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Catalytic Domains of Phosphodiesterase 5, 6, and 5/6 Chimera Display Differential Dynamics and Ligand Dissociation Energy Barriers

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Abstract

The enzyme phosphodiesterase 6 (PDE6) is a critical component of the visual signaling pathway, and functions to convert cGMP to GMP. The ability of PDE6 to affect cellular cGMP levels leads to deactivation of cGMP-gated ion channels in both rod and cone cells. PDE6 has been difficult to structurally characterize experimentally, though the structures of the closely related PDE5 and a PDE5/6 chimera have been determined by X-ray crystallography. In this work, we employ a computational approach to study the dynamics of the catalytic domains of PDE6, PDE5 and the PDE5/6 chimera. Through equilibrium molecular dynamics (MD) simulations we identify a region of PDE6 ($\alpha 12$) to be correlated to distal regions of the enzyme (H- and M-loops) which surround the catalytic center. These correlations are not observed for PDE5 and we speculate that these unique motions in PDE6 may relate to the high catalytic efficiency of PDE6 and the requirement for an endogenous inhibitory subunit ($P\gamma$). We further investigate the ligand binding pathways and energetics by enhanced sampling simulations (metadynamics) using the inhibitor sildenafil and GMP. The energetics and pathways of ligand binding are consistent with the high efficiency of PDE6 and further implicate the $\alpha 12$ region as an important regulatory element for PDE6 functional dynamics.

Graphical Abstract

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Supporting Information Available

Eight figures containing additional analyses on the homology model validation, ligand stability, and metadynamics

Introduction

The enzyme phosphodiesterase 6 (PDE6) is a critical component of the pathway that converts light into the electrical signals that result in vision.^{1,2} PDE6 is a member of the class I cyclic nucleotide phosphodiesterase superfamily, which contains 11 different PDE enzymes. PDE6 converts cyclic guanosine 3',5'-monophosphate (cGMP) to GMP, a critical and highly regulated step in the visual signaling pathway, and is found in both the rod and cone cells of the eye.³

This molecular signaling pathway that results in vision has several major steps. G-protein coupled receptor (GPCR) rhodopsin is activated by light and in turn activates G-protein transducin.⁴ The α -subunit of transducin then displaces the inhibitory PDE6 subunit γ (P γ) from its position blocking the active site of PDE6.⁴ This allows PDE6 to rapidly hydrolyze cGMP to GMP.⁴ The drop in cGMP concentration deactivates cGMP-gated ion channels, causing hyperpolarization of the cell and then activation of the sensory neurons responsible for vision.^{4,5} One of the properties of PDE6 that makes it unique amongst PDEs is that it has a naturally occurring inhibitory subunit, P γ .^{5,6} This relationship allows P γ to regulate the activity of PDE6 and P γ is known to selectively inhibit PDE6 and not other PDEs.⁵

PDE5 and PDE6 exist as dimers, with each monomer containing a catalytic domain and tandem GAF domains.⁷ The PDE5 catalytic domain can be expressed and purified as an active monomer,⁸ however there are no such reports for PDE6. In cone cells PDE6 is a homodimer ($\alpha\alpha$), and this work will focus on the catalytic domains in a monomeric state. PDE6 has not been as well characterized as other PDEs because it is difficult to express in bacteria and purify. As a consequence, a high resolution structure of PDE6 has yet to be determined, although recent work characterized the overall topology of PDE6 through an 11 Å resolution cryo-EM reconstruction.^{5,9} A model construct was developed by Barren et. al to gain information of PDE6 structure through the creation of a chimera of PDE5 and PDE6 referred to as PDE5/6.¹⁰ PDE5 and PDE6 have high sequence homology, so substituting sequences unique to PDE6 into the corresponding areas on the PDE5 gene allowed the expression and isolation of PDE5/6.¹⁰ The PDE5/6 chimera is functional, as it can hydrolyze cGMP while also being effectively inhibited by P γ , making it a reasonable experimental model to study PDE6.¹⁰ In comparing the structure of PDE5 and PDE5/6, it was found that PDE5/6's H-loop (residues 660 to 683) is 26 Å closer to the M-loop (residues 788 to 811) than in PDE5 (Fig. 1), despite the H-loop amino acid sequence being exactly the same in PDE5 and PDE5/6.¹⁰ The H- and M-loops in the PDE5/6 also display helical structure, whereas in PDE5 the loops are unstructured. In PDE6 the regions of the H- and M-loops are believed to be involved in P γ binding,^{10,11} as observed in the crystal structure of PDE5/6 bound to P γ (PDBID: 3JWR, Fig. 1D). Given that P γ is required for the regulation of PDE6 it is intriguing that structural differences are observed in between PDE5 and PDE5/6.

