the fused enzyme (PRAI:IGPS) to provide additional clarity. **(B)** Relative binding energy between PRAI:IGPS and CdRP during a 400-ns aMD simulation, shown in dashed green, using molecular mechanics Poisson-Boltzmann surface area (MM/PBSA). The blue line represents vdW interactions. During the first third of the simulation, binding is driven by hydrogen bonds and non-specific vdW

interactions. In the second third, despite fewer vdW interactions and hydrogen bonds, repulsion occurs between negatively charged residues in PRAI:IGPS and CdRP (with a net charge of -3). As CdRP approaches these residues, charge–charge repulsion is observed. However, because of persistent hydrogen bonding in the region and local arrangement, CdRP remains in close proximity to PRAI:IGPS. EEL interaction shows in red to highlight the repulsions. **(C)** Number of hydrogen bonds Between PRAI:IGPS and CdRP during successful binding. Consistent with the energy plot shown in **(B)**, the hydrogen bond analysis shows that binding in the first third of the simulation is primarily stabilized by multiple hydrogen bonds between the ligand and the enzyme.

interactions occur, they appear insufficient to retain CdRP in a stable orientation, and no sustained hydrogen bonding network forms, as seen in the successful trajectory. At approximately 212 ns, CdRP shifts toward the bottom surface of the PRAI domain. However, unlike the dual-enzyme interaction phase observed previously, the ligand fails to form bridging interactions with the IGPS domain. Shortly thereafter, at ~220 ns, CdRP fully dissociates into the solvent. This event marks a critical divergence from the productive pathway, during which persistent hydrogen bonding had helped counteract electrostatic repulsion. In this case, the lack of stabilizing interactions leads to loss of substrate–enzyme contact, and CdRP remains solvated and mobile for most of the remaining simulation time (~140 ns), as visualized by its transparent vdW representation in **Figure 6**. Only during the final ~10 ns of the simulation does CdRP reassociate with the protein surface—this time near the IGPS domain but only at its outer edge rather than within the active site pocket. This diffusion-controlled re-binding does not have clear long-range EEL steering and likely reflects non-specific CdRP–surface attractions. The random encounter process did not lead CdRP to enter the IGPS pocket, which further underscores the importance of a coordinated, stepwise transition facilitated by both electrostatic steering and sustained hydrogen bonding.

To further dissect the molecular underpinnings of unsuccessful substrate transfer, we analyzed the same unproductive trajectory using MM/PBSA binding energy calculation and hydrogen bond quantification. As shown in **Figure 7A**, the vdW energy profile reveals an early phase of a relatively stable interaction between CdRP and the PRAI domain. For the first ~220 ns of the simulation, the ligand remains loosely stabilized within the PRAI pocket and along its surface. These interactions are primarily mediated by transient hydrogen bonds and non-specific vdW contacts, sufficient to retain CdRP in proximity but lacking the strength and persistence observed in successful binding events.

