Protein Kinase A in the Neutron Beam:
Insights for catalysis from directly observing protons

Oksana Gerlits,[1]* Kevin L. Weiss,[2] Matthew P. Blakeley,[3] Gianluigi Veglia,[4,5] Susan S. Taylor[6], and Andrey Kovalevsky,[2]*

[1]Department of Natural Sciences, Tennessee Wesleyan University, Athens, TN 37303, USA.
[2]Neutron Scattering Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA.
[3]Large Scale Structures Group, Institut Laue–Langevin, 38000 Grenoble, France
[4]Department of Chemistry, University of Minnesota, Minneapolis, MN 55455, USA.
[5]Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, MN 55455, USA.
[6]Department of Chemistry and Biochemistry, University of California at San Diego, Lo Jolla, CA 92093, USA.

Corresponding authors email addresses: kovalevskyay@ornl.gov, ogerlits@tnwesleyan.edu

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Abstract (150 words)

Protein kinases transmit chemical signals by phosphorylating substrate proteins, thus regulating a multitude of cellular processes. cAMP-dependent protein kinase (PKA), a prototypical enzyme for the whole kinase family, has been the focus of research for several decades, however the details of the chemical mechanism of phosphoryl group transfer have remained unknown. We used neutron crystallography to map key proton sites and hydrogen bonding interactions in the PKA catalytic subunit (PKAc) in a product complex containing adenosine diphosphate (ADP) and the phosphorylated high affinity protein kinase substrate (pPKS) peptide. To improve neutron diffraction, we deuterated PKAc allowing us to use very small crystals. In the product complex, the phosphoryl group of pPKS is protonated whereas the catalytic Asp166 is not. H/D exchange analysis of the main-chain amides and comparison with the NMR analysis of PKAc complex with inhibitor peptide complex revealed exchangeable amides that may distinguish the catalytic and inhibited states.

Keywords: protein kinase A, product complex, neutron crystallography, protonation state, hydrogen bond, phosphoryl transfer, enzyme mechanism, proton transfer.

1. Introduction

Protein kinases regulate many cellular processes by transferring the γ-phosphoryl group of adenosine triphosphate (ATP), which acts as a chemical signal, to serine, threonine, tyrosine and histidine Ser, Thr, Tyr and His residues of various proteins. ~1.7% of human genes encode protein kinases, pointing to the crucial biological significance of these signaling proteins (Manning et al., 2002). Dysfunctional protein kinases are implicated in many diseases and have become major therapeutic targets for the design and development of anti-cancer drugs (Klaeger et al., 2017). The cAMP-dependent protein kinase is the most studied enzyme of the kinase superfamily and has become a paradigm for all other kinases (Adams, 2001). The catalytic domain of PKA – PKAc – consists of small N-terminal and large C-terminal lobes connected by a small linker region. The nucleotide binding site is located between the two lobes, while protein substrates sit on the ledge of the C-lobe. The phosphoryl transfer reaction requires binding of one or two divalent metal ions to the PKAc active site, and the physiological metal is magnesium (i.e., Mg^{2+}) (Taylor et al., 2013).

X-ray crystallographic (Madhusudan et al., 2002; Akamine et al., 2003; Bastidas, et al., 2013; Gerlits et al., 2013, 2014, 2015; Das et al., 2015), NMR spectroscopic (Masterson et al., 2010, 2011; Jin et al., 2012; Srivastava et al., 2014) and biomolecular simulation (Cheng et al., 2005; Valiev et al., 2007; Perez-Gallegos et al., 2015, 2017) studies have provided us with a good understanding of how PKAc functions. During the catalytic cycle, PKAc undergoes several conformational changes. It adopts an open conformation in the apo-form before ATP or protein (or peptide) substrate binds. It transitions to an intermediate, partially closed, state in binary complexes with the nucleotide or substrate. Finally, PKAc assumes a fully closed conformational state in the ternary complex, when both the nucleotide and substrate are present at the active site. Phosphoryl transfer occurs only in the closed conformation of the enzyme, whereas the MgADP product release is the rate-limiting step. The phosphoryl transfer chemical reaction involves several proton transfers, whose pathways can only be understood if protonation states of the active site residues, substrate and product functional groups are known. Among those are the catalytic residue Asp166, hydroxyl of the substrate serine, and the phosphate group on the phosphorylated product. Thus, understanding the mechanistic details of the chemical step remained challenging, because locations of protons have not been experimentally determined.

