Published in final edited form as:

FEBS J. 2011 July ; 278(14): 2579–2595. doi:10.1111/j.1742-4658.2011.08187.x.

Insight into the Phosphodiesterase Mechanism from Combined QM/MM Free Energy Simulations

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Summary

Molecular dynamics simulations employing a combined quantum mechanical and molecular mechanical potential have been carried out to elucidate the reaction mechanism of the hydrolysis of a cyclic nucleotide cAMP substrate by phosphodiesterase 4B (PDE4B). PDE4B is a member of the PDE superfamily of enzymes that play crucial roles in cellular signal transduction. We have determined a two-dimensional potential of mean force for the coupled phosphoryl bond cleavage and proton transfer through a general acid catalysis mechanism in PDE4B. The results indicate that the ring-opening process takes place through an S_N2 reaction mechanism, followed by a proton transfer to stabilize the leaving group. The computed free energy of activation for the PDE4B-catalyzed cAMP hydrolysis is about 13 kcal/mol and an overall reaction free energy is about -17 kcal/mol, both in accord with experimental results. In comparison with the uncatalyzed reaction in water, the enzyme PDE4B provides a strong stabilization of the transition state, lowering the free energy barrier by 14 kcal/mol. We found that the proton transfer from the general acid residue His234 to the O3' oxyanion of the ribosyl leaving group lags behind the nucleophilic attack, resulting in a shallow minimum on the free energy surface. A key contributing factor to transition state stabilization is the elongation of the distance between the divalent metal ions Zn^{2+} and Mg^{2+} in the active site as the reaction proceeds from the Michaelis complex to the transition state.

I. INTRODUCTION

Signal transduction plays an essential role in cellular functions.[1–3] One of the most vital classes of signaling proteins are enzymes catalyzing nucleotide dephosphorylation, such as

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Supporting information

The following supplementary material is available:

Doc. S1. B3LYP/6-31+G(d) optimized structure of a model of product-bound active site.

Doc. S2. Ensemble-average structure of selected 331 atoms for Michaelis complex.

Doc. S3. Ensemble-average structure of selected 331 atoms for transition state in hydrolysis.

Doc. S4. Ensemble-average structure of selected 331 atoms for product-bound complex.

This supplementary material can be found in the online version of this article.

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cyclic-nucleotide phosphodiesterases (PDEs),[3–6] with which many biological responses are mediated by the cellular concentrations of cyclic adenosine 3'-5'-monophosphate (cAMP) and cyclic guanosine 3'-5'-monophosphate (cGMP). By degradation of the secondary messengers, PDEs are responsible for promptly and effectively terminating cellular responses. Phosphodiesterases catalyzes the hydrolysis of cAMP and cGMP to form 5'-adenomonomophosphate (AMP) and 5'-guanomonophosphate (GMP), respectively (Scheme 1). Since the role of PDEs is to rapidly terminate the cellular response to a signal for specific function, several drugs have been developed to inhibit different members of the enzymes.[4] For example, the drug Viagra® (sildenafil citrate) for the treatment of erectile dysfunction, inhibits PDE5 to keep smooth muscles relaxed for the blood flow.[3, 7, 8] Another drug, Rolipram®, which has been commonly used to treat inflammation by inhibiting PDE4,[4, 9, 10] has recently been suggested to be beneficial to patients with Alzheimer's disease[11] because one of the cAMP-dependent protein kinases (PKAs) involves in the cellular processes associated with long-term memory.[4, 12] Owing to the importance in understanding signal-transduction pathways and the general interest in designing new drugs against phosphodiesterases, there have been extensive experimental and theoretical studies of the catalytic activities of PDEs.[3–38] Nonetheless, the reaction mechanism of PDEs is still not fully understood, particularly on the issues of concerted and stepwise pathways via S_N2 or S_N1 -like processes. In this work, we carried out molecular dynamics (MD) simulations employing combined quantum mechanical/molecular mechanical (QM/MM) potentials [39–53] to model the hydrolysis of cAMP by the enzyme PDE4B, which provides further insights on the general features of phosphate hydrolysis.

The PDE superfamily of enzymes can be classified into 11 members based on their genome and regulatory properties, although, these PDEs fall into three categories: (1). cAMP specific (PDE 4, 7, and 8), (2). cGMP specific (PDE 5, 6, and 9), and (3). dual specificity both for cAMP and cGMP hydrolysis (PDE 1, 2, 3, 10, and 11). While the structure of a small fragment of PDE4D was reported in 1996,[5, 13] key insights into the understanding of the catalytic active site of PDEs were obtained following the determination of the crystal structure of PDE4B in 2000.[14] Subsequently, crystal structures of seven other PDE members (PDE 1–5, 7, and 9) have been reported.[5] A variety of structures, including the unligated apo-enzyme and ligand-bound complexes, are now available, all of which show a conserved catalytic core with ~300 amino acids and ~14 α -helices. The structure of PDE4 and probably all other PDEs can be further divided into three subdomains.[6, 14] The active site of PDEs is buried in a deep pocket located at the junction of these three subdomains, composed of highly conserved residues. In the active site, there are two metal ions that are coordinated by residues from the three subdomains (Figure 1), which help to hold the subdomains together. The first metal, which is more deeply buried in the binding pocket, has been identified as a zinc (Zn^{2+}) ion, coordinating with a bridging hydroxide ion, a phosphoryl oxygen atom of AMP, and amino acid residues His238, His274, Asp275, and Asp392, as revealed in the product-bound PDE4B-AMP ternary complex.[15] These coordinating residues, which are absolutely conserved across all other PDE members, come from three subdomains. These observations confirm that the function of this Zn^{2+} ion plays a structural role and is indispensable for catalysis. The identity of the second metal ion, which is more solvent-exposed, could not be confirmed by X-ray diffraction, though it is often described as a magnesium (Mg^{2+}) ion (or a Manganese ion).[5] The second metal ion also shows six coordinations, including the Asp275 and the bridging hydroxide ion that coordinate with the Zn^{2+} ion. Three crystal water molecules together with another phosphate oxygen atom complete the octahedral coordination geometry for this metal ion.