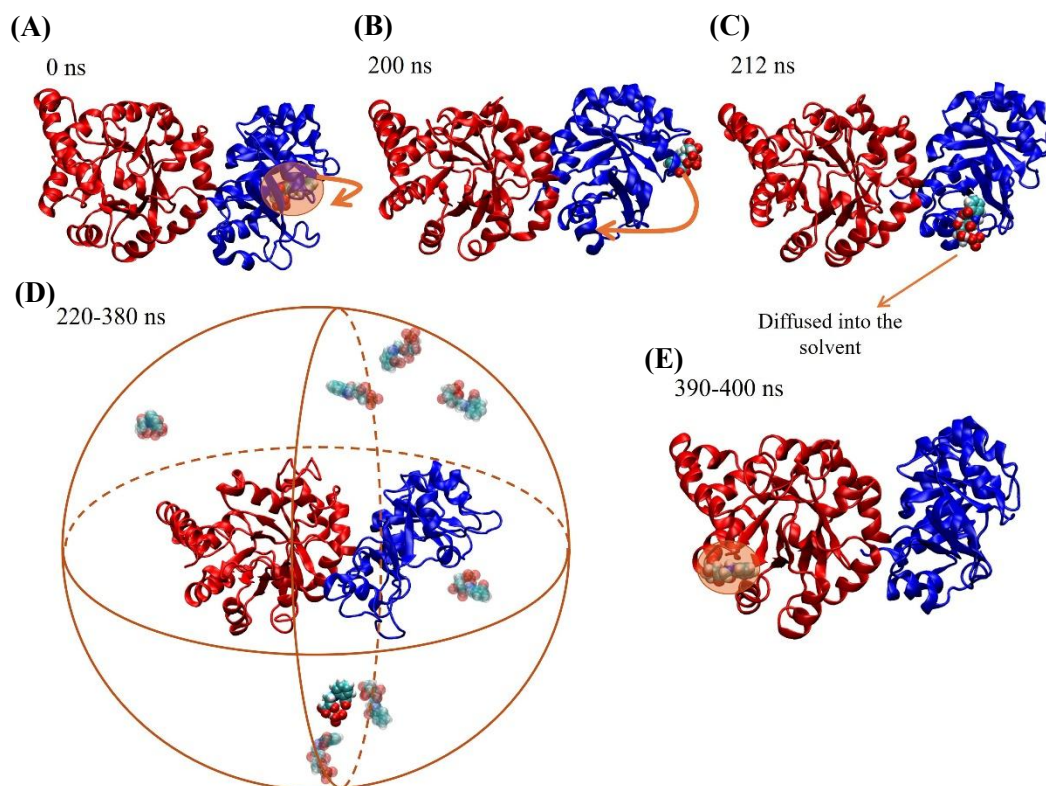


Figure 6. Representative unsuccessful binding event of CdRP in the fused system (TrpCF) during a 400-ns aMD simulation. CdRP is depicted in a vdW representation. CdRP is highlighted for enhanced clarity. PRAI and IGPS are in blue and red, respectively. Orange arrows show the next position of CdRP. Unsuccessful binding: CdRP undergoes dissociation to the solvent, attaching to the edge of the IGPS pocket at the end of the simulations. **(A-B)** From 0 to 200 ns, it fluctuates in the pocket and on the surface of the PRAI. **(C)** At 212 ns, it moves to the bottom of the PRAI. **(D)** CdRP dissociates in the solvent at ~220 ns (CdRP vdW representation) and keeps moving in the different parts of the solvent for about ~140 ns (vdW transparent representation). The movements of the ligand in the solvent are shown within the sphere for better visualization (the sphere does not represent the water box). **(E)** CdRP reassociates for the last 10 ns of the simulation to the edge of the IGPS close to its pocket.

Crucially, this interaction stability does not persist. As CdRP transitions toward the bottom region of PRAI, the number and strength of hydrogen bonds diminish. This situation is reflected in the decline of both vdW and electrostatic contributions, culminating in a sharp drop in total interaction energy as CdRP dissociates into the solvent. The shaded region in **Figure 7A** marks the point at which the energetic support for binding collapses, corresponding closely to the time of full dissociation (~220 ns). Unlike the successful trajectory, when hydrogen bonding compensated for electrostatic repulsion and helped steer the ligand toward the IGPS site, here the absence of persistent interactions leads to ligand escape and inefficient interdomain transfer.

The hydrogen bonding analysis in **Figure 7B** supports this observation. Although a modest number of hydrogen bonds are present early in the simulation—mainly between CdRP and residues on the PRAI surface—these interactions gradually decline without recovery. The lack of stable hydrogen bonding in the intermediate and later phases of the simulation contrasts sharply with the successful event, during which such bonds played a key role in maintaining substrate proximity and guiding its path. The failure to maintain these stabilizing interactions permits solvent dissociation and also prevents any meaningful engagement with the IGPS domain.

Overall, this unsuccessful binding illustrates the sensitivity of substrate transfer to the dynamic balance of intermolecular forces. In the absence of continuous stabilizing interactions—particularly hydrogen bonds—CdRP is unable to complete the transition between catalytic domains. This mechanistic failure reveals that passive diffusion alone is insufficient for effective substrate delivery within the bifunctional enzyme. Instead, successful transfer in systems like PRAI:IGPS depends on a finely tuned interplay of spatial orientation, surface electrostatics, and energetically favorable contacts. Comparison with the successful trajectory emphasizes this point: when stabilizing interactions are weak or short-lived, the substrate diffuses into the bulk solvent, resulting in a missed catalytic opportunity and reduced functional efficiency. Moreover, to examine whether successful substrate dissociation/association requires charge-mediated steering, we removed electrostatic contributions (EEL) by setting the substrate charge to zero. We then performed aMD simulations with the same parameters as the successful binding runs (four different random seeds, each 800 ns). In these trajectories, dissociation from PRAI did occur; however, the substrate remained strongly bound and trapped on the PRAI surface (**Figure S8A**). To further quantify these observations, we calculated the relative substrate–enzyme interaction energies using MM/PBSA, which confirmed the strong non-electrostatic interactions in the absence of charge-mediated steering (**Figure S8B, see for details**).