Neutron diffraction provides a means to directly observe hydrogen atoms and accurately determine their positions in proteins or other biological macromolecules (Niimra & Podjarny, 2011). Because, unlike X-rays, neutrons are scattered by atomic nuclei, instead of electron clouds, their scattering power does not depend on the number of electrons in an atom. Therefore, the lightest element hydrogen scatters neutrons nearly as well as do other atoms, such as carbon, oxygen and nitrogen that are commonly present in protein molecules, although the neutron scattering length for hydrogen is negative. Unambiguous determination of hydrogen atom positions then allows us to visualize hydrogen bonds and other non-covalent interactions, which is usually not possible even in ultra-high resolution X-ray structures (Oksanen et al., 2017). Moreover, the cold neutrons with wavelengths of ~2-5 Å used to perform crystallographic measurements do not cause radiation damage to protein crystals, thus permitting data collection at room temperature to afford radiation damage-free crystal structures at close-to-physiological conditions (Blakeley et al., 2015). One of the significant drawbacks of using neutrons is the very strong incoherent scattering by hydrogen (protium) that only contributes to background in the diffraction images, reducing the signal. To overcome this and increase the signal-to-noise ratio of the neutron diffraction from protein crystals, hydrogens are replaced with the heavy isotope deuterium. The neutron scattering length of deuterium is positive and its incoherent scattering cross section is very small. Hence, deuterium atoms appear in the neutron scattering length density maps as peaks, as do other atoms, unlike hydrogen atoms that are observed as troughs in the maps. Labile hydrogen atoms on OH, NH and SH chemical groups with pK_a values below 20 can be replaced using hydrogen/deuterium (H/D) exchange with the heavy water (D_2O), these make up ~15-20% of the hydrogen content in a protein. Non-labile hydrogen atoms on CH bonds, having pK_a values considerably higher than 20, cannot be displaced by deuterium from D_2O. Deuterium can be incorporated into the CH bonds by expressing protein in the deuterated media made with D_2O and a deuterated carbon source to produce perdeuterated protein as discussed below but this is currently limited to protein production in bacterial and yeast systems.

We have carried out a neutron diffraction experiment at room-temperature to obtain an atomic-level view of PKAc trapped in a ternary complex containing products adenosine diphosphate (ADP), a phosphorylated protein kinase substrate peptide (pPKS) and two metal ions (Figure 1) and gained unprecedented insight into the phosphoryl transfer reaction. We experimentally pinpointed locations of protons (observed as deuterium, D, atoms) in the active site

of PKAc and throughout the protein allowing us to revise the reaction mechanism, and to visualize hydrogen bonds enabling us to compare to those previously inferred from X-ray structures. Furthermore, we identified H/D exchange patterns on the main-chain amides gaining insight into the dynamics of PKAc (Gerlits et al., 2019).

2. Protein sample preparation and crystal growth

Sample preparation is not an easy task for neutron crystallographic experiments. The reasons are fundamental: 1) neutron sources are many orders of magnitude weaker than synchrotrons and 2) the probability of neutron scattering by a nucleus is substantially lower than the probability of X-ray photon scattering from an atom. Thus, for a successful neutron diffraction measurement, production of considerably larger crystals than those necessary for X-ray diffraction is required. But, the outcomes and insights into the biological function this technique offers very well justify the effort (Langan & Chen, 2013; Niimura et al., 2016; O'Dell et al., 2016).