The efficiency of cGMP hydrolysis also differs between PDE5, PDE5/6, and PDE6. PDE5 and PDE5/6 hydrolyze cGMP to GMP with low efficiency (~0.55 cGMP/second) while PDE6 is the most efficient member of the PDE family, hydrolyzing cGMP at a rate of ~6,000–8,000 cGMP/second.^{4,10,12} PDE6's high efficiency is needed for the near instantaneous process of vision to occur properly. Inhibitors of PDE5 have been developed

for the treatment of erectile dysfunction, the most notable being sildenafil (Viagra). However, given the high degree of structural similarity between PDE5 and PDE6, sildenafil can inhibit PDE6, which can cause vision impairment side effects.¹³

The aims of this study are to apply molecular dynamics (MD) simulations to the PDE5, PDE5/6, and PDE6 catalytic domains in order to develop a structure-function understanding of the enzyme. We seek to rationalize why slight sequence differences give rise to the structural changes in the H- and M-loops, generate hypotheses on the requirement of an inhibitory subunit for PDE6 and understand the origins of the vastly different catalytic efficiencies between PDE5 and PDE6. In this study, we perform equilibrium MD simulations on several systems comprising combinations of apo, sildenafil bound, GMP bound, and P γ bound for PDE5, PDE5/6 and PDE6. We analyze these simulations using standard structural metrics as well as principle component analysis (PCA) and mutual information analysis. From the equilibrium simulations we develop a hypothesis that the α -helix 12 (α 12, residues 674–696 in PDE6) is allosterically connected to the catalytic site in PDE6 based upon differences in the correlated motions between PDE5 and PDE6. We believe this connection may relate to the different catalytic rates of PDE5 and PDE6 and we conclude the study by exploring this hypothesis through the use of ligand unbinding metadynamics simulations, which allow us to estimate the free energy barriers for ligand (un)binding.

Methods

Coordinates for PDE5 and PDE5/6 were taken from X-ray crystal structures to initiate simulations of apo PDE5 (PDBID: 2H40), sildenafil bound PDE5 (PDBID: 2H42)¹⁴, apo PDE5/6 (PDBID: 3JWQ) and P γ bound PDE5/6 (PDBID: 3JWR).¹⁰ Zn⁺² and Mg⁺² ions bound in the active site were retained in all simulations, while waters observed in the crystal structures were removed. To study PDE6 despite the lack of an existing crystal structures in the PDB, we generated an apo homology model for PDE6 using I-TASSER¹⁵ and then modeled in P γ and sildenafil through structural alignments with PDE5/6 structures, which include a P γ bound structure (PDBID: 3JWR).¹⁰ Simulations containing GMP were based upon the PDE5 GMP bound structure (PDBID: 1T9S)¹⁶, but due to mutations in that PDE5 structure we aligned the GMP bound structure with the sildenafil bound PDE5 structure (PDBID: 2H42). Sildenafil was deleted and GMP was copied into the sildenafil bound structure. A PDE6 bound to GMP model was created by an alignment of our I-TASSER model with PDBID: 1T9S and copying over the GMP coordinates. 250 ns equilibrium simulations were performed for both PDE5 and PDE6 bound to GMP. The I-TASSER generated PDE6 model was validated by analyzing the backbone dihedral angles using PROCHECK¹⁷ (Fig. S1). Only three residues (SER143, GLN273 and LEU331) fell outside of allowed regions, and those violating residues were either in unstructured loops or near the terminus. The different systems and simulations times are listed in Table 1. All simulations were run using GROMACS 4.6.5^{18–20} with the CHARMM27 force field^{21,22} in the NPT ensemble. System sizes were approximately 9 nm X 9 nm X 9 nm and consisted of approximately 20,000 TIP3P waters and NaCl at 150 mM concentration. Langevin dynamics were performed with a 2 fs timestep and a friction factor of 1 ps⁻¹. Temperature was maintained at 300 K by the Langevin algorithm and the system pressure was isotropically

coupled to a 1 atm pressure bath with the Parrinello-Rahman barostat. Nonbonded Lennard-Jones interactions were unmodified out to 1.0 nm and then smoothly shifted to zero between 1.0 and 1.2 nm. The electrostatic interactions were computed by the PME method where the direct interactions were smoothly switched off between 0 and 1.2 nm. Force field parameters for sildenafil were generated using SwissParam.²³ Prior to the production simulations, all systems underwent an equilibration phase during which the protein heavy atoms, Zn^{2+} and Mg^{2+} ions and $\text{P}\gamma$ (if present) were restrained with a 1000 kJ/mol*nm² harmonic positional restraint. The equilibration involved three steps: i) energy minimization for up to 5,000,000 steps using the steepest descent algorithm, ii) NVT equilibration for 100 ps, and iii) NPT equilibration for 100 ps.

