To study the catalytic mechanism of phosphorylation catalyzed by cAMP-dependent protein kinase (PKA) a structure of the enzyme-substrate complex representing the Michaelis complex is of specific interest as it can shed light on the structure of the transition state. However, all previous crystal structures of the Michaelis complex mimics of the PKA catalytic subunit (PKAc) were obtained with either peptide inhibitors or ATP analogs. Here we utilized Ca^{2+} ions and sulfur in place of the nucleophilic oxygen in a 20-residue pseudo-substrate peptide (CP20) and ATP to produce a close mimic of the Michaelis complex. In the ternary reactant complex, the thiol group of Cys-21 of the peptide is facing Asp-166 and the sulfur atom is positioned for an in-line phosphoryl transfer. Replacement of Ca^{2+} cations with Mg^{2+} ions resulted in a complex with trapped product groups, a loose transition state, and metal movement. The present structural results in combination with the previously reported X-ray structures of PKAc complexes along the phosphoryl transfer reaction have been obtained.

**Background:** PKAc (catalytic subunit) catalyzes phosphorylation of protein substrates thereby regulating a myriad of cellular processes.

**Results:** X-ray structures of PKAc complexes along the phosphoryl transfer reaction have been obtained.

**Conclusion:** The phosphotransfer follows a multistep mechanism, including conformational changes of the substrate and product groups, a loose transition state, and metal movement.

**Significance:** Mechanistic knowledge about the phosphorylation by PKAc will contribute to understanding of the kinase function and regulation.

Protein kinases are signaling enzymes that regulate cellular processes by catalyzing phosphorylation of proteins. Chemically, protein kinases transfer the γ-phosphoryl group of a nucleotide triphosphate (e.g. ATP) to the hydroxyl group of a serine, threonine, tyrosine, or histidine residue of the substrate protein. Over 500 protein kinases have been identified in the human genome (~1.7% of genes), pointing to the biological importance of phosphoryl-transfer chemistry (1). Extensive studies of the cAMP-dependent protein kinase (PKA) that phosphorylates the side chains of Ser or Thr residues have made it a paradigm for the whole family of kinase enzymes (2, 3).

Being a regulatory enzyme, PKA is highly regulated itself. When inactive, PKA is a tetrameric holoenzyme, R_{2}C_{2}, composed of two catalytic (C) monomeric and regulatory homodimeric (R_{2}) subunits. An increase in cAMP concentration activates PKA; binding of four cAMP molecules to R_{2} causes the tetramer to dissociate, releasing two active C subunits (that we refer to here as PKAc)\(^{3}\) (4). In PKAc, the nucleotide-binding site is in the cleft between N-terminal and C-terminal lobes that are connected by a small linker region, but the nucleotide primarily interacts with the N-lobe. The substrate sits at the edge of the cleft on the surface of the large C-lobe. PKAc requires one or two divalent metal ions to bind to the active site to be active (5, 6). The physiological metal is magnesium, although others can support phosphotransferase activity (7, 8).

Crystallographic studies have provided a wealth of information on how PKAc functions (4, 9–11). Complexes of PKAc with nucleotide and/or substrate analogs are found in three major conformational states that differ in the relative orientation of the N- and C-lobes. With no ligands bound (apo form) PKAc adopts an open conformation; upon nucleotide or substrate binding (binary form), PKAc transitions to an intermediate, partially closed, state; last, PKAc assumes a closed conformation when all components for the reaction are in place (ternary form) (12–15).

Although the PKAc phosphoryl transfer step is fast, >500 s\(^{-1}\), the product turnover rate is at least an order of magnitude slower; \(k_{\text{cat}}\) is \(~20\) s\(^{-1}\) (16). The rate-limiting step at high magnesium concentrations (~10 mM) is the release of MgADP (17). Analysis of the crystallographic structures and solution kinetic data suggests that conformational changes, particularly those involved in the release of the nucleotide, might be essential for PKAc function. Recent nuclear magnetic resonance (NMR)
studies and molecular dynamics (MD) simulations have established a relationship between the PKAc conformational fluctuations and its turnover rate (18, 19). Specifically, the rate of the opening motion correlates with $k_{\text{cat}}$ and the rate-limiting step, MgADP product release. The chemical step occurs only in the closed form of PKAc and the release of product is concurrent with the enzyme returning to the open conformation. However, single molecule electronic measurements of PKAc catalysis indicate that not every open-close conformational cycle results in the phosphorylation reaction and/or product release, which can partially explain the relatively low catalytic efficiency of the enzyme (20). These experiments also caution us on correlating bulk kinetic values, such as the $k_{\text{cat}}$ with the time it takes each individual molecule to go through a particular conformational change or a chemical reaction.