In addition to the interactions of the phosphate group of AMP with the two metal ions, the adenine group and ribosyl ring of AMP are also bound subtly with the active site. The pentose ring has a configuration of O3' forming a hydrogen bond with His234 (Figure 1),

which could be an important integral part in catalysis. The adenine orients to the hydrophobic pocket and forms four hydrogen bonds with the side chain of Asn395, Gln443 (Figure 1). The hydrogen binding network around these two amino acids has been proposed to be important for substrate nucleotide selectivity (e.g., the 'glutamine-switch' mechanism). [4, 5, 16, 38]

Variations in crystal structures provide invaluable information on the PDE mechanism. For example, after soaking the substrate cAMP with unligated PDE4, the bridging hydroxide becomes part of the phosphate group in the PDE4-AMP complexes.[5, 15, 17] This clearly suggests that the hydroxide anion is the nucleophile in the hydrolysis of the cyclic phosphodiester bond, and is also consistent with quantum chemical calculations and MD simulations performed by Zhan and co-workers.[26–28] Moreover, His234 is the acidic residue to protonate the O3' leaving group, as implicated by the hydrogen bond between His234 and the O3' oxygen found in the PDE4-AMP and PDE5-GMP structures.[4] Not only His234 is strictly conserved, but also the three amino acids that His234 interacts with (e.g., Tyr233, His278, and Glu413 in the PDE4B-AMP complex; see Figure 5 in Ref. [15]) are functionally conserved. Therefore, at least four residues are required for the general acid site, which may reveal the significance of this protonation step. The similarities in the conserved residues in the active site, and in substrate-binding between AMP and GMP suggest that the above proposed mechanism could be universal for all PDE family members. [5]

On the theoretical side, several groups carried out molecular dynamics (MD) simulations and quantum chemical minimizations to understand various properties of PDEs.[26–38] These studies were performed either as ground state stable species in MD simulations, or as active site models to mimic the catalytic mechanism to gain knowledge about the potential energy surface. Useful information from these simulations has been obtained. For instance, Chen and Zhan employed *ab initio* molecular orbital calculations to show that the dominant reaction pathway for the cAMP hydrolysis in neutral solution is a direct nucleophilic attack on the phosphorus atom by a hydroxide anion,[29] and that the hydrolysis proceeds by an S_N2 -like mechanism. The theoretical results are consistent with experimental studies using isotopic labeling to show a direct attack by a hydroxide ion in the hydrolysis of phosphodiester substrates.[18] Zhan et al. published a series of papers, using density-function-theory (DFT) optimizations and classical force-field MD simulations either for a full PDE apo-enzyme or for simplified models, suggesting that a hydroxide anion, instead of a water molecule, is the bridging ligand between the two metal ions.[26–28] The same conclusion about the identity of nucleophile as a hydroxide ion has also been drawn for a similar binuclear metal enzyme, phosphotriesterase.[30, 40]

In this study, we incorporate protein dynamic and thermal contributions in MD simulations using a combined QM/MM potential to generate a two-dimensional (2-D) free-energy profile for the phosphate hydrolysis and leaving-group protonation steps in PDE catalysis. This technique has been successfully applied to a number of enzymes and ribozymes to gain insights into their reaction mechanisms,[40, 42–52] including our recent study of phosphotriesterase.[40] Based on the 2-D potential of mean force (PMF) and the structural changes of the active site during the catalytic process, we conclude that the PDE-catalyzed phosphate hydrolysis is an asynchronous S_N2 type. The nucleophilic attack on the cAMP by the bridging hydroxide is followed by the protonation on the phosphate dianion from His234. The corresponding ensemble-average structures of the reactant, transition state, and product in Cartesian coordinates are provided in Supporting information. Importantly, from the Cartesian coordinates, we can see that the hydrolysis reaction is accompanied by significant variations in the inter-metal distance along the reaction path. Similar metal breathing motions have been observed in other binuclear metal enzymes, including xylose

isomerase,[54–57] PTE,[40] alkaline phosphatases,[53] and Ribonuclease H.[58, 59] Binuclear metal enzymes constitute a growing family of enzymes, which are important in pharmacology and metabolisms,[60, 61] and have been investigated by Klein and coworkers on a number of systems.[58, 59, 62] Unlike the case of xylose isomerase, the changes in metal separation for either phosphotriesterase or PDE have not yet been determined by X-ray crystallography. It would be of particular interest to investigate experimentally the metal separation as a result of the enzymatic reaction.

II. RESULTS AND DISCUSSION

A. Two-Dimensional Free-Energy Profile

The two-dimensional (2-D) potential of mean force (PMF) for the coupled proton transfer and phosphate hydrolysis reactions catalyzed by PDE4B is shown in Figure 2. The horizontal axis represents the reaction coordinate for the nucleophilic attack by the bridging hydroxide ion:

$$z_1 = r_{\text{PO}_3'} - r_{\text{OH}P}, \quad (1)$$

where $r_{\text{PO}_3'}$ and $r_{\text{OH}P}$ are the distance of the leaving group O3' oxygen and the distance of nucleophile hydroxide oxygen from the phosphorus atom, respectively. The protonation coordinate is described by the vertical axis:

$$z_2 = r_{\text{NH}} - r_{\text{HO}_3'}, \quad (2)$$

where r_{NH} and $r_{\text{HO}_3'}$ are the separations of the His234 proton from the donor to the acceptor atoms, respectively. Figure 2 reveals that the mechanism of the by PDE4B proceeds as a step-wise process. Along the minimum free-energy reaction path (MFEP), the nucleophilic attack on the phosphorus atom of cAMP occurs first, followed by a proton transfer from His234 to the oxyanion leaving group of cAMP. The substrate-bound Michaelis complex is located at the coordinate of (−1.2, −1.0) in Figure 2, in angstroms throughout, with a free energy of 17.4 kcal/mol above the product state near (2.9, 2.0). The transition state for the hydrolysis is at (−0.1, −0.8), which is the rate-limiting step for the overall reaction with a free energy barrier of 13.2 kcal/mol. In contrast, for the concerted pathway, the free energy barrier at the coordinate (−0.1, 0.0) is more than 7 kcal/mol higher.

Although the protonation of the O3' oxygen of the ribosyl leaving group from His234 occurs after the formation of an intermediate in the 2-D PMF (Figure 2), the reaction path in which the proton is transferred to O3' concertedly without the intervention of intermediate at (2.3, −0.9) (red dotted curve in Figure 2) would have the same activation free energy as that along the MFEP reaction path. The significant thermodynamic driving force of the product complex, which is about 7.5 kcal/mol more stable than the intermediate, may help to branch the dynamic pathway in favor of the process without intermediate. Therefore, as the cyclic phosphate bond is cleaved, there could be no need for a transition state for the proton transfer of the general acid catalysis. Nonetheless, the relative free energies at the key stationary points (z_1 , z_2) following the MFEP reaction path are summarized in Figure 3, which are compared with the free energies branched through a hilltop barrier without the formation of the intermediate.