For each simulation, the following analyses were performed: root mean square deviation (RMSD), root mean square fluctuations (RMSF), calculation of H- and M-loop distances, principal component analysis (PCA), and mutual information analysis. For PDE5 and PDE6 apo systems, PCA was performed on a master trajectory combining the five 300 ns simulations, excluding the first 50 ns of each simulation to account for equilibration. These master trajectories contained 1.25 μs of data. Mutual information analysis was performed on the five individual 300 ns trajectories for each system and the results were then averaged.

Ligand stability was checked using a protein aligned RMSD measurement, which captures both rigid body movements and internal structural changes. Small molecules (sildenafil and GMP) displayed low (~ 3 Å) RMSDs for both PDE5 and PDE6 (Fig. S2A–D), indicating stable interactions and limiting concerns about docking of the ligands into the PDE6 homology model. For the PDE6- $\text{P}\gamma$ simulation the RMSD of $\text{P}\gamma$ is considerably higher reaching a steady value of ~ 8 Å in the last 300 ns of the 900 ns simulation (Fig. S2E). However, the orientation of $\text{P}\gamma$ with respect to PDE6, namely the C-terminal region of $\text{P}\gamma$ occluding entry to the active site of PDE6 is maintained in the simulation, as shown by an overlay of the initial and final orientation of $\text{P}\gamma$ (Fig. S2F).

Mutual information was calculated using the MDEntropy package version 0.3.²⁴ Mutual information ($I(X, Y)$), measures the extent the uncertainty in a given variable (Y) changes when the state of another variable (X) is known. The mutual information is defined as the difference between the Shannon entropies (S) of the marginal distributions of variables X and Y , and the joint Shannon entropy

$$I(X, Y) = S(X) + S(Y) - S(X, Y). \quad (1)$$

$I(X, Y)$ can be calculated from the marginal and joint probabilities as

$$I(X, Y) = \sum_{x \in X} \sum_{y \in Y} p(x, y) \log \frac{p(x, y)}{p(x)p(y)}. \quad (2)$$

The analysis is performed in internal coordinate space to avoid alignment issue artifacts. The dihedral angles of the backbone (ϕ and ψ) as well as sidechain dihedrals are used in the analysis and the mutual information between a pair of residues is calculated by summing

over all dihedral pairs between the residues.²⁵ Mutual information has been successfully used to find co-varying regions of proteins that may be involved in allosteric conformational change.²⁵ Dihedral information was calculated every 10 ps for each 300 ns apo trajectory then the five trials were averaged. In the mutual information figures, the data was filtered to provide clarity; in the figure presenting mutual information of the apo simulations the self I along the diagonal as well as all low I pairs ($I < 0.2$) were set to zero. Differences in mutual information between ligand bound and unbound systems was normalized by dividing through by the average value along the diagonal, effectively setting both bound and unbound states to have $I=1$ along the diagonal.

Metadynamics²⁶ simulations of sildenafil and GMP unbinding from the binding pockets of PDE5 and PDE6 were carried out to evaluate the free energy profile of binding. Initial conformations for sildenafil unbinding metadynamics were selected by first projecting the equilibrium sildenafil bound simulations onto the first two principal components of the respective apo enzyme. Structures were selected from the center and extreme positions in the PC projection map and were used as starting configurations for the metadynamics simulation trials (Fig. S3). For GMP metadynamics unbinding, PCA was performed on the backbone including the carbonyl oxygen of the final 100 ns for each simulations. The starting structures for the metadynamics were chosen one from the center and four extremes of the first two principal components, analogous to the sildenafil procedure.

In metadynamics, Gaussian potentials are deposited along a collective variable (CV) in a history dependent manner to bias the system towards more rarely sampled conformations. For sildenafil unbinding we define the distance between the center of mass (COM) of the protein and the COM of sildenafil as our reaction coordinate. In each trial a single unbinding event was observed (without rebinding) and the simulation was stopped when sildenafil reached more than 30 Å away from its initial binding pose. The PMFs were shifted to have the ligand-bound state be the reference free energy value ($G=0$). For PDE5 complete unbinding took between 54 ns and 84 ns with an average of 67.4 ns, and for PDE6 unbinding took between 21 ns and 65 ns with an average of 49.4 ns. Single pass ligand unbinding metadynamics has been used previously and shown to successfully determine unbinding pathways and associated kinetics^{27,28} and to score the stability of different docked drug poses.²⁹ Metadynamics was performed with a hill height of 0.01 kJ/mol, a Gaussian width of 0.03 nm with a Gaussian potential deposited every 500 steps. For GMP unbinding we found the strong interaction between GMP and the coordinating metal ions lead to large protein distortions during our unbinding events, and therefore we removed the Zn^{+2} and Mg^{+2} ions in these simulations. The CV for GMP metadynamics was the mean square displacement (MSD) of GMP heavy atoms from the starting structure after an alignment on the protein α carbons. A Gaussian width of 0.1 nm², a hill height of 0.05 kJ/mol and a deposition rate of 1/500 steps were used. The simulations were stopped when the MSD reached 45 nm². Traces of the collective variable versus time for both sildenafil and GMP unbinding trials are shown in Fig. S4. All metadynamics simulations were performed with GROMACS 4.6.6 patched with Plumed 2.1.³⁰

Sildenafil and GMP contacts with the enzyme during the transition path were analyzed using MDTraj v1.9.³¹ A residue was considered in contact with the ligand if any heavy atom on

that residue was within 3.5 Å of any heavy atom on the ligand. In order to exclude residues from the bound state, any residue with a heavy atom within 4 Å of a ligand heavy atom at the start of the simulation was not considered.