Despite these advances fundamental questions remain unanswered. In particular it is still not clear whether the phosphoryl transfer proceeds as a direct nucleophilic attack by the OH group of the substrate on the γ-phosphorus of ATP in the $S_{N}2$ fashion or through the metaphosphate intermediate as an $S_{N}1$ reaction. Stereochemical NMR studies suggest that PKAc facilitates a direct in-line displacement reaction with the pentacordinated phosphorus in the transition state (21). Yet, the possibility of the short-lived hindered metaphosphate has not been completely ruled out. Uncertainty also surrounds the roles of active site residues and metal ions, and the details of hydrogen transfer pathways during the chemical step. Specifically, different functions have been proposed for the catalytically important Asp-166 residue. In PKAc, Asp-166 is the nearest ionizable residue to the OH group of the substrate and may act as a catalytic base and/or to correctly position the nucleophile. Asp-166 is universally conserved in the active site of all protein kinases and can form hydrogen bond interactions with Ser of the substrate (14, 22, 23). Asp-to-Ala substitution produces a mutant variant with activity below 1% of the wild-type (24).

Several crystallographic structures of PKAc complexes that represent different stages of the phosphoryl transfer reaction have been determined previously. Those mimicking the reactive ATP, Ca$^{2+}$ ions capable of promoting phosphoryl transfer and a nucleophilic group in the substrate peptide, this complex is the closest model of the actual Michaelis complex to date. In addition we argue that PKAc-Mg$_{2}$ADP-PO$_{4}$-CP20 can represent a state just after the phosphoryl transfer reaction, but before the phosphorylated Ser-21$_{\text{SP20}}$ rotates out toward the bulk solvent. Thus, our current results taken together with previously published crystallographic work and theoretical calculations provide the most complete picture to date of the phosphorylation reaction catalyzed by PKAc.
Catalytic Mechanism of Protein Kinase A

Experimental Procedures

General Information—Pseudo-substrate peptides SP20 (TTY-ADFIASGRTGRRASIHD; residues 5–24 of the heat-stable PKAc inhibitor PKI, where positions 20 and 21 have been mutated to Ala and Ser) and CP20 (TTYADFIASGRTGRRACHI; SP20 derivative, where Ser-21 was substituted with a Cys residue) were custom synthesized by Biomatik (Wilmington, DE). ATP as the magnesium or disodium salts and AMPPNP as a lithium salt were purchased from Sigma. GRRACIHD; SP20 derivative, where Ser-21 was substituted to Ala and Ser) and CP20 (TTYADFIASGRTGRRASIHD; residues 5–24 of the heat-stable PKAc inhibitor PKI, where positions 20 and 21 have been mutated to Ala and Ser) and CP20 (TTYADFIASGRTGRRACHI; SP20 derivative, where Ser-21 was substituted with a Cys residue) were custom synthesized by Biomatik (Wilmington, DE). ATP as the magnesium or disodium salts and AMPPNP as a lithium salt were purchased from Sigma. Protein purification supplies were purchased from GE Healthcare. Crystallization reagents were purchased from Hampton Research (Aliso Viejo, CA).

Protein Expression and Purification—His6-tagged recombinant mouse PKAc was expressed in Escherichia coli using LB or minimal medium at 18–20 °C for 16–18 h. The recombinant enzyme was purified by affinity chromatography using HisTrap fast-flow chromatography columns supplied by GE Healthcare. The enzyme was then buffer exchanged with 50 mM MES, 250 mM NaCl, 2 mM DTT, pH 6.5, on a desalting column. Isoforms of PKAc were not separated, without any obvious effect on crystallization trails. First, the concentrated PKAc solution was mixed with a solution of metal chloride salt to reach the final metal concentration of ~20 mM. Then, the nucleotide was added. The peptide substrate was introduced to the mixture last. The molar ratio of PKAc:nucleotide:peptide was kept at 1:10:10. Crystals were grown in sitting drop microbridges or in 9-well glass plates using well solutions consisting of 100 mM MES, pH 6.5, 5 mM DTT, 15–20% PEG 4000 at 4 °C. For complexes with different metal ions, the corresponding metal chloride salts were introduced to the well solutions at 50 mM concentrations prior to setting up crystallization drops.

Data Collection, Structure Determination, and Refinement—X-ray crystallographic data were collected at 100 K using a Rigaku HomeFlux system, equipped with a MicroMax-007 HF generator, Osmic VariMax optics, and an RAXIS-IV++ image plate detector. Diffraction data were collected, integrated, and scaled using HKL3000 software suite (30). The structures were refined using SHELX-97 (31). A summary of the crystallographic data and refinement is given in Table 1. Similar to our previous observations (25) all the structures were of isoform 2, and contained three post-translationally phosphorylated residues: Ser-139, Thr-197, and Ser-338. The structure of the ternary complex of PKAc with 2Mg2+, ATP, and peptide inhibitor IP20 (PDB code 4DH3) (25) was used as a starting model to solve all the structures described here. The structures were built and manipulated with the program Coot (32), whereas the figures were generated using the PyMol molecular graphics software (version 1.5.0.3; Schrödinger LLC).