The estimated reaction energy from the reactant to product in Figure 3 is −17.4 kcal/mol, whereas the free energy change from the intermediate to the product is −7.5 kcal/mol. This relatively large exergonicity for the overall cyclic phosphate hydrolysis is consistent with DFT calculations in the gas phase (−17.9 kcal/mol) [31], and the experimental results

ranging from -11 to -14 kcal/mol in aqueous solution determined by calorimetry and measuring equilibrium constants.[19, 20] These results suggest that the PDE4B-AMP complex is much more energetically favorable than the substrate-bound complex, which is reflected by the observation that the product-bound crystal structure is obtained after it is soaked with cAMP substrate.

To elucidate the catalytic power of PDE, we have also examined the uncatalyzed hydrolysis of a model for cAMP (cAMPm) and trimethylene phosphate (TMP) in aqueous solution, represented by a $40 \text{ \AA}$ cubic box with periodic boundary conditions. To reduce computational cost in the present QM/MM simulations, the adenine base of cAMP is replaced with a hydrogen atom in the cAMPm model. The computed free energy barriers for the cAMPm and TMP hydrolysis reactions in water are about 27 and 32 kcal/mol, respectively, in good agreement with experimental values (~ 29 kcal/mol for cAMP and ~ 32 kcal/mol for TMP) and with *ab initio* calculations using an implicit solvent model (~ 29 kcal/mol for cAMPm and ~ 32 kcal/mol for TMP).[32] Note that Tunon and Moliner and coworkers used the AM1/d-PhoT model to determine the kinetic isotope effects for the hydrolysis of another substrate, p-nitrophenylmethylphosphate, in water with good agreement with experimental data.[63] This further demonstrates that the present AM1/d-PhoT QM model for phosphate hydrolysis reactions is adequate.

On the experimental side, the rate constants k_{cat} for phosphate hydrolysis by PDE4 enzymes vary from 3.9 s^{-1} for PDE4D[21] to 3702 s^{-1} for PDE4A.[22] Using transition state theory, [64] we obtain free energy barriers of 12.8 to 16.6 kcal/mol for PDE4-catalyzed cAMP hydrolysis, which may be compared with our simulation result (13.2 kcal/mol). Overall, PDE4B lowers the free energy of activation for the hydrolysis of cAMP by about 14 kcal/mol, in comparison with the uncatalyzed process in water. The tremendous catalytic power originates from the interactions of cAMP and the nucleophile with residues in the binuclear metal center, which will be discussed in the following sections.

Recently, Salter and Wierzbicki found that the phosphodiesterase reaction is concerted,[33] using Gaussian 03[65] with the ONIOM method at the B3LYP/6-31G(d) and PM3 levels. The authors located the reactant state, the transition state, and the product state geometries by energy minimization on a truncated model. However, the optimized reactant and transition states exhibit quite unusual characters. For their reactant state, the phosphorus atom has five coordinates with distances of 1.94 and $1.84 \text{ \AA}$ for the forming (r_{OHP}) and breaking ($r_{\text{PO}_3'}$) bonds to the phosphorus atom, respectively (see Figure 1), whereas they are 1.72 and $2.87 \text{ \AA}$ at the transition state, suggesting an exceedingly late transition structure. By contrast, penta-coordinated phosphorus intermediate is not found for the hydroxide nucleophilic attack of cAMP in solution in the work of Chen and Zhan.[29] Further, in the exceedingly late transition state, the location of the proton from the general acid is about halfway between His234 and the O3' oxygen with an imaginary frequency is $844i \text{ cm}^{-1}$. The latter is consistent with a proton transfer process indicating that the transition structure in Ref. [33] actually supports a stepwise mechanism with the proton transfer as the rate limiting step.

B. Michaelis Complex Structure

The ensemble-average structure of the substrate-bound or Michaelis complex is depicted in Figure 4A. This structure is obtained by computing the ensemble average of nuclear Cartesian coordinates corresponding to the reactant state in the 2-D PMF (Supporting information). Selected ensemble average of internuclear distances and angles from the reactant to the product states are listed in Table 1. The internuclear distances and angles based on the ensemble average of atomic Cartesian coordinates are also provided in parentheses. Note that the definitions of these two types of ensemble averages are different.

For example, the ensemble average of internuclear distance D between atoms #1 and #2 is defined as follows:

$$D = \left\langle \sqrt{(x_1 - x_2)^2 + (y_1 - y_2)^2 + (z_1 - z_2)^2} \right\rangle, \quad (3)$$

where x , y , z are the Cartesian coordinates and $\langle \dots \rangle$ represents an ensemble average. On the contrary, the internuclear distance Δ between atoms #1 and #2 based on the ensemble average of their Cartesian coordinates is defined as follows:

$$\Delta = \sqrt{\langle x_1 \rangle - \langle x_2 \rangle^2 + \langle y_1 \rangle - \langle y_2 \rangle^2 + \langle z_1 \rangle - \langle z_2 \rangle^2}. \quad (4)$$

Nevertheless, the differences of the computed values between the two approaches are about 0.1 Å in distance and about 1 degree in bond angles in present case. However, for the case of a methyl group rotating during MD simulations, the values of Δ between two hydrogen atoms of the methyl group are shorter than that of D . Similarly, for the case of a water molecule, in which the donor of a hydrogen bond is switching back and forth from one hydrogen atom to another one, the value of Δ between the two hydrogen atoms can be so short that their positions are possibly overlapped.

The cAMP-bound complex from the present QM/MM MD simulations is found to be in good agreement both with the optimized structure of the active site models[26–28] and with the unligated crystal structures.[5, 14] The average distance between the bridging hydroxide oxygen nucleophile and the phosphorus atom of cAMP is 2.9 Å, whereas the O3'—P distance is ~1.7 Å (Table 1). The substrate cAMP is anchored in the active site through coordination to the two metal ions by O2P and O3P oxygen atoms, respectively. Figure 1 shows that the nucleophile hydroxide ion is perfectly aligned with the O3'—P bond of the leaving group, with an average angle of 165°.

His234, which serves as the general acid in the active site, is in close proximity to hydrogen bond with the O3' oxygen in the Michaelis complex. The average separation between the HE2 atom of His234 and O3' is 2.0 Å. The residue Glu413, which is hydrogen bonded to HD1 of His234, ensures that His234 is in an ideal position throughout the enzymatic reaction.