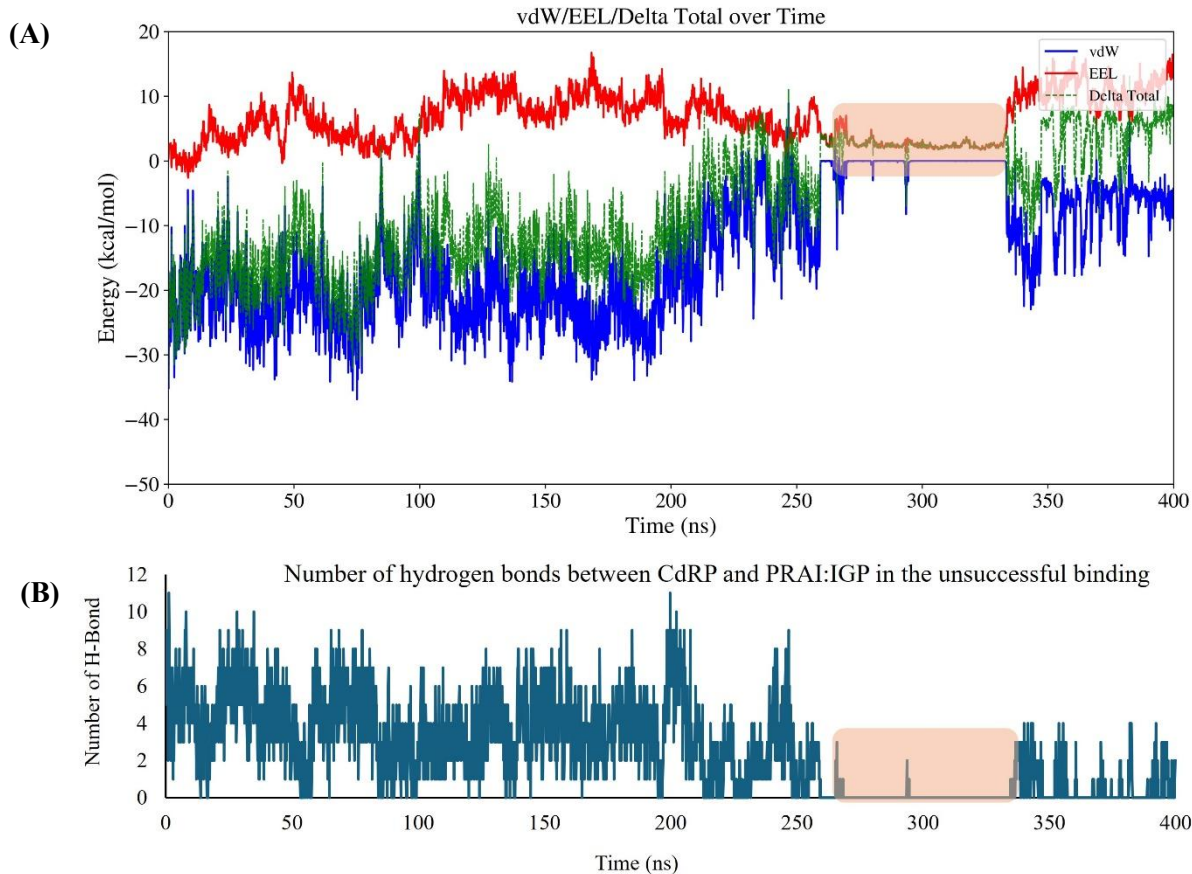


Figure 7. Quantifying the interactions between CdRP and PRAI:IGPS (TrpCF) in unsuccessful binding. **(A)** Binding energy between PRAI:IGPS and CdRP during a 400-ns aMD simulation using MM/PBSA. Blue represents vdW interactions, EEL in red, and dashed green delta total in kcal/mol. From 0 to 220 ns, the ligand remains stabilized in the PRAI pocket and surface region through hydrogen bonding and non-specific vdW interactions. Once these interactions are lost, the ligand diffuses into the solvent. The highlighted region indicates minimal interactions and loss of the hydrogen bonds at the same time of the simulation, leading to CdRP dissociation into the solvent. **(B)** Number of hydrogen bonds between PRAI:IGPS and CdRP during unsuccessful binding during 400 ns simulation.

Although our aMD simulations sampled local energy minima, as reflected by trajectories in which CdRP remained transiently associated with the TrpCF surface, while other runs showed complete dissociation into solvent. Multiple dissociation routes from PRAI to IGPS were observed, but no single dominant diffusion pathway was identified (**Figure S7**, see for more details).⁵⁴ We concluded that TrpCF does not use substrate channeling to transport its intermediate CdRP. Nevertheless, a substrate channeling guiding mechanism is commonly seen in other multi-enzyme systems, such as an electrostatically mediated channel in sulfate-activating complexes to interconnect two active sites⁴⁶ and malate dehydrogenase (MDH) and citrate synthase (CS) metabolons^{36,39,41,45,79}. Of note, the enzyme system catalyzing the next reaction to biosynthesize Trp, TRPS, has a physical tunnel connecting a pair of active sites in subunits α and β to effectively prevent the substrate from leaving TRPS⁷⁴. Notably, our simulations showed the functional