Results

Equilibrium Simulations

We begin by comparing the apo PDE5, PDE6 and PDE5/6 simulations. Since the PDE6 system is based upon a homology model, we wanted to evaluate if the model displayed structural stability in a range consistent with the experimentally determined PDE5 and PDE5/6 structures. The RMSD probability distributions are presented in Fig. 2 and the individual simulation traces in Fig. S5. It can be observed that PDE5/6 is highly stable with the structure staying within 2 Å of the initial structure, though this is based upon a single long trajectory, whereas PDE5 and PDE6 data came from five shorter (300 ns) simulations. The high degree of stability of PDE5/6 may explain why the catalytic domain of the chimera could be crystallized. PDE5 and PDE6 both display good stability and have a consistent range between of RMSD values between the two systems, with all structures of PDE6 remaining within 4 Å of the initial configuration. PDE5 is slightly less stable than PDE6 and in one simulation the RMSD deviated to beyond 6 Å. Based upon the RMSD distribution we can conclude the homology model of PDE6 has the same range of structural stability as the experimentally determined structures for PDE5 and PDE5/6 and we believe it can serve as an informative model for PDE6.

We next examined the root-mean-squared-fluctuations per residue (RMSF) during the equilibrium simulations to evaluate if the different systems had different regions of flexibility. Fig. 3A presents the RMSF comparison between apo PDE5, PDE6 and PDE5/6 and we observe significant differences. The major regions of flexibility are in the H- and M-loop regions, though PDE6 has considerably lower fluctuations in these regions than PDE5. Interestingly, PDE6 displays a region of high flexibility peaking around residue 699 (shown as residue 215 in Fig. 3A), which is towards the base of α -helix 12 (α 12). PDE5/6 has low fluctuations throughout the structure, including in the H- and M-loops, which is consistent with the low RMSD of this structure (Fig. 2). We also examined the effect of the inhibitor (sildenafil) on both PDE5 and PDE6 and the effect of the inhibitory subunit (P γ) on PDE6 and PDE5/6, the bound location of sildenafil and P γ are shown in Fig. 3E and Fig. 3F, respectively. Sildenafil has a significant effect on PDE5 by reducing the flexibility in the M-loop (Fig. 3B). However, PDE6 does not display much variation in the RMSFs between apo and inhibitor bound simulations (Fig. 3C). Sildenafil is known to be a 10-fold more potent inhibitor of PDE5 than PDE6 (IC₅₀ of 2.9–6 nM and 74 nM, respectively),^{32–34} so if PDE5 relies on enzyme flexibility for catalysis, then the more dramatic effect seen in PDE5 may correlate with the enhanced inhibitory efficacy against PDE5. The small effect P γ has on PDE6 fluctuations is somewhat unexpected, though as a control the effect of P γ on PDE5/6 was also examined. P γ does function to inhibit PDE5/6 but similarly to PDE6 the effect of P γ on the fluctuations of PDE5/6 is relatively minor (Fig. 3D)

Correlated Motions

While the atomic fluctuations show little variation between the apo and the inhibitor bound simulations of PDE6 (Fig. 3C), this does not preclude the possibility that the direction of motion could be different between these systems. We have performed principal component analysis (PCA) on the equilibrium simulations of PDE6 in apo and inhibitor bound states, as well as for PDE5 apo. Both PDE5 and PDE6 apo simulations are well characterized by a few low frequency modes, as the first 10 modes account for 78% of the total variance for PDE6 and 84% in the PDE5 system. We examine the projection of the apo and inhibitor bound simulations onto the space of the PDE6 apo first two modes in Fig. 4A. We observe significant restriction of the motion of PDE6 in the PC1-PC2 subspace when PDE6 is bound by either sildenafil or P γ . This constriction of sampling in the PC1-PC2 subspace can be interpreted as the inhibitor bound systems having different collective dynamics than the unbound native enzyme.