The adenine base of cAMP forms four hydrogen bonds with residues Asn395 and Gln443 in the Michaelis complex (Figure 1 and Table 1). The orientation of Gln443, which is anchored through an ion-pair interaction with Tyr403 was proposed to be a key factor to the nucleotide specificity across the PDE family in the 'glutamine switch' mechanism.[4, 5, 16, 38] For example, in the cGMP-specific PDE5A (PDB ID: 1T9S[16]), the Gln443-equivalent residue in PDE5A (i.e., Gln817) is rotated by ~180° relative to the orientation of Gln443 in PDE4B due to interactions with the Gln775 (i.e., the equivalent residue for Tyr403 in PDE4B). Nevertheless, the glutamine-switch mechanism is only supported by some of structural data.[5, 38]

It is of importance to note that several crystal water molecules have stable hydrogen bonds with key residues in the active site of the Michaelis complex. For example, the crystal water molecule, H₂O66, is hydrogen bonded both to His389 and to Asp392 (Figure 1), which helps to keep it in a stable position throughout the phosphate hydrolysis reaction. The three ligand water molecules to Mg²⁺ (H₂O2, H₂O24, and H₂O26) also have a subtle H-bond network with other residues (Figure 1). The hydrogen atoms of H₂O2 form hydrogen bonds

with the side chain of Glu304 and the backbone of Thr345. Interestingly, the side chain of Thr345, together with the backbone of His274, forms a stable H-bond with the two hydrogen atoms of H₂O24 (note that the side chain of His274 is bound to Zn²⁺). One hydrogen atom of H₂O26 also forms an H-bond to His307. This H-bond network provides a key structure role to stabilize the three crystal waters throughout the catalysis.

C. From the Reactant to the Transition State

The structural variations of the binuclear metal center and the associated ligands accompanying the chemical processes from the reactant to the product state underlie the catalytic mechanism of PDE. In addition to the geometrical parameters listed in Table 1, Figure 5 shows the changes of some of the geometries as a function of the MFEP reaction coordinates. At the transition state (TS), the distances of $r_{\text{PO}_3'}$ and r_{OH_P} , the breaking and forming bonds, are 1.8 and 1.9 ± 0.1 Å, respectively, while the angle θ between these two bonds is 168°. The TS structure illustrated in Figure 4B depicts a concerted S_N2 reaction mechanism for the hydrolysis of cAMP by PDE4.

The nucleophilic attack by the bridging hydroxide ion is accompanied by significant changes in the Zn-coordination sphere. In the reactant state, the distance (**a**₁) between the hydroxide oxygen and zinc is 2.1 Å, which changes to 3.2 Å in the TS. In contrast, the coordination between the hydroxide and Mg²⁺ remains little changed throughout the enzymatic reaction (Figure 5A). We note that a similar transition has been reported in the phosphate hydrolysis by the binuclear metal enzyme, phosphotriesterase (PTE).[40] Moreover, similar to the reaction in PTE, we found that the internuclear distance between the two metal ions in PDE also undergoes a breathing motion in the catalytic cycle.[40] Thus, the separation between Zn²⁺ and Mg²⁺ ions of PDE increases from 3.8 Å in the Michaelis complex to 4.5 Å in the transition state (Figure 5B), which would be restored in the next catalytic cycle when a new substrate is bound in the active site.[54–57] One important energetic advantage in the stabilization of the transition state as a result of the coupled motions of the metal ions accompanying the reaction pathway is that the elongated metal distance helps to relieve the electrostatic repulsion between the two metal centers, which is stored in the Michaelis complex due to the attractive ligation from the bridged hydroxide ion. Recently, Lopez-Canut investigated the alkaline hydrolysis of methyl p-nitrophenylphosphate by nucleotide phosphatase, making use of the AM1/d-PhoT model, in which the distance between the two active-site zinc ions was found to correlate with the basicity of leaving group such that a greater separation is found to stabilize a charge-localized leaving group than a more delocalized leaving group.[53] One final note is that it is interesting to notice that the average TS structure is similar to the ‘reactant’ complex in the Salter-Wierzbicki paper, although their optimized complex in a truncated mode was obtained by fixing the separation of the two metal ions at 4.0 Å.[33]

D. From the Transition State to the Product State

Following the MFEP reaction path in Figure 2, an intermediate is produced by the hydroxide ion attack prior to the full proton transfer from His234 to the oxyanion leaving group. In the intermediate state, the cyclic phosphate bond is completely broken at a distance of 4.0 Å between O3' and P (Table 1). The separation between the two metal ions is further increased to 4.8 Å. The initial tetrahedral configuration about phosphorus is now entirely inverted. This Walden inversion of configuration is reflected by the positive values of ϕ_1 and ϕ_2 (Figure 5B and Table 1). Although the O3' atom of the ribosyl ring of AMP is quite far away from the phosphorus and the phosphorus is bonded with the nucleophile, the strong hydrogen bonds of the adenine base of AMP with Asn395 and Gln443 do not alter significantly during the reaction from cAMP to AMP (Table 1). The dihedral angle ϕ_3 between the pentose ring and the adenine base provides a flexible degree of freedom to

accommodate the variations (Figure 1). Its value decreases from 119° in the substrate-bound complex to 92° in the intermediate state (Figure 5B and Table 1).

For the transition state of the subsequent proton transfer process, the overall structure of the active site is very similar to the intermediate, but the HE2 atom of His234 is now halfway between the O3' oxygen and the NE2 atom (Table 1). This structure somewhat resembles the geometry determined by Salter and Wierzbicki for the transition state in the concerted process.[33] The proton-transfer process is likely to occur after the intermediate is formed in view of the small free-energy barrier. In fact, it is also entirely possible that the intermediate is by-passed altogether to directly form the final product from downhill trajectories in the transition state of the nucleophilic substitution ring opening step.

In the product complex, the distance $r_{\text{PO}_3'}$ is further increased to 4.6 Å (Figure 5C and Table 1) and ϕ_3 is 87°. Overall, the PDE4B-AMP complex from the present simulations is in good agreement with the crystal structure, except the position of the bridging hydroxide ion. In the crystal structure, the OH:O is nearly equidistant from Zn^{2+} and Mg^{2+} with separations of 2.6 and 2.7 Å, respectively.[15] However, our ensemble-average structure shows that the hydroxide is shifted towards Mg^{2+} . The distances of OH:O—Zn and OH:O—Mg in the complex from our simulations are 3.6 and 2.2 Å, respectively (Table 1). To confirm that this discrepancy from the crystal structure is not due to an artifact of the semiempirical method, we have performed density-functional-theory (DFT) calculations using B3LYP/6–31G+(d) to optimize an active site model with a simple phosphate group PO_4 mimicking the product AMP.[66, 67] The histidine residues in the active site are replaced with ammonia NH_3 molecules, while the aspartic acids are replaced with formate anions. This simplified active site model and the level of DFT optimizations have been employed by Zhan and Zheng to validate that the bridging oxygen in the crystal structure of unligated PDE is a hydroxide ion rather than a water molecule.[26] All DFT calculations were carried out with Gaussian 03.[65] Our initial geometry for the optimization is from the crystal structure of PDE4-AMP complex, i.e., we placed the hydroxide in the middle between the two metals. However, within 10 steps of optimizations, the hydroxide already loses the coordination with Zn^{2+} , and shifts towards Mg^{2+} . The optimized DFT-product structure is available in Supporting information, and selected internuclear distances and angles are also presented in Table 1. The optimized OH:O—Zn is 3.7 Å, whereas OH:O—Mg is 2.2 Å. These two distances and other geometries optimized at the B3LYP/6-31+G(d) level are in excellent agreement with the product-bound complex from QM/MM simulations of the full enzyme.