motions of the loops near the catalytic sites of both enzymes, where the open/closed conformations (**Figure S3**) regulated product CdRP release from PRAI and substrate CdRP binding to the catalytic site of IGPS. However, unlike other multi-enzyme complexes that use allosteric regulation to control intermediate transportation, such as the fused α and β subunits of TRPS^{75,76}, or protein motions to assist substrate binding, such as the tetrameric form of acetylcholinesterase⁷⁷, we did not observe correlated or regulated motions between the fused IGPS and PRAI to facilitate CdRP binding^{82,83}.

Modeling substrate diffusion in test-tube and cell-like environments using BD simulations

Although we did not identify a major pathway guiding CdRP diffusion on the TrpCF surface, the formation of a fused enzyme complex may still offer other advantages. To explore the factors influencing intermediate CdRP diffusion and their impact on the final product synthesis, we used BD simulations to model substrate travel between the active sites of TrpCF and isolated PRAI and IGPS. Because our BD simulations treated CdRP as a rigid body, we first calculated its diffusion coefficient to ensure that this approximation did not distort the modelled diffusion behavior. The BD runs yielded a translational diffusion coefficient $D_{\text{substrate}} = 7.2 \pm 0.74 \times 10^{-6} \text{ cm}^2/\text{s}$ for CdRP (**Table S2**, see for details), consistent with the analytical value, $D_{\text{analytical}} = 5.0 \times 10^{-6} \text{ cm}^2/\text{s}$, derived using the CdRP radius of gyration ($\sim 3.71 \text{ \AA}$, see Supplementary materials).

To compare substrate travel between the two catalytic sites, we selected an open conformation of the loop near the PRAI active site and initiated BD simulations for TrpCF, the fused PRAI:IGPS complex. We simply separated the PRAI:IGPS complex to isolate PRAI and IGPS. We maintained the same enzyme orientation as in TrpCF and varied the inter-enzyme distances to 50 $\text{\AA}$, 100 $\text{\AA}$, and 150 $\text{\AA}$ (**Figure 2A**). Using $D_{\text{substrate}}$ to compute the theoretical travel time for CdRP to diffuse 50 $\text{\AA}$, 100 $\text{\AA}$, and 150 $\text{\AA}$, the estimated association time was $\sim 25 \text{ ns}$, $\sim 100 \text{ ns}$, and $\sim 220 \text{ ns}$, respectively. However, CdRP took significantly longer to reach the IGPS catalytic site in our BD simulations, with a median association time $> 8.4 \text{ }\mu\text{s}$ in the test-tube environment (**Table 1**). The large variance suggested that CdRP rarely took the shortest path to travel from PRAI to IGPS, with $< 2\%$ of the binding contributed from CdRP diffused directly from PRAI to IGPS without deviating the paths into the solvent (**Table 1**). Notably, TrpCF, the fused PRAI:IGPS complex, did not accelerate the substrate association. Instead, the average association time is $\sim 3\text{-}5 \text{ }\mu\text{s}$ longer than the median because the substrate was trapped between the PRAI:IGPS interface, thus resulting in excessively longer simulation time. Moreover, as the BD simulations employ a rigid-body approximation that does not incorporate protein motions or conformational flexibility, this methodological limitation may also influence the association and dissociation dynamics observed. As in aMD simulations, our BD runs demonstrated that weak and non-specific vdW attractions (**Figure S9**) were insufficient to

retain CdRP on the enzyme surfaces. As a result, CdRP diffused into the solvent in most cases and then rebounded to the IGPS active site. Because PRAI, IGPS, and CdRP all carry a negative charge, there was no long-range electrostatic attraction to facilitate CdRP binding. As shown in **Table 1**, the most effective binding occurred when PRAI and IGPS were 50 Å apart. Reducing the inter-enzyme distance or fusing PRAI:IGPS did not significantly increase direct binding. The distributions of association times across the different BD setups are non-Gaussian (**Figure S10**); therefore, we analyzed the data using the median as well as the 25th and 75th percentiles for each setup (**Figure S11**). These statistics allow direct visualization of the distributions and highlight whether a larger fraction of trajectories reaches association more quickly in one setup compared with others.

Table 1. Presents the simulation results conducted under varying conditions, highlighting the dynamics between enzyme distances and association times. Median T_{asso} in microseconds (μs) and the percentage of ligand consumed due to the side reactions for different inter-enzyme distance setups across three simulation conditions. Median values were computed after the completion of 500 replicates. P_{Direct} represents the percentage of direct binding for the substrate to the enzyme in the different setups.