We also examined the dynamics of the H- and M-loops as these motifs form a surface which is adjacent to enzyme active site. We measured the distance between a Ser-Leu pair at the tips of the H- and M-loops (Ser617-Leu756 in PDE6 and Ser667 - Leu796 in PDE5) to examine the loop separation. These residue pairs were chosen based upon the close proximity of the corresponding residues in the PDE5/6 crystal structure (PDBID: 3JWQ). The distance distribution (Fig. 4B) for PDE6 shows a sharply peaked distribution with a peak at less than 1 nm, indicating the H-M loops are in contact and either are relatively static or moving in a positively correlated fashion. In contrast, PDE5 shows a broad distribution with peaks in the range of 2–3 nm. The observed flexible separation in the H-M loops for PDE5 is consistent with a previous modeling study,³⁵ though the simulation length was 100 times shorter in the previous work. This difference in the H-M distance distributions is informed by examination of the first PC for each system. In PDE6 (Fig. 4C), the first PC accounts for 27% of the variance and shows the most significant motion is in the H-loop, M-loop, α 12 and the loop at the base of α 12. The motion can be described as a clam shell motion where the H- and M-loops are moving down together and α 12 and is moving up to close (or open) access to the binding site. In PDE5 (Fig. 4D) the first PC is very robust and accounts for 40% of the variance. The motion in the PDE5 PC1 is concentrated in the H- and M-loops, but shows the loops move in opposite directions causing separation between the loops, consistent with the separation distance distribution in Fig. 4B.

To further examine the correlations within PDE5 and PDE6, mutual information analysis was performed. Mutual information shows how knowledge about the state of one residue (X) reduces the uncertainty in the state of another residue (Y). The reduction in uncertainty indicates the conformational distribution of the two residues are correlated and therefore is an effective approach to identifying allosteric sites within proteins.³⁶ In apo PDE5 (Fig. 5A) significant mutual information is observed for the intersection of the H- and M-loop as well as the M-loop with itself. While the regions of significant mutual information are well defined in PDE5, there is wide-spread correlations within PDE6 (Fig. 5B). The strong correlation between the H- and M-loops present in PDE5 is greatly reduced in PDE6. The interpretation of this difference may be that the H- and M-loops are cooperatively folding in PDE5, while they are rigid in PDE6. In PDE6 α 12 shows high mutual information across

many different regions of the protein. The rigidity of the structured H-loop allows global bending and hinging motions consistent with the motion of PDE6 PC1, which we suspect may allow global communication through the protein. This indicates that the conformation of $\alpha 12$ is highly connected to the conformation of the rest of the protein and therefore may serve as a critical regulatory element of the enzyme. There is significant mutual information between PDE5 $\alpha 12$ and other regions of the protein, but this communication is more localized. The increased flexibility and disorder of the H-loop allows the sidechains of the H-loop to interact with $\alpha 12$ without transferring information through the rigid core of the protein. The correlations of $\alpha 12$ with spatially distant regions of both PDE5 and PDE6 is not intuitively obvious based upon structural considerations and it implies an allosteric regulation mechanism is active.

The effect of ligand (sildenafil and $P\gamma$) binding on the enzyme mutual information was also analyzed. The difference between the apo and ligand bound systems are presented in Fig. 6. The effect of sildenafil on PDE5 shows both increases and decreases in mutual information throughout the protein (Fig. 6A). The most significant change in mutual information occurs between the H- and M-loops where the mutual information is reduced upon ligand binding. The effect of sildenafil on the mutual information of PDE6 was less pronounced, showing slight reductions through the protein (Fig. 6B). The effect of $P\gamma$ binding on PDE6 was widespread, with more significant reductions in mutual information than the reductions in mutual information caused by sildenafil binding and displaying a consistent reduction in the $\alpha 12$ region (Fig. 6C). Overall the results point toward a consistent picture of sildenafil being more effective against PDE5 than PDE6 and that $P\gamma$ is an effective regulator of PDE6 dynamics.

Ligand (un)Binding

Based upon the analysis of the principal components and mutual information of PDE5 and PDE6, we speculated that the collective motions of each enzyme could have an effect on ligand access to the active site. The correlated motions in PDE6 of the H-loop, M-loop and $\alpha 12$ region appear to generate an opening to the binding site, whereas the motion of the H- and M-loops in PDE5 are not concerted with regions below the binding pocket. We hypothesized that the high catalytic efficiency of PDE6 would be reflected by a relatively flat energy surface for ligand binding and we have utilized metadynamics²⁶ to estimate the free energy profiles. By performing unbinding simulations of sildenafil from PDE5 and PDE6 we can evaluate the energetics of ligand unbinding by detecting barriers in the binding pathway that could affect kinetic aspects of catalysis.