E. Comparison with phosphotriesterase

Although there are many similarities between phosphodiesterase (PDE) and phosphotriesterase (PTE)[40] active sites, there are also significant differences between the two enzymes. For instance, PDE is a hybrid metal protein. Zn^{2+} is the metal ions more buried into the protein, while Mg^{2+} ion is more exposed to the solvent. For the wild-type PTE, both metals are zinc ions. Additionally, the binding of cAMP with the PDE active site is through the coordinations of the two phosphoryl oxygen atoms with Zn^{2+} and Mg^{2+} , while the binding of paraoxon is only through the coordination of the phosphoryl oxygen with the more exposed Zn^{2+} ion. Furthermore, general acid catalysis by protonating the O3' oxygen leaving group of cAMP is an integral element in the PDE reaction, whereas the protonation on the oxyanion of the leaving group in the PTE-catalyzed reaction is not essential to the catalytic step.

Among the differences, the most significant is that the substrates for PDE and PTE have different charge states. cAMP and cGMP are negatively charged nucleotides, but a substrate for PTE, e.g., paraoxon or sarin, is neutral. This could explain the finding that there is a lack of a stable product-bound complex in previous simulations of the paraoxon hydrolysis by

PTE. A stable product-bound complex is inconsistent with the fact that PTE catalysis can reach the diffusion limit.[68, 69] On the contrary, we obtained a product-bound complex in the PDE simulations. However, the dissociation of a negatively charged product from the binuclear active site could be difficult. Thus, we conjecture that His234 could be protonated again by nearby water molecules, which may serve again as an acid to protonate one of the two bridging phosphoryl oxygen atoms to dissociate from metal binding in the product-release step. We are currently investigating this plausible protonation process.

F. Phosphodiesterase Mechanism

Based on the 2-D free energy profile and the structural changes of the active site during the catalysis, we summarize the reaction mechanism for the phosphodiesterase-catalyzed cAMP hydrolysis. The substrate cAMP first binds to the active site by coordinating its two phosphoryl oxygen atoms with the two metal ions, respectively. This makes cAMP in a position ready for an in-line nucleophilic attack by the bridging hydroxide ion. In turn, relatively to the barrier in the uncatalyzed reaction, this position reduces the free energy difference between the Michaelis complex and the rate-limiting transition state. The two metal ions are bridged by the hydroxide ion and the aspartic acid Asp275; both metals are hexa-coordinated. His234 is in a position stabilizing the substrate-bound complex through hydrogen bonding interactions with the O3' of cAMP and the phosphoryl oxygen O3P. The adenine base of cAMP forms is hydrogen bonded to Asn395 and Gln443. The structural features of the Michaelis complex are consistent with both the optimized structures on simplified models without a substrate [26–28] and the unligated crystal structures.[5, 14]

The first chemical step occurs as a direct nucleophilic attack on the phosphorus center of cAMP by the bridging hydroxide. This chemical process proceeds by an S_N2 -like mechanism, which is predicted to be the rate-limiting step for the chemical transformations with a free energy barrier of about 14 kcal/mol (which is in accord with the experimental values of 13–16 kcal/mol for various PDE enzymes). In the nucleophilic substitution, a number of interactions undergo substantial changes along the reaction pathway. First, the binding of the phosphoryl substrate in the active site weakens the interaction between OH^- Zn^{2+} , which facilitates an S_N2 attack at the phosphorus center. The nucleophilic substitution process effectively transfers a negative charge to the leaving group O3' oxygen, resulting in an elongation of the binuclear separation about one angstrom. The latter provides an important mechanism for the stabilization of the transition state by reducing electrostatic repulsions between the two metal centers at a short distance in the Michaelis complex. Concomitantly, the configuration of the phosphate group is inverted as a result of the S_N2 mechanism.

The second chemical step is the protonation of the leaving group O3' oxyanion by His234. Although the minimum free energy reaction path (MFEP) in the 2-D PMF suggests that an intermediate is formed and there is a barrier for the proton transfer from the intermediate, the proton transfer requires a backward movement associated with the O3' oxygen and the ribosyl ring. Therefore, it is plausible that the S_N2 reaction intermediate is not kinetically accessible in the enzymatic reaction. The proton-transfer process could occur immediately along the downhill trajectory from the substitution transition state.

The residue His234 not only protonates the O3' oxygen after the hydrolysis, it also initiates the product-release step. This idea is consistent with the structural facts that His234 is absolutely conserved across the PDE family, and that the three surrounding residues (Tyr233, His278, and Glu413; see Figure 5 in Ref. [15]) are conserved.[4] Currently, we are computing the PMF regarding our proposed product-release and water-recycle steps.

III. CONCLUSIONS

Molecular dynamics simulations employing a combined QM/MM potential have been performed to study the reaction mechanism of the hydrolysis of the cyclic nucleotide cAMP by phosphodiesterase4B (PDE). The superfamily PDE enzymes play an important role in the signal-transduction pathways to effectively terminate the second messenger in response to signals. Thus, PDEs have become an important target for drug design. To compute the two-dimensional potential of mean force (PMF) associated with the catalysis, we made use of three set of semiempirical parameters which are specifically designed for phosphorylation and active sites containing zinc or magnesium ions.

From the 2-D PMF, we found that the catalysis by PDE proceeds in an S_N2 mechanism, followed by proton transfer to stabilize the leaving group. We estimate the free energy of activation for the hydrolysis step is ~ 13 kcal/mol and the overall reaction free energy is about -17 kcal/mol. Both are in good agreement with experimental and DFT results. In comparison with the uncatalyzed reaction in water, the enzyme PDE4B provides strong stabilization of the transition state, lowering the free energy barrier by 14 kcal/mol. A key contributing factor is the elongation of the separation of the divalent metal ions as the reaction proceeds from the Michaelis complex to the transition state, and the activation of the hydroxide ion as a nucleophile. In particular, after cAMP binds with the active site through the bonds between the phosphoryl oxygen atoms and the two metal ions, these two cations in the Michaelis complex enjoy an octahedral coordination sphere in a compact conformation with their separation at about ~ 3.8 Å with a bridging hydroxide ion. However, the Zn^{2+} ion loses its coordination to the nucleophile hydroxide in the S_N2 attack. This results in a loose binuclear conformation, characterized by an elongated Zn—Mg distance of ~ 4.8 Å. Thus, the structural variations of the two metal ions are closely correlated to the reaction coordinates. This feature has also been reported in the previous studies on the other two binuclear metal enzymes: xylose isomerase[54–57] and phosphotriesterase.[40]