Simulation Conditions	Simulation index	Inter-enzyme distance	Median T_{asso} (μs)	P_{Direct}	Consumed by side reactions
Test-tube environment	A1	PRAI:IGPS(TrpCF)	10.4	2%	0%
	A2	50Å	8.4	3.1%	0%
	A3	100Å	9.6	0.17%	0%
	A4	150Å	11.5	0.37%	0%
Cell-like environment	B1	PRAI:IGPS(TrpCF)	18.4	0.40%	0%
	B2	50Å	15.5	0.37%	0%
	B3	100Å	14.8	0.17%	0%
	B4	150Å	15	0%	0%
Cell-like environment with side reaction	B5	PRAI:IGPS(TrpCF)	3.2	0.58%	47.5%
	B6	50Å	3.4	0.76%	44.4%
	B7	100Å	3.7	3.5%	44.3%
	B8	150Å	4.4	0.58%	45.1%

Surprisingly, TrpCF did not enhance substrate travel efficiency from PRAI to IGPS. One possible explanation is that fusing PRAI and IGPS partially occludes surface regions on IGPS that would otherwise attract CdRP. As reported in existing studies^{75–79}, protein surface can play a role in non-specifically attracting its binding partners, which yields increased local ligand concentration to facilitate the association rate. We used our GeomBD3 program to model the probability density map to reveal potential non-specific attractions between substrate CdRP and IGPS. As illustrated in **Figure 8B**, several concave regions on the surface of IGPS (Concave area are shown in surf representation in **Figure 8c**) with high substrate retention probability become inaccessible when the enzyme was fused with PRAI. These results suggest that simply

fusing enzymes into a larger complex may not improve intermediate transfer between the catalytic sites, unless a surface channel or enclosed tunnel may be created to efficiently guide intermediate transportation. This finding aligns with previous *in vitro* enzymatic measurements, which showed that TrpCF did not enhance catalytic efficiency⁵⁷.

Another distinction between TrpCF and isolated PRAI and IGPS is their relative orientation. Although the fused enzyme adopted a fixed orientation, isolated enzymes can rotate freely. Previous studies have suggested that optimizing inter-enzyme orientations can enhance catalytic efficiency^{12,39,44,80,81}. Therefore, we rotated the enzyme pair with a fixed inter-distance of 100 Å and modelled the CdRP association time (**Figure 2C** and **S7**). Enzyme rotation significantly affected the CdRP ability to directly diffuse to IGPS, the fastest association occurring when the catalytic sites faced each other (i.e., BD simulation index C4). In this orientation, CdRP exhibited the shortest median and average association time (8.2 μs and 12.6 μs, respectively) (**Table 2**) and the highest direct binding probability (17.4%). In contrast, rotating the catalytic site 180° (matching the TrpCF orientation) resulted in not much change in the direct binding events as compared with the original orientation of TrpCF. Of note, because of the shape of the IGPS active site, rotations of ±90° yielded different association times. The +90° orientation (index C5) allowed easier CdRP access, leading to more direct binding events (10.0%) (**Figure 2C**), and a shorter median binding time (9.6 μs) as compared with the -90° orientation (index C2), which had a 3.5% direct binding probability and a median binding time of 10.6 μs. This study highlights that the specific geometric details of the enzymes play a crucial role in optimizing overall catalytic rates via spatial organization. If optimal inter-enzyme orientations cannot be maintained, simply co-localizing enzymes to allow free rotation may suffice to create a local environment that efficiently facilitates intermediate transfer and enhances the overall product synthesis rate. These observations correspond to the BD simulations C1–C6, in which we manually adjusted the relative orientations of the enzyme pockets under test-tube–like conditions. The findings suggest that even in the absence of optimal inter-enzyme orientations, co-localization of non-fused enzymes with sufficient freedom to reorient can establish a local environment that facilitates substrate association and enhances overall catalytic efficiency. In contrast, for the other BD indexes where only the inter-enzyme distance was varied, co-localization alone did not lead to a significant improvement in catalytic efficiency under either test-tube–like or cell-like conditions.