Molecular Dynamics Simulations—MD simulations were performed for PKAc-Mg2+,ATP-IP20, PKAc-Mg2+AMPPNP-SP20, and PKAc-Ca2+ADP-pSP20. Briefly, the PKAc-Mg2+,ATP-IP20 crystal structure (PDB code 1ATP) (33) was used as a template to set up the simulations. In all three systems, Thr-197 and Ser-338 are phosphorylated with fully ionized phosphate groups. Gromacs-4.6.1 (34) was utilized to conduct all the simulations with the Amber ff99SB (35) force field with ILDN (36) and NMR (37) modifications. Each of the systems was first energy minimized then a total of 50 ns production simulation was performed under isobaric and isothermal conditions (298 K and 1 atm).
Results

Ternary Pseudo-Michaelis Complexes PKAc-Ca\textsubscript{2}ATP-CP20 and PKAc-Ca\textsubscript{2}AMPPNP-SP20—The electron density maps clearly indicate that intact peptide and nucleotide molecules are trapped in the enzyme active site, confirming the formation of pseudo-Michaelis complexes (Fig. 1, A and B). The two structures are similar, with the r.m.s. deviation on the main chain atoms being 0.42 Å, but there are several important structural differences.

In PKAc-Ca\textsubscript{2}ATP-CP20, M1 and M2 metals have coordination numbers of seven and six, whereas in PKAc-Ca\textsubscript{2}AMPPNP-SP20 both metals are surrounded by eight ligands (Fig. 1C). In both structures the coordination spheres around each metal site involve protein residues (Asn-171, Asp-184) and ATP groups, which are identical to those in the coordination spheres of magnesium, observed previously including the PKAc-Mg\textsubscript{2}ATP-IP20 complex (PDB code 4DH3) (15, 25, 38). The remaining coordination sites around each metal, with the exception of Ca2 in PKAc-Ca\textsubscript{2}AMPPNP-SP20, are provided by water molecules. A slight displacement of ∼0.5 Å of γ-phosphorus in AMPPNP relatively to ATP puts the former’s γ-phosphate closer to M2 site, allowing formation of the second coordination contact between M2 and the γ-phosphate in PKAc-Ca\textsubscript{2}AMPPNP-SP20. In addition, a sliding shift of the glycine-rich loop of ∼1 Å in PKAc-Ca\textsubscript{2}AMPPNP-SP20 toward the αB helix relatively to its position in PKAc-Ca\textsubscript{2}ATP-CP20 makes the active site of the former more accessible for water molecules, which explains the increased coordination number of 8 for both Ca\textsuperscript{2+} cations.

FIGURE 1. A, electron density map for the active site components in PKAc-Ca\textsubscript{2}ATP-CP20 contoured at 1.5σ level (4σ for calcium cations). B, electron density map for the active site components in PKAc-Ca\textsubscript{2}AMPPNP-SP20 contoured at 1.5σ level (4σ for calcium cations). C, superposition of the active sites in PKAc-Ca\textsubscript{2}ATP-CP20 (colored by atom type, carbon is green, Ca\textsuperscript{2+} ions are dark cyan, H\textsubscript{2}O molecules red) and PKAc-Ca\textsubscript{2}AMPPNP-SP20 (light magenta, carbon atoms; light cyan, Ca\textsuperscript{2+} ions; magenta, H\textsubscript{2}O molecules), showing metals Ca1 and Ca2 bound at sites M1 and M2, respectively, nucleotides ATP and AMPPNP, Cys-21\textsubscript{CP20} and Ser-21\textsubscript{SP20} of the substrate peptides CP20 (blue carbon atoms) and SP20 (orange carbon atoms), respectively, and the residues of the enzyme that are important for metal binding or catalysis. Metal coordination, as black solid lines, and possible hydrogen bonds, as dashed lines, are shown for PKAc-Ca\textsubscript{2}ATP-CP20. Distances are in Å. D, superposition of the active sites in pseudo-Michaelis complexes PKAc-Mg\textsubscript{2}ATP-IP20 (PDB: 4DH3, dark magenta for all atoms including Mg\textsuperscript{2+} ions and water molecules), and PKAc-Ca\textsubscript{2}ATP-CP20 (carbon, green; Ca\textsuperscript{2+} ions, dark cyan; and water molecules, red). Metal coordination are shown as black solid lines, and possible hydrogen bonds as dashed blue are shown for PKAc-Mg\textsubscript{2}ATP-IP20. Distances are in Å.
In PKAc-Ca\textsubscript{2}ATP-CP\textsubscript{20}, the thiol group of Cys-21CP\textsubscript{20} is facing Asp-166. In this orientation the S atom is located in close proximity to the Asp-166 carboxyl (S . . O distance is of 3.2 Å). In contrast, in the PKAc-Ca\textsubscript{2}AMPPNP-SP\textsubscript{20} structure the C\textsubscript{\beta}-O\textsubscript{\gamma} bond of Ser-21SP\textsubscript{20} is rotated by \sim 110° away from Asp-166, pointing toward the bulk solvent (Fig. 1C).