Although a stable intermediate is possible after the above S_N2 attack according to the 2-D free-energy contour map, the protonation step may directly follow the nucleophilic attack since there is a barrier for the proton transfer from the intermediate and it requires a re-orientation of the ribosyl ring too. By adjusting the relative orientation between the adenine base and the pentose ring, the strong hydrogen bonds associated with the adenine remain stable throughout the simulations. Although in the product-bound crystal structure, the OH^- ion is equidistant from both metals, the hydroxyl group in the product-bound complex is only coordinated with Mg^{2+} , which is supported by DFT optimized structures. To release the negatively charged product AMP from the two divalent metals, we propose that an extra proton is needed to neutralize the product by protonating one of the two phosphoryl oxygen bound to the metals. This protonation could be initiated from the reprotonated His234. Since the motions of the three water molecules bound with Mg^{2+} are restricted by their hydrogen networks with other residues, the crystal water H₂O66, which is found in a position between His389 and Asp392 and is not yet bound with metal ions, is an ideal candidate for restoring the leaving nucleophile. To further quantify our proposed product-release step, we are currently computing the associated free-energy profile.

IV. METHODS AND COMPUTATIONAL PROCEDURES

A. Product-Bound Complex

The X-ray crystal structure of the PDE4B-AMP complex (at pH 6.5 and 4 °C) determined at 2.0 Å resolution (PDB ID: 1ROR[15]) was used to construct the solvated product-bound complex. Usually, for MD simulations of an enzymatic reaction, we start with the solvated Michaelis (substrate-bound) complex.[70] Since the product-bound complex was formed by

soaking the protein with the real substrate cAMP, we employed this structure as the starting geometry in MD simulations for generating the two-dimensional free-energy profile. All ionic amino acid residues are set in a protonation state corresponding to pH 7. The protonation state of each histidine residue was determined by considering the possible hydrogen-bond network with its neighboring groups. As a result, His247 and His278 are neutral with the proton at the epsilon nitrogen (NE2) atom. His238, His274, His307, His350, His389, and His435 are also neutral but with the proton is located on the delta nitrogen (ND1) atom. The rest of the eight histidine residues, including His234, are protonated. Consequently, there are 36 positively charged residues (13 Arg, 15 Lys, and 8 His), 50 negatively charged residues (28 Asp and 22 Glu), one hydroxide anion, a negatively charged cAMP, and two divalent metal cations. We added 16 sodium and 4 chloride counterions to bring the total charge of the simulation neutral.

To solvate the PDE4B-AMP complex, periodic boundary conditions were used for a $65 \times 65 \times 65 \text{ \AA}^3$ cubic box consisting of water molecules in which the product-bound complex is immersed along with 254 crystal water molecules. The structural preparation was carried out using VMD.[71] Overall, the solvated PDE4-AMP complex contains a total of 30,198 atoms including 8,249 water molecules and 20 counterions.

B. Potential Energy Function

We used a combined quantum mechanical and molecular mechanical (QM/MM) method[42–49] to construct the potential energy function for the hydrolysis and protonation reactions of cAMP by PDE. A total of 97 atoms in the active site (Figure 1), consisting of one Zn^{2+} and one Mg^{2+} ions, the bridging hydroxide ion, the substrate cAMP, His234, His238, His274, Asp275, Asp392, and the three crystal water molecules coordinated with the Mg^{2+} ion are included in the QM region.[51, 72–86] The generalized hybrid orbital (GHO) method[87, 88] was employed to couple the QM region with the MM region through the C_α atoms of the five residues listed above. The CHARMM22 all-atom empirical force field[89] and the three-point-charge TIP3P model[90] were used to represent the rest of the protein and water molecules, respectively. Long-range QM/MM electrostatic interactions were calculated using the QM/MM-particle-mesh Ewald method[91] with a maximum reciprocal lattice component k_{max} of 7 and its squared k_{max}^2 of 49.

In general, *ab initio* molecular orbital theory[67, 92, 93] or density-functional theory (DFT) [58, 59, 62, 94] would be ideal for electronic structure calculations. However, these methods are still too time-consuming, particularly for two-dimensional free energy simulations, limiting applications to minimizations of smaller model systems,[95] and short full MD simulations.[96, 97] Yet, it is essential to include protein and solvent dynamics in free-energy simulations of biocatalysis,[48, 49] particularly for phosphate hydrolysis in enzyme active site containing two metal ions in the present PDE catalysis. We note that Zhang and coworkers have utilized Born-Oppenheimer *ab initio* QM/MM techniques to study a number of enzymes, demonstrating that these applications are becoming feasible with multiple processors.[98–101] To this end, we employed the approximate molecular orbital model specifically parameterized for modeling phosphoryl transfer reactions, i.e., the AM1/d-PhoT method based on the NDDO (neglect diatomic differential overlap) approximation.[86] The AM1/d-PhoT can provide an accuracy for phosphoryl transfer reactions comparable to DFT/B3LYP results at a fraction of its costs. This method has been successfully applied to phosphate hydrolysis reactions in water,[51, 86, 102] including the computation of phosphorus kinetic isotope effects,[63] and in biological systems to shed light on the mechanisms of hairpin and hammerhead ribozymes,[39, 51, 52] and on the phosphodiester hydrolysis by nucleotide pyrophosphatase.[53] For the metal ions, we used the newly derived AM1/d parameters for Mg^{2+} ion coordinating with oxygen atoms.[85], and the zinc model of Merz and coworkers in the context of the PM3 method.[78]

C. Molecular Dynamics Simulations

Molecular dynamics (MD) simulations of the solvated PDE4B–AMP complex were performed using periodic boundary conditions along with the isothermal-isobaric ensemble (*NPT*) at 1 atm and 298 K. The *NPT* ensemble was maintained by the Andersen algorithm[103] and the Nosé-Hoover thermostat[104, 105] with effective mass of 500 amu and 1000.0 ps²-kcal/mol, respectively. The smooth particle mesh Ewald method[106, 107] was employed for treating long-range electrostatic interactions. The value of the Gaussian screening parameter κ for the real space Ewald summations is 0.34 Å⁻¹. 64 grid points in each side of the cubic box and a 6th order B-spline interpolation were used for the fast Fourier transforms in the reciprocal space summations. Nonbonded interactions were treated using a group-based cutoff of 12 Å with a shifted van der Waals potential. Numerical integrations for the Newtonian equations of motion were performed using the leapfrog-Verlet algorithm[108] with a time step of 1 fs. Covalent bond lengths involving hydrogen were constrained with the SHAKE algorithm.[109] Throughout the MD simulations, the nonbonded and image atom lists were updated in every 25 time steps. All the simulations were performed using the CHARMM program (version c33a2).[110, 111]