2.1. Partial protein deuteration *versus* perdeuteration

Although both hydrogen and deuterium atoms scatter neutrons strongly, as described above, it is advisable to deuterate the protein sample (replace H by D) for a neutron diffraction experiment. Although full protein deuteration can be very beneficial for the crystallographic experiment, it has several drawbacks. First, protein expression in a fully deuterated media is very costly, as it requires all the components of the expression media including the carbon source to be deuterated. Second, in many cases perdeuteration significantly decreases the yield of the expressed protein. Lastly, it may adversely affect the size of crystals; fully deuterated proteins may, as we have observed for PKAc, not produce large enough crystals for neutron diffraction experiment. We discovered that partial protein deuteration is effective in providing us with significant amounts of the protein and in leading to the growth of sufficiently large crystals. In our study PKAc was expressed in 80% (v/v) D_2O using minimal medium and hydrogenous glycerol as a sole carbon source for the *Escherichia coli* growth. With this partially deuterated sample of PKAc in hand, we were able to grow crystals of ~0.1 mm^3 in volume (Figure 2) and to successfully collect a 2.5 Å room temperature quasi-Laue neutron diffraction dataset.

The His_6-tagged recombinant mouse PKAc subunit was partially deuterated using the following protocol. In the initial step, BL21 (DE3) competent cells containing the PKAc plasmid (carbenicillin resistance, 100 μg/mL) were grown in LB medium made with H_2O until OD_{600} reached ~2. The cells were spun down and resuspended in the *Enfors* minimal medium made with 80% D_2O. 3L of fresh minimal medium containing 80% D_2O was inoculated with the 100 mL of this pre-culture and grown in a bioreactor (BioFlo 3000, New Brunswick Scientific). Because bioreactors allow *E. coli* to grow to very high cell densities due to automatic maintenance of cell growth parameters and feeding, our culture was allowed to reach OD_{600} of ~10 at 35 °C before it was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 20 hours at 18 °C to express PKAc. The sole carbon source for the bacterial growth and protein expression was hydrogenous glycerol. The cell suspension was then spun down at 6000g at 4 °C for 30 min to yield ~ 90g of total wet cell mass. All subsequent protein purification steps were performed using buffer solutions made with H_2O. The enzyme was purified by Ni-affinity chromatography, concentrated to ~ 10 mg/mL, and loaded on to a gel filtration column (HiLoad 16/60 Superdex 75) equilibrated with 50 mM TRIS, 20 mM $SrCl_2$, 250 mM NaCl and 2 mM dithiothreitol (DTT). The purified partially deuterated protein, henceforth called dPKAc, was again concentrated to 10 mg/mL and used for crystallization.

2.2.Crystallization

Protein crystallization is not always a straightforward process, and it is especially tedious when preparing crystals necessary for a neutron crystallographic experiment. This technique requires crystals of orders of magnitude larger than those used at synchrotrons. If a crystal of ~ 0.001 mm^3 (100 microns on each side) would be considered quite large and would diffract X-rays well in most cases, it would have to be at least 100 times larger than that to be suitable for neutron diffraction. Therefore, crystal growth conditions must be optimized much more carefully, and crystallization approaches may have to be altered (for example, much larger crystallization drops are usually required to grow neutron quality crystals). Also, initially a variety of conditions needs to be tested during crystallization trials on hydrogenous protein and in aqueous solutions before attempting work with the deuterated samples. Variables such as protein concentration, type of complexes (reactants *vs.* products or intermediate analogs) and ligand, ligand-to-protein ratio, pH,

precipitant type and concentration must to be adjusted/selected to find those which will produce crystals of the desired size.