Five unbinding trials were performed for both PDE5 and PDE6 and the averaged free energy profiles of sildenafil binding are presented in Fig. 7A and the individual trial profiles are presented in Fig. S6. The profile is generated from averaging five metadynamics trials, in which each trial was initialized from a different starting configuration, extracted from the equilibrium sildenafil bound simulations. The free energy profiles show that there is a more favorable G of binding for PDE5 (-13 kcal/mol) than PDE6 (-9 kcal/mol), which is not only qualitatively consistent with the lower IC_{50} of sildenafil against PDE5 than PDE6, the calculations are also quantitatively consistent with the experimental $G_{binding}$ based upon IC_{50}

values (PDE5: -11.9 kcal/mol; PDE6: -9.8 kcal/mol).^{32,34} While the free energy change of binding is smaller for PDE6, the barriers along the binding pathway are also smaller, which would lead to faster binding kinetics (k_{on}). On the other hand, the PDE5 profile displays a large barrier (~ 4 kcal/mol) at around 1 nm. Also, the unbinding barrier is much smaller for PDE6 (~ 4.5 kcal/mol) than PDE5 (~ 10 kcal/mol), which may reflect shorter residence times (faster k_{off}), which would also indicate less effectiveness of sildenafil against PDE6.

The pathways of sildenafil unbinding are visualized in Fig. 7B–C, by tracing the ligand coordinates during the metadynamics runs. In PDE5, sildenafil interacts with several parts of the protein during unbinding. In some trials sildenafil interacts with α -helix ($\alpha 15$, residues 809–836 in PDE5), on the right side of the binding pocket, briefly and then moves closer to the $\alpha 12$ region where it exits. Other PDE5 trials move toward the H-loop and exit on the left side of the binding pocket. For PDE6 all trials move toward $\alpha 15$ and wrap around this helix before exiting the binding pocket. Sildenafil does not make contact with the H-loop in any of our PDE6 trials and only contacts the $\alpha 12$ region in one trial. The additional rigidity and helical stability of the H-loop, M-loop and $\alpha 12$ region seen in both the metadynamics simulations as well as the apo equilibrium simulations, appears to create a larger binding pocket with lower barriers to unbinding.

The contacts sildenafil makes with PDE5 and PDE6 as it leaves the binding pocket were quantified using the MDTraj compute neighbors utility. This data is presented in Fig. 8 and is based upon the metadynamics unbinding trajectories. The number of frames in which sildenafil is in contact with a PDE5 or PDE6 residue is tabulated over all five trajectories and converted into a contact percentage. In PDE5 simulations, sildenafil makes contact with the H-loop and $\alpha 12$ and also to a lesser extent is in contact with the M-loop and $\alpha 15$. In PDE6 simulations, sildenafil makes significant contacts with $\alpha 12$ and $\alpha 15$, while it does not significantly contact the H- or M-loops. The contact analysis is consistent with a model in which PDE6 provides a focused binding pathway, while PDE5 appears to allow a broader range of pathway directions.

Comparing the free energy profiles and unbinding pathways, we are able to correlate some of the energetic features with aspects of the unbinding pathways. In PDE5, sildenafil has to cross a large barrier about 5 Å from its starting location, which appears to arise as the ligand becomes constricted between the flexible H-loop, M-loop and $\alpha 15$. Sildenafil then encounters a free energy well as it gets past $\alpha 15$, but then faces another large free energy barrier as it crosses over the $\alpha 12$ region while the H-loop sterically hinders its exit path. In PDE6 the H-loop is further away from sildenafil than in PDE5 making it easier for the ligand to leave its initial binding pose. The unbinding paths in PDE6 are characterized by a pathway contacting $\alpha 15$, $\alpha 12$, or in between these two helices. Sildenafil also stays in contact with the enzyme surface longer in PDE6 as it is still in contact at a COM distance of 29 Å, whereas in PDE5 it breaks contact at about 22 Å. During binding this feature may correspond to a larger area of first encounter that can “catch” sildenafil, then the correlated motions (PC1) may serve to shuttle the ligand into the binding pocket and/or provide accessibility to the binding pocket.

While understanding the ligand binding pathway of sildenafil to PDE5 and PDE6 may provide some insights into to general ligand binding and differences in catalytic rates, to probe the biological function more directly we performed metadynamics unbinding simulations of GMP from PDE5 and PDE6. We observe that PDE6 has a flat landscape with no significant barriers (within the uncertainty of calculation) along the pathway in going from unbound to bound (Fig. S7–S8). This observation is consistent with a diffusion-limited catalytic rate which PDE6 is known to have. In contrast the PDE5 pathway does have a deep well at an intermediate state around 5.5 nm² in MSD space (~ 23 Å in RMSD).