D. Potential of Mean Force

To shed light on the PDE mechanism of the hydrolysis and protonation steps, we obtained a two-dimensional (2-D) potential of mean force (PMF),[112] as a function of the two reaction coordinates, which are defined as follows. The reaction coordinate z_1 [i.e., Eq. (1)] describing the nucleophilic attack of cAMP by a hydroxide ion is defined as the difference between the distances of the cleaving and making bonds.[49] The second reaction coordinate z_2 is associated with general acid catalysis for the proton transfer from His234 to O3' oxygen [i.e., Eq. (2)]. The 2-D PMF G is obtained from molecular dynamics simulations using umbrella sampling:[113]

$$G(z_1, z_2) = -k_B T \ln \rho(z_1, z_2) + G_0, \quad (5)$$

where k_B is Boltzmann's constant, T is temperature, U_0 is a normalization constant, and $\rho(z_1, z_2)$ is the probability density of finding the system at the reaction coordinate (z_1, z_2) .

The full system was first equilibrated for more than 1 ns, in which the active site is harmonically restrained at the crystal structure and gradually released. Next, a series of umbrella sampling MD simulations were carried out to span the entire range of the two reaction coordinates from the product to the reactant states. To enhance sampling efficiency, a harmonic biasing potential was applied with a force constant ranging between ~20 and ~100 kcal/mol-Å⁻² in accordance with different regions of the configuration samplings. The total of 831 umbrella sampling windows was used. The choice of the force constants and the number of windows ensure sufficient overlap of the probability distribution with neighboring windows. Following the initial 1-ns equilibration, each window was further equilibrated for more than 10 ps after the system was equilibrated in the adjacent window. Subsequently, a 25-ps of configuration samplings was performed in each window, resulting in a total of ~30 ns MD simulations. The weighted histogram analysis method[114–117] was used to combine the sampled configurations (collected at every time step in a bin size of 0.1 Å) to compute the unbiased 2-D PMF as a function of the two reaction coordinates [i.e., Eq. (5)].

E. Visualization

The two software packages used to visualize the computational results and to generate the figures in this paper were Visual Molecular Dynamics (VMD)[71] and GaussView.[118]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

AM1	Austin model 1
NDDO	neglect diatomic differential overlap
cAMP	cyclic adenosine 3'-5'-monophosphate
cGMP	cyclic guanosine 3'-5'-monophosphate
DFT	density functional theory
GHO	generalized hybrid orbital
MD	molecular dynamics
NPT	isothermal-isobaric ensemble
PMF	potential of mean force
PDE	phosphodiesterase
PTE	phosphotriesterase
QM/MM	quantum mechanical and molecular mechanical
TMP	trimethylene phosphate

Acknowledgments

This work has been generously supported by the National Institutes of Health (grant number GM46736).

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Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Hydrolysis of cyclic adenosine 3'-5'-monophosphate (cAMP) by phosphodiesterase

Hydrolysis of cyclic guanosine 3'-5'-monophosphate (cGMP) by phosphodiesterase

Scheme 1.

Selected Ensemble Average Internuclear Distances and Bond Angles at the Reactant, Transition, Intermediate, and Product States in the Active Site of Phosphodiesterase.^a

Table 1

Label ^b (ligand:atom)	Reactant ^c	Transition 1 ^d Distance (Å) or Angle (degree)	Intermediate ^e	Transition 2 ^f	Product ^g	DFT-product ^h	IROR ⁱ
<u>Hydrolysis</u>							
r _{PO3} (cAMP:O3'—P)	1.7±0.0 (1.7)	1.8±0.1 (1.8)	4.0±0.0 (4.0)	3.5±0.0 (3.5)	4.6±0.0 (4.5)	--	3.9
r _{OH} (OH:O—P)	2.9±0.0 (2.9)	1.9±0.1 (1.9)	1.7±0.0 (1.7)	1.7±0.0 (1.7)	1.7±0.0 (1.7)	1.6	1.5
θ (OH:O—P—cAMP:O3')	165±5 (165)	168±5 (169)	143±5 (144)	150±6 (151)	130±6 (129)	--	136
φ ₁ (O2P—P—O5'—O3P)	-144±4 (-144)	175±3 (175)	136±5 (136)	140±4 (140)	139±5 (140)	134	121
φ ₂ (O5'—O3P—O2P—P)	-28±4 (-28)	-3±3 (-3)	32±4 (32)	29±3 (29)	33±4 (33)	34.3	34.1
<u>Zn-Mg interaction</u>							
c ₁ (Zn—Mg)	3.8±0.1 (3.7)	4.5±0.2 (4.5)	4.8±0.1 (4.7)	4.7±0.1 (4.7)	4.7±0.1 (4.7)	4.6	4.4
<u>Interaction with Zn²⁺</u>							
a ₁ (OH:O—Zn)	2.1±0.1 (2.1)	3.2±0.4 (3.2)	3.5±0.2 (3.5)	3.5±0.2 (3.5)	3.6±0.1 (3.6)	3.7	2.6
a ₂ (cAMP:O2P—Zn)	2.1±0.1 (2.1)	2.1±0.0 (2.0)	2.0±0.0 (2.0)	2.1±0.0 (2.0)	2.0±0.0 (2.0)	2.1	2.0
a ₃ (Asp275:OD2—Zn)	2.4±0.4 (2.4)	2.1±0.3 (2.1)	2.1±0.0 (2.1)	2.1±0.0 (2.0)	2.1±0.0 (2.1)	2.0	2.2
<u>Interaction with Mg²⁺</u>							
b ₁ (OH:O—Mg)	2.1±0.0 (2.0)	2.1±0.1 (2.1)	2.2±0.1 (2.2)	2.2±0.1 (2.2)	2.3±0.1 (2.4)	2.2	2.7
b ₂ (cAMP:O3P—Mg)	2.1±0.1 (2.1)	2.1±0.1 (2.1)	2.1±0.1 (2.1)	2.1±0.0 (2.1)	2.1±0.1 (2.0)	2.1	2.6
b ₃ (Asp275:OD1—Mg)	2.1±0.1 (2.1)	2.1±0.1 (2.1)	2.1±0.1 (2.0)	2.1±0.1 (2.0)	2.1±0.1 (2.0)	2.0	2.4
<u>Protonation</u>							
r _{HN} (His234:HE2—His234:NE2)	1.0±0.0 (1.0)	1.0±0.0 (1.0)	1.0±0.0 (1.0)	1.2±0.0 (1.2)	3.0±0.0 (3.0)	--	--
r _{OH} (His234:HE2—cAMP:O3')	2.0±0.0 (1.9)	1.8±0.0 (1.8)	1.9±0.0 (1.9)	1.4±0.0 (1.4)	1.0±0.0 (0.9)	--	--
<u>Relative orientation between</u>							
<u>adenine and pentose ring of cAMP</u>							
φ ₃ (C4—N9—C1'—C2')	119±9 (119)	119±10 (119)	92±12 (92)	104±9 (104)	87±10 (89)	--	97
<u>Interaction with His234</u>							
d ₁ (His234:HE2—cAMP:O3P)	2.7±0.3 (2.7)	2.6±0.3 (2.7)	2.7±0.2 (2.6)	2.9±0.2 (2.9)	3.7±0.3 (3.7)	--	--
d ₂ (His234:HD1—Glu413:OE1)	1.9±0.3 (1.9)	2.0±0.3 (2.0)	1.9±0.2 (1.9)	2.0±0.3 (2.0)	2.1±0.3 (2.1)	--	--