The interactions formed by Lys-168 are similar in both structures reported here, but they differ relative to the previously reported PKAc complexes with ATP and IP\textsubscript{20} (Fig. 1D) (25). Specifically Lys-168 loses direct H-bond interactions with C\textsubscript{\beta}-phosphate oxygen of ATP or AMPPNP observed in structures with IP\textsubscript{20}. The smaller side chain of Ala-21IP\textsubscript{20} lacks a substituent in the C\textsubscript{\beta}-position, which allows ATP to move closer to the substrate peptide and knock out W4 in PKAc-Mg\textsubscript{2}ATP-IP\textsubscript{20} (Fig. 1D).

Comparison of PKAc-Ca\textsubscript{2}ATP-CP\textsubscript{20}, Transition State Mimic PKAc-Mg\textsubscript{2}ADP-MgF\textsubscript{3}-SP\textsubscript{20}, and Product PKAc-Ca\textsubscript{2}ADP-pSP\textsubscript{20}—The previously reported crystal structure of the PKAc with MgADP, SP\textsubscript{20}, and Mg\textsubscript{3}F\textsubscript{4} is considered to be a transition state mimic in the phosphoryl transfer reaction (23). Mg\textsubscript{3}F\textsubscript{4}, the transition state analog in place of C\textsubscript{\beta}-PO\textsubscript{3}, has been reassigned as MgF\textsubscript{3} anion by Jin et al. (39) based on \textsuperscript{19}F NMR measurements. Thus, we designate this structure as PKAc-Mg\textsubscript{2}ADP-MgF\textsubscript{3}-SP\textsubscript{20} (PDB code 1L3R). In addition, we recently obtained an x-ray structure of the PKAc-Ca\textsubscript{2}ADP-pSP\textsubscript{20} complex, in which the ADP and phosphorylated SP\textsubscript{20} products were captured in the active site (8). Comparison of our current pseudo-Michaelis complex with the transition state mimic and the product allows us to visualize structural changes that may accompany the catalysis and to identify mechanistically important structural information.

The superposition of the active sites of PKAc-Ca\textsubscript{2}ATP-CP\textsubscript{20} and PKAc-Mg\textsubscript{2}ADP-MgF\textsubscript{3}-SP\textsubscript{20} is shown in Fig. 2. The catalytically important residues occupy very similar positions in both structures, which are aligned with r.m.s. deviations of 0.55 Å. The main difference is the conformation of the phosphate groups of the nucleotide and the displacement of the glycine-rich loop. In PKAc-Ca\textsubscript{2}ATP-CP\textsubscript{20} the C\textsubscript{\beta}-PO\textsubscript{3} of ATP and the oxygen in Ser-21SP\textsubscript{20} is shown as black dashed double arrow. The red dashed arrow demonstrates the difference in the position of C\textsubscript{\beta}-PO\textsubscript{3} group before and after the reaction. Distances are in Å.
is coordinated to both metals and hydrogen bonds to Ser-53. In the product complex, γ-PO₃ group moves 2.7 Å, relative to its position in ATP to bind to Ser-21SP₂₀. The transferred PO₃ group retains its interactions with Ca₁ and Ser-53, but loses coordination to Ca₂. Ca₁ maintains 7 ligands in its coordination sphere, whereas Ca₂ gains an extra water in the product structure. Another striking difference is the orientation of C₇⁻S₉ and C₉⁻O₇ bonds of Cys-21CP₂₀ and Ser(P)-21SP₂₀ before and after the reaction. In PKAc-Ca₂ATP-CP₂₀ the side chain of Cys-21CP₂₀ is oriented toward Asp-166, whereas in PKAc-Ca₂ADP-pSP₂₀ C₇⁻O₇ is rotated away from the active site toward the bulk solvent, which prevents the hydrogen bond from forming between the phosphate group on Ser(P)-21SP₂₀ and Asp-166. The γ-PO₃ transfer is also accompanied by a ~2 Å gliding shift of the glycine-rich loop away from the αB helix and toward the metals and ADP (Fig. 3).