Discussion

We believe that this work has provided evidence of a region of structural and functional significance in PDE6, the α 12 helix. The RMSFs of all of the PDE6 systems highlight that in addition to the H- and M-loops showing high flexibility, the α 12 region also shows high flexibility. This flexibility is absent from the corresponding residues in PDE5. The motions of the first principal component support this, highlighting that the α 12 region and the H- and M-loops cohesive ‘breathing’ movements are representative of PDE6’s overall motions. The importance of α 12 and the α 12 basal loop to PDE6 function has also been indicated experimentally. In a chimera in which residues 676–741 in PDE6 were substituted by the corresponding residues in PDE5 (residue 723–741) the resulting chimera was inactive and had poor solubility.³⁷

Our hypothesis is that this concerted motion helps provide access and possibly recruit cGMP to the catalytic pocket to allow hydrolysis, which results in the high activity observed in PDE6. The lack of fluctuations and correlations in the corresponding residues in PDE5 and PDE5/6 along with the lack of the breathing motion in the first principal component indicates a difference in collective motions between PDE5 and PDE6. The correlated motions in PDE6 which couples the H-loop, M-loop and α 12 regions may influence the catalytic rates in several ways, e.g. i) providing a binding surface for initial ligand interactions, ii) providing an opening motion that facilitates access to the binding site and iii) providing motions which could shuttle the ligand towards the binding site. It should be noted that the relationship between enzyme catalysis and protein conformational dynamics is a debated one. Theoretical studies have argued that the enzyme dynamics do not couple to the chemical reaction rate in enzymes.^{38,39} However, the overall catalytic rate can be effected by protein dynamics, as it has been shown the conformational changes associated with product release can be rate-limiting in some systems. Other studies have supported a link between dynamics and catalysis, in particular for dihydrofolate reductase.⁴⁰

Determination of a PDE6 structure by X-ray crystallography has yet to be achieved, though a recent study generated a full-length rod PDE6 dimer structure using cryo-EM density and crosslinking restraints.¹¹ In the present study we have generated a homology model to study the cone PDE6 catalytic domain, which is an approach that has been used in previous modeling studies to examine small molecule interactions with PDE6.^{33,41,42} In all studies, including the current one, the PDE6 catalytic domain was studied as a monomer despite the full-length protein being known to exist and function as dimer.³ In the first such study (Cahill et al.)⁴², the PDE6 modeling consisted of small molecule docking and protein

dynamics and stability were not evaluated. In a subsequent study (Huang et al.)³³ the PDE6 model was unstable in the absence of bound ligand, but through MM-PB(GB)SA calculations they were able to show different affinities for sildenafil and tadalafil (another PDE5 inhibitor) towards PDE5 and PDE6. They employed three different variants of the MM-PB(GB)SA calculations and the results were not always consistent across the methods, for example the IC_{50} for tadalafil is two orders of magnitude higher than sildenafil against PDE6, but only two of the three calculations predicted a more favorable $G_{binding}$ for sildenafil. Simulations timescales in that study ranged from 30 – 150 ns. The most recent study (Kayık et al.)⁴¹ did produce a stable apo PDE6 model ($< 5 \text{ \AA}$ RMSD) during a 50 ns simulation, and they also observed increased fluctuations in the $\alpha 12$ region consistent with our findings. That study was focused on identifying compounds that could have selectivity for PDE5 over PDE6 or PDE11, based upon MM-PB(GB)SA. Our study is different to previous studies in that we have put more emphasis on understanding the protein dynamics and how PDE5 and PDE6 display different motions. Furthermore, we employ a different approach to understanding the ligand-protein interactions by using metadynamics which allows us to observe pathways and barriers, rather than just free energy differences.

This investigation has only examined monomers of PDE5 and PDE6 and this is a limitation to the current work. Both PDE5 and PDE6 exists in vivo as dimers and there are allosteric interactions between the subunits.^{43,44} While the computational cost will rise significantly in moving from monomer to dimer simulations, especially if one is to consider a full-length structure containing the GAF domains, this is an important endeavor that is becoming more feasible with rising computational power.

Conclusions

When PDE6 is active, it is necessary for the enzyme to hydrolyze cGMP rapidly so that the signal can be passed on quickly and vision can occur nearly instantly. Our hypothesis is that $\alpha 12$ works in concert with the H- and M-loops to cause active transport of cGMP to the catalytic pocket for hydrolysis. When there is no light, there should be no signal propagation and no vision, so PDE6 should be completely off. The γ -subunit not only blocks access to the catalytic pocket, it also disrupts any allosteric interactions between the H- and M-loops and $\alpha 12$ by physically blocking direction connections between the regions (see Fig. 1D). The results of the equilibrium simulations have been further explored through additional analyses on the mutual information shared through the enzymes and through enhanced sampling (metadynamics) simulations which allow for the ligand binding mechanism and energetics to be estimated. We hope this study may inform future experimental efforts to study PDE6 possibly through creation of a new chimera that accounts for importance of $\alpha 12$ for PDE6 and its functionality. Additionally, $\alpha 12$ may present a possible allosteric therapeutic target for retinitis pigmentosa or other diseases in which PDE6 is implicated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Structure and sequence of PDE catalytic subunits.