During such trials with hydrogenous PKAc we learned that, although we can successfully grow crystals of several PKAc complexes, trapping all stages of the catalytic cycle (Gerlits et al., 2013, 2014, 2015), it was the complex of hydrogenous PKAc with the products of phosphorylation reaction that crystallized the best. The procedure has been described in detail previously in Gerlits et al., 2013. In brief, the reaction mixture was prepared by addition of the solid reagents, the nucleotide ATP, and the 20-residue peptide substrate, PKS, to the solution of the enzyme, PKAc, in presence of ~20 mM Mg_2Cl. In the formed ternary complex, the phosphoryl transfer from ATP to the reactive serine residue of PKS catalyzed by the enzyme generated the reaction products, ADP and the phosphorylated substrate (pPKS). The mixture was incubated for 30 min at 4 $^{\circ}$C to ensure completion of the reaction, filtered and combined at 1:1 ratio with the crystallization solution. The PKAc:ATP:PKS molar ratio, in these experiments, was kept at 1:10:10 with a PKAc concentration of ~10 mg/mL. We also exploited effects of different metal ions, Na^+, K^+, Ca^{2+}, Sr^{2+} and Ba^{2+} in place of the physiological Mg^{2+} and observed that the complex with Sr^{2+} reliably produced the largest crystals. Subsequent X-ray structures of the hydrogenous PKAc complexes, with different metals including Mg^{2+} obtained through the same procedure, revealed both phosphoryl transfer products, ADP and pPKS, captured at the PKAc active site including two metal ions in all studied complexes. Thereby we confirmed the formation of the product complexes with every studied metal ion. Once the proper system capable of producing crystals of the desired size was identified for hydrogenous PKAc, the conditions were tuned to obtain similar results for the deuterated sample.

Purification and crystallization of the deuterated sample were carried out in H_2O-based solutions. The product complex was prepared in a similar manner to that for hydrogenous protein prior to crystallization. Specifically, the dPKAc:ADP:pPKS complex was formed by adding sodium ATP and PKS in solid form to the solution of dPKAc in the molar ratio of 1:10:10. The reaction mixture was incubated at 4 °C for 30-40 min, allowing the phosphoryl transfer reaction to proceed to completion, filtered through 0.2 µm VWR spin filters and combined at a 1:1 ratio (v/v) with the crystallization solution containing 19.5% w/v PEG6000, 0.1 M MES (pH 5), 5 mM DTT and 50 mM $SrCl_2$. The resulting dPKAc:Sr_2ADP:pPKS complex produced several crystals

of ~ 0.1 mm^3 in volume in the same crystallization drop. The crystals were mounted in quartz capillaries containing the reservoir solution made with 100% D_2O to allow for H/D vapor exchange of the labile H atoms (Figure 2). The H/D exchange was allowed to proceed for several weeks prior to neutron diffraction data collection. It is important to note that in some cases H/D exchange can be accomplished directly in the crystallization drop through replacement of the reservoir solution with the solution of the same composition but prepared using 100% D_2O, which ultimately replaces H_2O with D_2O in the crystallization drops. In addition, crystals can be directly grown from solutions in D_2O. To our surprise, neither method was successful for the crystals of our dPKAc complexes. We noticed that the crystal quality quickly deteriorated when we replaced the reservoir solution with the one made in D_2O, indicating that the dPKAc:Sr_2ADP:pPKS crystals did not tolerate D_2O. Moreover, only tiny needles could be grown when we attempted crystallization of our dPKAc complexes in D_2O solutions.

3. Structural analysis

This section will focus on specific insights gained from the neutron structure of dPKAc product complex that illustrate the unique contribution of neutron crystallography to the understanding of protein function that otherwise would be impossible to infer from X-ray diffraction. Specifically, the previously unknown protonation states of several residues, assessment of hydrogen bonding interactions predicted from X-ray structures and applicability of H/D exchange patterns observed for main-chain amides to protein dynamics will be described. The neutron diffraction from a dPKAc:Sr_2ADP:pPKS crystal was first tested at room temperature using the IMAGINE beamline (Meilleur et al., 2013, 2018) at the High Flux Isotope Reactor (Oak Ridge National Laboratory, USA) and subsequently the complete dataset was collected on the LADI-III diffractometer (Blakeley et al., 2010) at the Institut Laue Langevin (France).