**Ternary Complex PKAc-Mg₂ADP-PO₄-CP₂₀**—Although this complex was crystallized using excess ATP the omit map unmistakably shows that only its hydrolysis products, ADP and PO₄, are present at the active site of the enzyme (Fig. 4A). Refinement suggested 100% occupancy for ADP but 64% for the free phosphate. The partial occupancy of PO₄ is not surprising because small inorganic ions can easily diffuse into the bulk solvent. Additionally, the side chain of Cys-21 of CP₂₀ clearly displays two conformations as indicated by the omit difference electron density map, which would not be possible with 100% PO₄ retention. Conformation A (64% occupancy), in which the Cβ-Sγ bond rotated toward the bulk solvent and away from Asp-166, is similar to that observed in all product complexes (8, 14), and in PKAc-Ca₂AMPPNP-SP₂₀ complex. Conformation B (36% occupancy) with the SH group pointing toward Asp-166, which is identical to the position of Cβ-Sγ in PKAc-Ca₂ATP-CP₂₀ and clashing into the free phosphate (Fig. 4B). The PKAc-Mg₂ADP-PO₄-CP₂₀ structure agrees very well with the recently published room temperature structure of PKAc-Mg₂ADP-PO₄-IP₂₀, which also demonstrated complete hydrolysis of ATP (25). The two structures are superimposed with a r.m.s. deviation of 0.34 Å.

**Molecular Dynamics Simulations**—Root mean square deviations for the three simulations showed that all complexes are stable and converged well during our simulation time (Fig. 5A). Using the PKAc-Mg₂ATP-IP₂₀ crystal structure as reference, the PKAc-Ca₂AMPPNP-SP₂₀ has the largest r.m.s. deviation of the three simulations performed. Root mean square fluctuations of the PKAc residues in the complexes are generally small with the exception of the following regions: Lys-28, Ala-70, Pro-236, His-260, Val-275, Lys-295, and Asp-328. We calculated the distances between the S atom of Cys-21CP₂₀ and closest O atom in the Asp-166 carboxyl in PKAc-Ca₂ATP-CP₂₀ as well as the distance between the O atom in Ser-21SP₂₀ and closest O atom in Asp-166 carboxyl in PKAc-Ca₂AMPPNP-SP₂₀. The S...O distance distribution has a primary peak at 3.5 Å and a minor peak at 7.4 Å, whereas the O...O distance distribution is broad, spanning from 4.0 to 9.0 Å.
For the product complex, the distance between O and the Asp-166 carboxyl is 6.5 ± 0.9 Å. Finally, the PKAc-Ca$_2$AMPPNP-SP20 complex has the largest radius of gyration, whereas PKAc-Ca$_2$ADP-pSP$_2$0 is the most compact structure (Fig. 5C). A characterization of the global motions using principle component analysis indicated that PKAc-Ca$_2$ATP-CP$_2$0 and PKAc-Ca$_2$ADP-pSP$_2$0 have comparable movements related to the opening and closing of the cleft (the dominant motion) that differ from those found in PKAc-Ca$_2$AMPPNP-SP$_2$0 and PKAc-Ca$_2$ADP-pSP$_2$0 systems.

Discussion

In this study we utilized Ca$^{2+}$ ions in place of Mg$^{2+}$ and replaced the nucleophilic oxygen in Ser-21 of SP$_2$0 with sulfur by substituting Cys for Ser to produce a better mimic of the Michaelis complex for the phosphotransfer reaction catalyzed by PKAc. Although a thiol group is a stronger nucleophile than a hydroxyl, thiol analogs are poor kinase substrates (40). Replacement of Ca$^{2+}$ with Mg$^{2+}$ ions in the presence of the CP$_2$0 substrate analog afforded a complex with trapped phosphate ion. These structures in combination with the previously reported structures of the transition state mimic (23) and product complexes (8, 38) complete the snapshots of the phosphoryl transfer reaction in PKAc.

According to the proposal of Mildvan (41) the upper limits of two mechanistically relevant distances, reaction coordinate and axial distances, can be estimated from crystal structures of phosphotransferase reactant and transition state mimic complexes. Analysis of the present results may help discriminate between the dissociative ($A_2D_{N2}$ or $S_{N2}$) mechanisms, and predict the transition state nature, i.e. loose or tight, for the $S_{N2}$ mechanism. In the case of PKAc, the reaction coordinate distance can be measured between the nucleophile and γ-P$_{ATP}$ atoms in a Michaelis mimic. The axial distances can be estimated from the Michaelis and transition state mimic structures by dividing the distances between the nucleophile and leaving γ-P$_{OADP}$ atoms in half, assuming fully symmetric reaction. As estimated by Mildvan (41), the reaction coordinate values of ≤4.9 Å and the axial distances of ≤3.3 Å would indicate the $S_{N2}$ mechanism is in operation, whereas longer separations would point to the $S_{N1}$ mechanism. The other important parameter is the O-P–O angle between the P-O bond of the phosphoryl and the nucleophile atom in the reactant state. If this angle is close to 180°, the reactants are positioned in the near attack configuration for the $S_{N2}$ mechanism.