The structures of PDE5 (A), PDE5/6 (B) and PDE6 (C) are shown with coloring of the H-loop (blue), M-loop (red) and α -helix 12 (yellow). Mg^{2+} (pink) and Zn^{2+} (grey) ions in the catalytic site are shown as spheres. The PDE5 and PDE5/6 structures are taken from PDBIDs: 2H40 and 3JWQ, respectively, while the PDE6 structure is a homology model. D) The interactions of the $P\gamma$ (orange) with PDE5/6 is displayed from three perspectives to highlight interactions with H- and M-loops and α 12. (PDBID: 3JWR) E) The sequence alignment of all three sequences are presented with colored overbars matching the structural regions highlighted in panels A-C. In addition, the green overbar region is the region in which the PDE6 sequence was inserted into the PDE5 sequence to generate the PDE5/6 chimera.

Figure 2.
RMSD comparison between PDE5, PDE6 and PDE5/6.

Figure 3. PDE5, PDE5/6 and PDE6 RMSF comparisons.

A) RMSF comparison between apo PDE5, PDE6 and PDE5/6, residue numbering is 1-based since the constructs start at different residue numbers. B) RMSF comparison of PDE5 apo and inhibitor bound (sildenafil). C) RMSF comparison of PDE6 apo and inhibitor bound (sildenafil or P γ). D) RMSF comparison between PDE5/6 apo and P γ bound. The underbars in A-D are the locations of the H-loop (blue), α 12 (green) and M-loop (red). The binding location of the inhibitor sildenafil is shown in (E) in green, and the inhibitory subunit (P γ) is shown in (F) in orange. The H- and M-loops are colored in a consistent manner with Figure 1.

Figure 4. Collective motions in PDE6 and PDE5.

A) The simulations of PDE5 apo, sildenafil bound and P γ bound are projected on the PDE6 apo principal components 1 (PC1) and 2 (PC2) subspace. B) The H-M loop distance probability distribution for apo PDE5 and PDE6. The distance is defined by the center of mass distance between residues SER617 and LEU756 in PDE6 and the corresponding residues (SER667 and LEU796) in PDE5. C) The first PC of PDE6 apo accounts for 27% of the total variance. D) The first PC of PDE5 apo accounts for 40% of the total variance.

Figure 5. Mutual information of apo systems.

The mutual information for PDE5 (A) and PDE6 (B) is presented. In both figures the H-loop, M-loop and $\alpha 12$ regions are denoted by horizontal and vertical boxes.

Figure 6. Change in mutual information upon ligand binding.

A) The difference in mutual information between PDE5 bound to sildenafil and PDE5 apo.

B) The difference in mutual information between PDE6 bound to sildenafil and PDE6 apo.

C) The difference in mutual information between PDE6 bound to P γ and PDE6 apo.

Figure 7. Energetics and pathways of sildenafil unbinding from metadynamics.

A) The free energy as a function the distance between the center of mass of the drug and the center of mass of the protein. The average of 5 PMFs for each system are represented by the lines and the shaded regions represent the standard error over the five runs. Externalization pathways are shown from two vantage points for PDE5 (B) and PDE6 (C). Each pathway is traced by a different color and $\alpha 12$ is colored yellow in all structures to provide a common reference point.

Figure 8. Sildenafil contacts with PDE during unbinding pathways.

Contacting residues are indicated on the PDE5 (A) and PDE6 (B) structures. Color scale goes from blue (no contacts) to red (highest percentage of contacts). The contacting percentage, averaged over 5 metadynamics trajectories, is shown for PDE5 (C) and PDE6 (D). In C-D the residues which are contacting sildenafil in the bound state are given a contact percentage of zero, to highlight the residues which make contact in the transition out of the binding pocket. The underbars in C-D are the locations of the H-loop (blue), α 12 (green) and M-loop (red).

Table 1.

Systems and simulation runs and lengths

System	Type	Runs	Run length	Total Sampling
PDE5 apo	Equilibrium	5	300 ns	1.5 μ s
PDE6 apo	Equilibrium	5	300 ns	1.5 μ s
PDE5/6 apo	Equilibrium	1	900 ns	900 ns
PDE6-P γ	Equilibrium	1	900 ns	900 ns
PDE5/6-P γ	Equilibrium	1	100 ns	100 ns
PDE5-Sildenafil	Equilibrium	1	200 ns	200 ns
PDE6-Sildenafil	Equilibrium	1	200 ns	200 ns
PDE5-GMP	Equilibrium	1	250 ns	250 ns
PDE6-GMP	Equilibrium	1	250 ns	250 ns
PDE5-Sildenafil	Metadynamics	5	54 –84 ns	337 ns
PDE6-Sildenafil	Metadynamics	5	21–65 ns	247 ns
PDE5-GMP	Metadynamics	5	27–58 ns	220 ns
PDE6-GMP	Metadynamics	5	10–40 ns	93 ns
			Total	6.7 μ s