Label ^b (ligand:atom)	Reactant ^c	Transition 1 ^d	Intermediate ^e	Transition 2 ^f	Product ^g	DFT-product ^h	IROR ⁱ
d ₃ (His234:HD1—Glu413:OE2)	2.0±0.2 (1.9)	1.9±0.2 (1.8)	2.0±0.2 (2.0)	1.9±0.2 (1.9)	2.0±0.2 (1.9)	--	--
<i>Interaction with adenine of cAMP</i>							
d ₄ (cAMP:N7—Asn395:HD21)	1.8±0.2 (1.8)	1.8±0.1 (1.7)	1.9±0.2 (1.8)	1.9±0.2 (1.8)	1.8±0.1 (1.7)	--	--
d ₅ (cAMP:H61—Asn395:OD1)	1.9±0.2 (1.8)	1.8±0.2 (1.8)	1.9±0.2 (1.8)	1.8±0.2 (1.8)	1.8±0.1 (1.7)	--	--
d ₆ (cAMP:H62—Gln443:OE1)	2.0±0.3 (2.0)	2.0±0.2 (1.9)	2.1±0.3 (2.0)	2.1±0.3 (2.0)	1.9±0.2 (1.9)	--	--
d ₇ (cAMP:N1—Gln443:HE21)	1.7±0.1 (1.7)	1.7±0.1 (1.7)	1.7±0.1 (1.7)	1.7±0.1 (1.7)	1.7±0.1 (1.7)	--	--
d ₈ (Tyr403:HH—Gln443:OE1)	1.8±0.2 (1.8)	1.9±0.2 (1.8)	1.9±0.1 (1.8)	1.8±0.1 (1.8)	1.9±0.1 (1.8)	--	--
<i>Interaction with recycling water candidate</i>							
c ₂ (H ₂ O66:O—OH:O)	5.0±0.3 (5.0)	4.4±0.3 (4.4)	4.3±0.3 (4.2)	4.1±0.3 (4.1)	4.4±0.3 (4.2)	--	4.2
d ₉ (H ₂ O66:O—His389:HD1)	2.1±0.3 (2.0)	2.0±0.2 (2.0)	2.0±0.2 (2.0)	2.0±0.1 (1.9)	2.1±0.4 (2.1)	--	--
D ₁₀ (H ₂ O66:H1—Asp392:OD2)	3.1±0.3 (3.0)	2.1±0.5 (2.0)	3.0±0.6 (2.9)	2.1±0.5 (2.0)	2.1±0.8 (2.4)	--	--
d ₁₁ (H ₂ O66:H2—Asp392:OD2)	1.9±0.3 (1.8)	3.0±0.5 (2.9)	2.3±0.6 (2.2)	3.0±0.5 (2.9)	3.3±0.4 (3.3)	--	--
<i>Interaction with crystal waters bound with Mg²⁺</i>							
d ₁₂ (H ₂ O2:H1—Thr345:O)	3.2±0.6 (3.1)	3.2±0.5 (3.2)	2.6±0.7 (2.5)	3.3±0.3 (3.3)	2.7±0.7 (2.5)	--	--
d ₁₃ (H ₂ O2:H1—Glu304:OE2)	2.1±0.6 (2.0)	2.5±0.7 (2.4)	2.4±0.7 (2.4)	1.8±0.3 (1.7)	2.4±0.7 (2.4)	--	--
d ₁₄ (H ₂ O2:H2—Thr345:O)	2.5±0.7 (2.4)	3.3±1.0 (3.1)	2.7±0.7 (2.6)	2.2±0.4 (2.1)	2.6±0.7 (2.6)	--	--
d ₁₅ (H ₂ O2:H2—Glu304:OE2)	3.0±0.6 (2.9)	2.4±0.7 (2.3)	2.4±0.7 (2.4)	3.1±0.3 (3.1)	2.5±0.7 (2.4)	--	--
d ₁₆ (H ₂ O24:H1—Thr345:OG1)	1.9±0.2 (1.9)	1.9±0.1 (1.8)	1.8±0.1 (1.8)	1.9±0.1 (1.8)	1.8±0.1 (1.8)	--	--
d ₁₇ (H ₂ O24:H2—His274:O)	1.9±0.2 (1.9)	1.9±0.2 (1.9)	1.8±0.2 (1.8)	1.9±0.1 (1.8)	1.9±0.2 (1.8)	--	--
d ₁₈ (H ₂ O26:H1—His307:NE2)	2.9±0.6 (2.9)	2.7±0.7 (2.6)	2.5±0.8 (2.5)	3.1±0.7 (3.0)	3.4±0.2 (3.3)	--	--
d ₁₉ (H ₂ O26:H2—His307:NE2)	2.3±0.6 (2.3)	2.6±0.7 (2.5)	2.8±0.8 (2.7)	2.3±0.7 (2.3)	1.9±0.2 (1.9)	--	--

^aValues given in parentheses are based on ensemble average of Cartesian coordinates [e.g., Eq. (4)].

^bSee Figure 1 for the schematic diagram representing the internuclear distances and angles.

^cAverage values over the configurations (z1, z2) corresponding to (−1.2, −1.0).

^dAverage values over the configurations (z1, z2) corresponding to (−0.1, −0.8).

^eAverage values over the configurations (z1, z2) corresponding to (2.3, −0.9).

^fAverage values over the configurations (z1, z2) corresponding to (1.8, −0.2).

g Average values over the configurations (z_1, z_2) corresponding to (2.9, 2.0).

h Optimized product-bound structure on a simplified active site model at B3LYP/6-31+G(d) level.

i From the first monomer of the PDE4B-AMP crystal structure in Ref. [[15]]