The concerted mechanism has been suggested for PKAc by stereochemical studies (21). Of the two pseudo-Michaelis complex structures reported here the positioning of reactants in the active site of PKAc-Ca$_2$ATP-CP$_2$0 is more consistent with the $S_{N2}$ mechanism (Fig. 1, A and B). Specifically, the angle between the O$_{N2}$P$_{O}$ bond of ATP and the SCys-21 atom of CP$_2$0 is close to linear (∠O$_{N2}$P$_{O}$SCys-21 = 161°). The C$_{P}$S$_{N2}$ bond of Cys-21CP$_2$0 is rotated toward Asp-166, making a 3.2-Å hydrogen bond with the carboxylic side chain. Importantly, similar orientation of the side chain of the substrate serine was observed in the structure of the transition state mimic PKAc-Mg$_2$ADP-MgF$_3$-SP$_2$0 (Fig. 2). By contrast in PKAc-Ca$_2$AMPPNP-SP$_2$0 the respective angle (∠N$_{SP2}$P$_{O}$OSer-21) is 136° and the serine residue is flipped.

![FIGURE 5. A, r.m.s. deviation of the PKA catalytic domain backbone for PKAc-Ca$_2$ATP-CP$_2$0 (red), PKAc-Ca$_2$AMPPNP-SP$_2$0 (blue), and PKAc-Ca$_2$ADP-pSP$_2$0 (PDB code 4IAX, black) systems is plotted as a function of time. The color scheme is the same for all the following figures. B, red line shows distribution of distances between the S atom of Cys-21CP$_2$0 and closest O atom in the Asp-166 carboxyl in PKAc-Ca$_2$ATP-CP$_2$0 system. Blue line shows distribution of distances between the O atom in Ser-21SP$_2$0 and closest O atom in Asp-166 carboxyl in PKAc-Ca$_2$AMPPNP-SP$_2$0 system. C, distributions of the radius of gyration calculated for PKAc-Ca$_2$ATP-CP$_2$0, PKAc-Ca$_2$AMPPNP-SP$_2$0, and PKAc-Ca$_2$ADP-pSP$_2$0 systems. D, the distributions of trajectory projections onto the first and second principal components for PKAc-Ca$_2$ATP-CP$_2$0, PKAc-Ca$_2$AMPPNP-SP$_2$0 and PKAc-Ca$_2$ADP-pSP$_2$0 systems.](image-url)
away from Asp-166, facing the bulk solvent. MD simulations demonstrate that $O_{\gamma}(\text{Ser}_{21})-O_{\beta}(\text{Asp}_{166})$ separation covers a wide range from 4.0 Å to 9.0 Å in PKAc-Ca$_2$AMPNP-SP20, whereas $S_1$(Cys$_{21}$)-O$_{\beta}$1(Asp$_{166}$) distance has a primary peak located at 3.5 Å in PKAc-Ca$_2$ATP-CP20 (Fig. 5B). Based on previous mechanistic studies, which indicated a possible catalytic role of Asp-166 (42, 43), we propose that in PKAc-Ca$_2$ATP-CP20 the thiol group of Cys$_{21}$CP20 has the correct near-attack conformation that the serine of the substrate would assume in the actual Michaelis complex. Notably, only when the C$_{γ}$-SH bond assumes the “inward” conformation and interacts with Asp-166, sulfur is primed for an in-line phosphoryl transfer, with the $O_{β}^{-}$-$P$-$γ$-$S$$_{\text{Cys_{21}}}$ angle close to 180°. The reaction coordinate distance $P_{γ}$-$S$$_{\text{Cys_{21}}}$ measured in PKAc-Ca$_2$ATP-CP20 is 5.4 Å, which correlates well with that estimated from NMR measurements for the PKAc complex with AMPPPCP and Kemptide (5.3 ± 0.7 Å) (44). The distance between the nucleophilic sulfur and leaving oxygen atoms, $O_{β}$-$S$$_{\text{Cys_{21}}}$, is 7 Å, which gives an estimated axial distance of 3.5 Å. These values suggest a very loose transition state and even recommend the formation of the metaphosphate intermediate in the dissociative mechanism $S_{2,1}$. However, NMR measurements established the inversion of stereochemistry around the $γ$-P atom in the product that points to the $S_{2,2}$ mechanism (21). Yet, $S_{2,1}$ reactions can also proceed with inversion if an inti-