3.1. Observing important protons for PKAc function

The direct observation of protonation states on the side chains of ionizable amino acids in a protein structure is the most obvious application of neutron crystallography. Although a good number of hydrogen atom locations can be reliably predicted from an X-ray structure, on the basis of the observed distances between the adjacent heavy atoms of neighboring residues, water molecules, ligands, *et cetera*, the protonation states of some chemical groups usually remain hard to determine unequivocally. Ironically, very often those residues, functional groups on ligands and

water species are of the most importance to the biological function of the macromolecule, and therefore are of the most interest for a researcher.

Proteins kinases have a conserved fold and, consequently, their structures and active sites are very similar. PKA has become a prototypical kinase enzyme and the structural and functional knowledge gained for PKA has been applied to understand structure-function relationships of other kinases. The proton distribution at the site of the phosphoryl transfer has always been debated. Specifically, the protonation states of the conserved catalytic residue Asp166, which is the closest ionizable residue to the OH group of a substrate that could act as a catalytic base, and of the phosphate group of the peptide product have remained unknown. In the X-ray structure of the PKAc product complex we can observe that the carboxyl group of Asp166 and the phosphorylated Ser21 of the substrate pPKS ($pS21_{PKS}$) participate in numerous interactions having distances suggestive of numerous hydrogen bonds. The $pS21_{PKS}$ phosphate makes five such interactions - three with water molecules, one with its own main-chain amide and one with hydroxyl group of Ser53. Asp166 has four interactions - three with water molecules and one with the Thr201 side chain (Figure 3A). However, this does not answer the important questions such as what the Asp166 and $pS21_{PKS}$ protonation states are, and which interactions are true hydrogen bonds, because these structural details cannot be inferred with confidence from the X-ray data. We cannot unequivocally distinguish between hydrogen bond donors and acceptors in these networks. Indeed, each one of the two contributors in a given interaction can act as either or both. Because the position of hydrogen atoms on the hydroxyl groups of Ser53 and Thr201 and orientation of water molecules in the networks are unknown, at least two plausible diagrams can be sketched for each of the groups that differ in the specific locations of known hydrogen atoms for the observed contacts. This is illustrated in Figure 3B and 3C for Asp166, one diagram can describe the situation when Asp166 is deprotonated, whereas the other depicts Asp166 being protonated in the same environment. Something similar, only more complicated, could be drawn for $pS21_{PKS}$; this functional group can have more than one protonation state. Only by using neutron crystallography, we were able to unambiguously determine the hydrogen atom positions.

The neutron structure of the dPKAc product complex provides a complete picture with experimentally determined positions of hydrogen atoms (observed as D) and water (D_2O) orientations. The neutron data clearly show that in the product-bound state Asp166 is deprotonated and the $pS21_{PKS}$ phosphoryl group is monoprotonated (Figure 4). Analysis of these observations

generated conclusions that are not obvious without seeing the observed protons: 1) the protonation of the phosphoryl group on the peptide product probably induces its rotation toward the bulk solvent noted previously from comparison of X-ray structures of the product and the pseudo-Michaelis complexes (Gerlits et al., 2015); 2) this rotation, very possibly, triggers early stages of the product peptide release. Having visualized the protonation states of these chemical groups in the PKAc active site, we can further suggest the possible mechanism of the proton transfer events occurring during the phosphoryl transfer reaction. The OH group of the substrate Ser21 residue should be protonated when the PKS binds to PKAc. The hydroxyl hydrogen can then be transferred to the Asp166 carboxylic group during the phosphoryl transfer step. Thus, Asp166 acts a general base during this reaction step. Once the phosphoryl group is transferred to PKS, the protonated Asp166 donates the proton to an oxygen of the $pS21_{PKS}$ phosphate, now acting as a general acid. The protonated $pS21_{PKS}$ side chain later rotates away from the metal ion to the position detected in our crystal structures. Therefore, it appears that Asp166 may have a dual role in the PKAc catalysis – it can first act as a general base to abstract a proton from the substrate hydroxyl in sync with the nucleophilic attack on the ATP γ-phosphorus, but after the phosphoryl transfer chemical step has occurred it can change its role to become a general acid donating its proton to the $pS21_{PKS}$ phosphate.