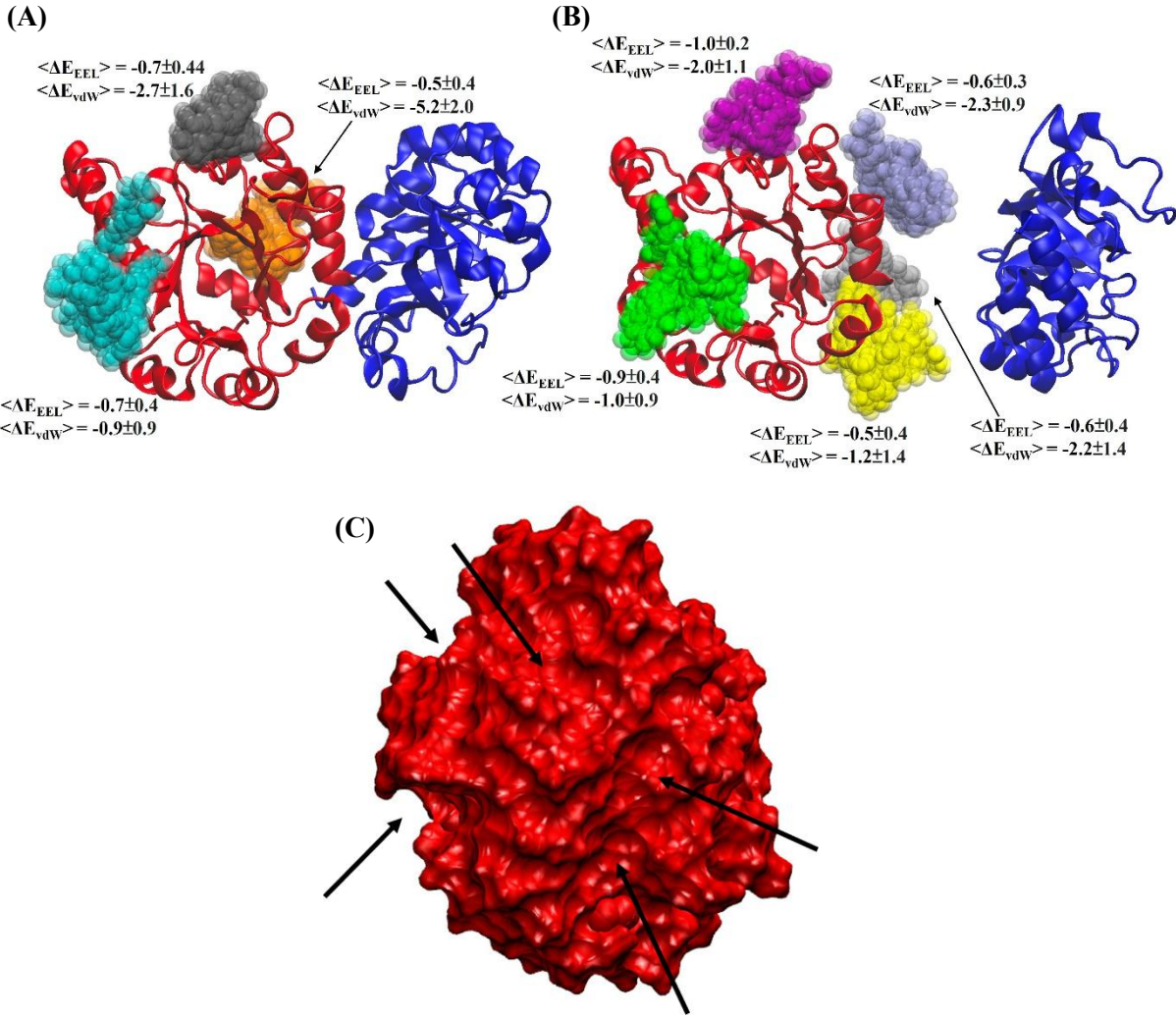


Figure 8. Ligand–enzyme intermolecular non-specific electrostatic (ΔE_{EEL}) and vdW (ΔE_{vdW}) interaction energies in kcal/mol were extracted from BD simulations when CdRP is on different parts of the IGPS surface areas shown in gray, orange, cyan, yellow, green, ice-blue, and purple beads in the (A) fused system and (B) 50 Å distance system in the test tube environment. ΔE values are reported as mean $\pm$ standard deviation across the analyzed simulation frames. (C) Surface representation of IGPS. For better visualization, the enzyme is shown from the back angle, with concave regions on the IGPS surface highlighted by black arrows.

Modeling substrate diffusion in cell-like environments without side reactions

Unlike the dilute conditions typically used in biochemistry assays, intracellular environments are densely packed with approximately 20% to 40% of the cytoplasmic volume occupied by various macromolecules. To explore how such a crowded, cell-like environment might influence the CdRP association with IGPS,