mation, or tight, ion pair is formed along the reaction coordinate (45). According to this concept, the metaphosphate intermediate would not diffuse into solvent and would have a short lifetime, enough only for vibrational relaxation. Although this idea is appealing, the extended reaction coordinate and axial bond distances can be explained by the larger van der Waals radius of sulfur (1.9 Å) compared with oxygen (1.3 Å) in our study and the use of AMPPCP in the previous NMR measurements. Indeed, in PKAc-Mg$_2$ADP-MgF$_3$-SP20 the axial distances $β_{\text{O_{ADP}}}$-$Mg_{F_3}$ and Mg$_{F_3}$-$O_{\text{Ser}_{21}}$ are equal, each of 2.3 Å. Assuming the positions of ATP in PKAc-Ca$_2$ATP-CP20 and Ser-21 of SP20 in PKAc-Mg$_2$ADP-MgF$_3$-SP20 are representative of the actual Michaelis complex, alignment of the two structures offers considerably shorter distances: 4.4 Å for the reaction coordinate $γ$-$P_{\text{ATP}}$-$O_{\text{Ser}_{21}}$ and 3 Å for the axial distances (Fig. 2). These values are similar to those predicted by theoretical calculations for the loose transition state in the $S_{2,2}$ phosphoryl transfer (46). The ternary complex PKAc-Mg$_2$ADP-PO$_4$-CP20 was prepared in an effort to produce a Michaelis complex mimic containing the physiological Mg$^{2+}$ metal ions. Unexpectedly, products of ATP hydrolysis, ADP, and the free phosphate, were found trapped in the active site of PKAc, even though the efficiency of ATP hydrolysis is <1% of the phosphotransferase activity. Acceleration of ATPase activity was previously observed in the presence of histones, which are natural kinase substrates (47). Moreover, increased ATPase activity has been reported with a pseudo-substrate Leu-Arg-Arg-Ala-Cys-Leu-

Gly, whose sequence is similar to that of CP20 and Kemptide (48). Tight binding of IP20 is very efficient in excluding water from the active site resulting in the full inhibition of ATPase activity, which allowed crystallization of ternary PKAc complexes with the unhydrolyzed ATP molecule (15, 25, 26).

theless, two previous crystallographic studies captured the free phosphate in the presence of IP20. Full ATP hydrolysis was observed in the room temperature structure of the wild-type PKAc ternary complex, and was attributed to the x-ray radiation damage (25). Partial ATP hydrolysis was detected in the 100 K structure of the PKAc mutant variant Y204A (49). In this instance, the reaction was attributed to altered conformational dynamics in the mutant.

As a possible explanation for the PKAc ATPase activity it has been suggested that instead of reacting with the nucleophile on the substrate, ATP can be attacked by a water molecule diffused into the enzyme active site and positioned by Asp-166 to initiate the phosphoryl transfer (49). Close inspection of PKAc-Mg$_2$ADP-PO$_4$-CP20 and PKAc-Ca$_2$ATP-CP20 revealed a water molecule that may facilitate ATP hydrolysis by serving as a nucleophile. In the reactant complex, this water molecule, labeled as W4 is only 3.9 Å from the $γ$-P atom of ATP, forms a hydrogen bond with Asp-166, and is also coordinated by Ca2 (Fig. 1, A and C). This water is located at a position nearly identical to an oxygen of the free phosphate in the PKAc-Mg$_2$ADP-PO$_4$-CP20 active site (Fig. 6). If W4 is, in fact, the nucleophilic water, our structures suggest that prior to the attack this water is activated by the metal ion at M2 and Asp-166. In support of this hypothesis, this water molecule is conserved in all of the previously determined product structures, but is absent in ternary PKAc complexes with IP20 (Fig. 1D) (25). This observation and the fact that histones increase the PKAc ATPase activity suggests that binding of PKAc natural substrates might not shield its active site from solvent molecules as effectively as does the high-affinity IP20. This is demonstrated in our MD simulation of PKAc-Ca$_2$ATP-CP20. In the simulation W4 water molecule is dynamic. It occupies the crystallographic position 29% of the
simulation time, but can move to nearby positions where it either loses hydrogen bonding to the $\gamma$-phosphate of ATP or coordination to Ca2, always keeping its hydrogen bonding to Asp-166. The water molecule can also leave the active site into the bulk solvent and then return to its original position. It is possible that both the water molecule and the nucleophilic group of the substrate are positioned in the reactant complex to attack the $\gamma$-P atom of ATP.

Further analysis of PKAc-Mg$_2$ADP-PO$_4$-CP20 leads us to propose that this complex mimics a product state immediately after the phosphoryl transfer takes place, providing insights into the initial step of the product release process. The free phosphate in PKAc-Mg$_2$ADP-PO$_4$-CP20 occupies a position similar to that of MgF$_3$ in the transition state mimic. In this position the P atom of the PO$_4$ ion is only 1.7 and 2.5 Å away from the nucleophilic atoms of the substrate analogs in the transition state mimic and PKAc-Mg$_2$ADP-PO$_4$-CP20 structures, respectively. Furthermore, two oxygen atoms of the free phosphate make short interactions with O$_2$ of Asp-166, which implies protonation of either the phosphate ion or Asp-166 residue. These interactions are consistent with Asp-166 acting as a base before the phosphoryl is transferred, and also suggests that the proton on Asp-166 moves to the phosphoryl group in the product, as predicted by recent QM/MM calculations (50). Protonation of the phosphoryl group would lower the charge on PO$_3$ from $-2$ to $-1$, possibly weakening its interaction with metal ions. This scenario agrees well with the observation that the side chain of Ser(P)-21 rotates away from the metals and Asp-166 toward the bulk solvent in PKAc-Ca$_2$ATP-CP20 after the phosphoryl transfer (8). Comparison of the active sites in PKAc-Ca$_2$ATP-CP20, PKAc-Mg$_2$ADP-PO$_4$-CP20, and PKAc-Ca$_2$ADP-pSP$_20$ demonstrates that free phosphate maintains interactions with the metal ions similar to the $\gamma$-phosphate of ATP, but the phosphoryl group on Ser(P)-21 loses its coordination to M2 (Fig. 6). If Asp-166 protonates the phosphoryl group of the product, it may be an essential first step in the cascade of

**FIGURE 7. Snapshots of the phosphoryl transfer reaction based on crystallographic structures.** A close-up view of the enzyme active site in (I) pseudo-Michaelis complex PKAc-Ca$_2$ATP-CP20 (green, dark cyan Ca$^{2+}$ ions), (II) transition state mimic PKAc-Mg$_2$ADP-MgF$_3$-SP$_20$ (PDB code 1L3R, pink, magenta Mg$^{2+}$ ions), (III) PKAc-Mg$_2$ADP-PO$_4$-CP20 ternary complex (yellow, dark magenta Mg$^{2+}$ ions), and (IV) product complex PKAc-Ca$_2$ADP-pSP$_20$ (PDB code 4IAK, cyan, cyan Ca$^{2+}$ ions). For II–IV, superposition of the present step (colored by atom type as described above) with the structure of the preceding step is shown in blue lines. Water molecules are represented by red and blue spheres for the present and preceding steps, respectively. Metal coordination is shown as solid lines, whereas possible hydrogen bonds are represented as dashed lines.
events necessary for product release, specifically by driving the rotation of the phosphoryl group toward the bulk solvent and opening the glycine-rich loop.

Based on crystallographic evidence, Bastidas et al. (38) have suggested that with natural substrates following the phosphorylated product release M1 ion may be expelled from the active site before ADP bound to M2 leaves. Detailed examination of the metal sites’ coordination in the previously reported product structures (8) provides additional support for this hypothesis. In the product structures containing Mg\(^{2+}\) or Ca\(^{2+}\) bound to both M1 and M2 sites the metals are surrounded by the same number of ligands, 6 for Mg\(^{2+}\) and 7 for Ca\(^{2+}\). M2 is chelated by the α- and β-phosphates of ADP; M1 has one bond to the β-phosphate of ADP and also is coordinated to the transferred phosphoryl group. Therefore, after the peptide product is released, M1 would have fewer interactions than M2. M1 would interact with Asp-184 and the β-phosphate of ADP, whereas M2 would still be chelated by ADP and keep coordination to Asn-171 and Asp-184.

Analysis of the x-ray structures reported here in combination with the previous structures of the transition state mimic and product complexes permits a detailed description of a possible phosphorylation reaction mechanism. In the Michaelis complex the P-site residue is in the near attack conformation when the side chain faces and makes hydrogen bond with Asp-166, as observed in PKAc-Cα2ATP-CP20 (Fig. 7, panel I). The reaction is initiated by the hydroxyl nucleophilic attack of the substrate at the y-P\(_{\text{ATP}}\) and its concurrent deprotonation by Asp-166 acting as a base. The reaction proceeds via the concerted S\(_{2}\)2 mechanism with a loose transition state, having the geometry similar to that in PKAc-Mg\(_2\)ADP-MgF\(_3\)-SP20 (Fig. 7, panel II). After the phosphoryl group has been transferred, the side chain of the phosphorylated product occupies a position similar to that found for the free phosphate ion in PKAc-Mg\(_2\)ADP-PO\(_4\)-CP20 (Fig. 7, panel III). In this orientation the transferred phosphoryl group would be within hydrogen bonding distance from Asp-166. In the next step, Asp-166 switches roles to act as an acid that protonates the phosphoryl group of the product, which in turn triggers its rotation away from the active site toward solvent (Fig. 7, panel IV). The rotation of the phosphoryl group reduces the number of interactions with the active site, including severing coordination to M2 and hydrogen bond with Asp-166. As a result a quick product release accompanied by opening of the glycine-rich loop is possible. Following the product release, M1 dissociates. In the final and rate-limiting step, ADP bound to M2 exits the active site, with M2 losing a number of coordination bonds and ADP breaking several hydrogen bonds with the enzyme. The final step would require substantial energy, involving synchronous motions of structural elements in the small lobe (51) and possibly demanding local unfolding of the enzyme (52).

Note Added in Proof—Susan Taylor was listed as an author on the version of this article that was published on April 28, 2015 as a Paper in Press but has withdrawn herself as an author on the final version.

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