we conducted a series of BD simulations (indices B1 to B4). In these simulations, we introduced 19 ribonucleases as crowders, occupying ~20% of the simulation box volume. The results, in **Table 1**, revealed that fusing PRAI and IGPS did not enhance substrate association efficiency. Instead, the average association time increased by approximately 5–8 μ s as compared with the median, primarily due to a subset of events that took significantly longer. The most efficient binding occurred when the enzymes were separated by 100 Å. Increasing the distance between the enzymes delayed the association distribution, and decreasing the distance of fusing PRAI and IGPS did not significantly increase direct binding events. Notably, < 0.5% of the events were direct binding. Although the simulation volume was reduced ~20% because of the presence of crowders, the dominant effect of crowding was to slow down substrate diffusion. In theory, the reduced volume in a confined space should shorten the substrate's traveling time from PRAI to IGPS in the absence of interactions with macromolecules. However, trajectory analysis revealed that the substrates frequently bound to the crowders anisotropically for an extended period before returning to the bulk solution and resuming diffusion (**Figure S7**). This observation suggests that although the substrates spent less time traveling, the prolonged interactions with crowders offset this advantage. These results indicate that the non-specific interactions with crowder biomolecules were the primary driver of the increased average association time^{82–86}.

Of note, the fused PRAI:IGPS complex, which has the shortest substrate travel distance between active sites, exhibited the longest median association times. This finding suggests that although the proximity may reduce the substrate's travel distance, it does not facilitate substrate association to yield sustained enhancement in catalytic efficiency^{54,55,57,87}. The same was seen in the test-tube environment: simply co-localizing or fusing two enzymes in a multienzyme cascade did not inherently improve catalytic efficiency in a cell-like environment. Although hydrodynamic interactions are critical for accurately modeling macromolecular diffusion^{87–89}, we omitted them in our BD runs to significantly accelerate computational throughput. This simplification is justified because the enzymes and crowders were held rigid, and the substrate CdRP is relatively small compared with other macromolecules. As demonstrated in a previous study, the effects of hydrodynamic interactions are negligible under these conditions⁹⁰. However, in real cells, biomolecules are mobile and undergo conformational changes, which may influence substrate diffusion properties. Therefore, future studies using classical MD simulations with all-atom, explicit solvent models could provide deeper insights into enzyme co-localization and intermediate transport in multi-step biosynthesis pathways within cell-like environments.

Table 2. Median association time (T_{asso}) in μs and direct binding percentage (P_{Direct}) for different orientations in the BD simulation setups in diluted conditions. C1 represents the TrpCF complex (fused PRAI:IGPS) orientation separated by 100 Å.

Simulation index	Median T_{asso} (μs)	P_{Direct}
C1 (-180°)	9.7	5.9%
C2 (-90°)	10.6	3.5%
C3 360°)	10.8	1.5%
C4 (0°)	8.2	17.2%
C5 (90°)	9.6	10.0%
C6 (180°)	10.0	5.3%

Modeling substrate diffusion in cell-like environments with side reactions

In cellular environments, substrates such as CdRP may react with other enzymes, thus reducing their effective concentration and potentially affecting the overall rate of product synthesis. In particular, CdRP contains a phosphate group, which is susceptible to dephosphorylation by various endogenous phosphatases present in the cytoplasm. This situation introduces competing side reactions that can rapidly consume CdRP before it reaches its next active site in IGPS. Investigating the CdRP association with IGPS when both crowders and side reactions are present is crucial to evaluate the advantage of bifunctional enzymes (i.e., TrpCF) in transporting the substrate in cells^{56,91}.

To simulate these competitive scenarios, we used the same crowders presented in B1-B4 BD simulations and selected four crowders as competing enzymes that could consume CdRP upon contact to mimic intracellular side reactions (B5-B8). In this model (**Figure 2B**), the four competing enzymes were distributed around the IGPS and PRAI complex: two located proximally (~ 100 Å from the PRAI active site) and two placed at a further distance (~ 200 Å). During the simulation, if CdRP reached the active site of one of the four crowders, the trajectory was terminated to reflect irreversible substrate consumption.

The results (**Table 1**) show a marked reduction in median association times when side reactions are introduced. In this setup, the substrate could bind not only to the target enzyme but also to other crowders designated as side-reaction elements. Once the substrate was consumed through these irreversible side reactions, the corresponding BD trajectories were terminated. As a result, the fused system exhibited a comparatively shorter association time.

Across all competitive setups, CdRP was consumed at similar rates—ranging from 44% to 47.5%—thus indicating that substrate loss to side reactions is substantial regardless of inter-enzyme distances. However, we observed differences in direct binding efficiency. In most configurations, the percentage of direct binding events between CdRP and IGPS decreased to nearly zero. Notably, in Index B7, with crowders positioned within ~ 100 Å of the PRAI domain, a modest 3.5% direct binding rate was retained, marking it as an outlier as compared with the other setups.

Trajectory analysis further revealed that CdRP often interacts non-specifically with nearby crowder surfaces. Because the crowders carry an overall positive charge (+2), they attract the negatively charged CdRP, leading to its consumption via side reactions. In such cases, the travel time between CdRP leaving the PRAI pocket and reaching IGPS was shorter than in simulations without side reactions. This situation is due to the non-specific vdW and EEL interactions between crowders and the substrate, which prevent CdRP from diffusing into the bulk solvent (see **Table 1**). Hence, both the spatial positioning and local interactions of crowders critically affect binding outcomes.

Overall, the simulations demonstrate that in crowded, reactive environments resembling the cytosol, substrate competition from unrelated enzymes significantly impairs productive binding. Nonetheless, spatial arrangement plays a nuanced role—proximity between crowders and target enzymes can modulate diffusion paths and transient interactions, occasionally increasing the likelihood of successful substrate delivery. These findings suggest that although the bifunctional TrpCF complex does not strongly protect the substrate from competing reactions, its structural organization may help mitigate substrate loss under certain spatial configurations.

Conclusions

In this study, we used a combination of aMD and BD simulations to investigate how structural fusion, spatial arrangement, and environmental effects (i.e., crowders and side reactions) affect substrate transfer between catalytic domains in the TrpCF bifunctional enzyme system. Our primary focus was the intermediate CdRP, which must diffuse from the PRAI domain to the IGPS active site during Trp biosynthesis in *E. coli*.

Our aMD simulations revealed that CdRP diffusion is not facilitated by a guided surface diffusion because there is no well-defined electrostatic channel in TrpCF. Instead, successful substrate transfer depends on transient stabilizing interactions—particularly hydrogen bonding and non-specific vdWs—that retain the ligand on the protein surface. Notably, during CdRP surface diffusion, although the enzyme complex did

not change the overall conformations, local sidechains as well as CdRP rearrangements allowed the formation of favorable interactions that kept CdRP in a few locations on the surface of PRAI:IGPS. Trajectories lacking these interactions resulted in CdRP becoming trapped in local minima or diffusing into the solvent without reaching IGPS.

Using BD simulations, we modeled the CdRP association under both idealized *in vitro* ("test-tube") and complex *in vivo*-like ("cell-like") environments. In test-tube conditions, reducing the inter-enzyme distance to <50 Å improved binding efficiency, but fusing PRAI and IGPS (as in TrpCF) did not yield significant improvements over optimally spaced, freely diffusing enzymes. These results highlight that proximity alone does not guarantee enhanced association unless accompanied by favorable catalytic site orientation and surface accessibility.

In crowded, cell-like environments, crowder macromolecules slowed down the substrate diffusion via non-specific binding. The introduction of side reactions, mimicking intracellular substrate degradation, further reduced CdRP availability. Notably, in these simulations, even TrpCF failed to preserve high binding efficiency, so without electrostatically steered diffusion or an enclosed tunnel, intermediate transfer remains vulnerable to spatial interference and competition. However, specific spatial arrangements, such as placing crowdiers near both PRAI and IGPS, modestly increased direct binding, which suggests that local geometry can still play a supportive role in keeping a substrate within the proximity.

Overall, the BD simulation results demonstrate that efficient substrate association in TrpCF is highly sensitive to both spatial arrangement and environmental context. Although domain fusion ensures physical proximity, it does not inherently optimize alignment or protect intermediates from loss. Maximum efficiency was observed in compact, well-oriented configurations, which emphasizes the importance of local geometry and interaction surface compatibility. These findings highlight the limitations of passive diffusion in crowded intracellular conditions and provide quantitative insight into the design principles required for optimizing multienzyme systems. Although rigid-body BD studies cannot precisely duplicate conditions in *in vitro* experiments or cells, our studies suggest no advantages in substrate diffusion in a fused PRAI:IGPS complex as compared with the arrangement with the inter-enzyme distance 50 Å in the test-tube environment. Nevertheless, in a cell environment, without fusing the two enzymes, co-localizing the two enzymes to bring them in proximity can be challenging. In brief, our studies demonstrated that maximum intermediate substrate transfer was achieved with small (<50 Å) inter-enzyme separation and catalytic sites oriented toward one another. Efficient substrate handoff in multienzyme systems relies on a synergistic balance of proximity, orientation, local binding landscapes, and environmental context. These

findings underscore the value of computational modeling in dissecting complex enzymatic systems and inform future designs of engineered enzyme assemblies for optimized catalysis under biologically relevant conditions.

Supporting information

Accelerated MD sampling information, Diffusion coefficient calculation for BD simulation, Diffusion coefficient of the substrate in different BD setups, different dissociation pathways for aMD runs, method information and results.

Author contribution

P.F. and K.W. contributed to the project equally. P.F. and K.W. performed computational simulations and data analysis. S.K. helped in optimizing the GeomBD3 package. C.C. supervised the project. P.F. and C.C. wrote the manuscript with the support of all authors.

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“TOC Graphic”