ATP binds in an exquisitely tailored nucleotide binding site that accommodates three parts of the molecule – the adenine group, the ribose sugar ring and the phosphates tail (Figure 5). There are also two metal ion binding sites, with the cations coordinating with both the phosphates and PKAc active site residues. The adenine group is located in a sterically tight subsite, interacting with Ala70, Val57 and Leu73 through hydrophobic C-H…π contacts. Its exocyclic N6 amino group makes a single hydrogen bond with the main chain carbonyl of Glu121, which is conserved in all kinases. The phosphates tail stretches over to the phosphoryl transfer site, interacting with the conserved protonated Lys72 and deprotonated Glu91. Importantly, we found that interactions made by the ribose are in agreement with this complex being a product complex, poised for product release. Both 2'- and 3'-OH groups of the sugar ring do not participate in strong hydrogen bonding. The D atom of 2'-OH rotated away from the carboxylic oxygen of Glu127, even though the O…O separation of 2.7 Å implies hydrogen bonding. Moreover, 3'-OH forms only a weak hydrogen bond with the main-chain carbonyl of Glu170.

The catalytic cycle of PKAc is highly regulated and dynamic (Nolen et al., 2004). In the active form, which is facilitated by autophosphorylation of the activation loop Thr197 (pThr197), the kinase catalytic core fluctuates between open (apo-PKAc), intermediate (nucleotide bound PKAc) and closed (nucleotide and substrate bound) conformations (Johnson et al., 2001). The phosphorylation reaction only happens in the closed state. To release the products of the reaction PKAc transitions back to the open conformation and the catalytic cycle is repeated. A contact between pThr197 in the activation loop and His87 at the tip of the αC-helix is one of the key interactions that regulates the active site mechanics (Figure 6). This contact is always present in the structures of PKAc in the intermediate or closed conformations, but the interaction does not form in the open conformation (Akamine et al., 2003). The interaction of His87 with the phosphorylation site of the activation loop has been shown to act as an anchor that slows down opening of the active site cleft and consequently the catalytic cycle. We found that His87 is doubly protonated, carrying a positive charge which stabilizes the negative charge on the deprotonated phosphate of pThr197. It is likely that in the open conformation of apo-PKAc His87 would be not protonated and have a neutral charge. Thus, His87 is a crucial part of the switch mechanism that slows down catalysis and makes the release of ADP the rate-limiting step. Of note is the lack of neutron scattering length density on the phosphate, which is canceled due to the smaller scattering length for P relative to other atoms and low resolution of the dataset. This can be compared with the observation of the strong neutron scattering length density on the phosphate of $pS21_{PKS}$, which may be related to its deuteration. Another important observation afforded by our neutron structure is that Cys199 (in the activation segment) is protonated and is a thiol. In the apo-PKAc, however, it is highly reactive and can be easily chemically modified leading to loss of the kinase activity (Jimenez et al., 1989). In this case, Cys199 is likely deprotonated (thiolate), but protonated Cys199 (thiol) is less reactive than thiolate, ensuring catalytic activity of PKAc is not blocked by chemical modification.

3.2. Dynamic features through H/D exchange pattern and comparison to NMR

Apart from the obvious implications related to protein function, the ability to visualize H/D exchange patterns can be used to determine certain aspects of protein dynamics. This is normally done by examining the H/D exchange levels of the main chain amides, extracting information about the protein molecule regions that are more flexible to allow exchange or more rigid where

exchange is very slow. The crystal of dPKAc:Sr_2ADP:pPKS complex was allowed to H/D exchange for several weeks in order to ensure significant H/D exchange occurred for the sites with rates of > 10^{-6} s^{-1}, and also taking into account that exchange rate decreases at acidic pH (Connelly et al., 1993). The H/D exchange pattern in the dPKAc:Sr_2ADP:pPKS complex is depicted in Figure 7. We consider sites to be fully exchanged when the refined D atom occupancies are in the range of 0.61 – 1.0, partially exchanged D atoms have occupancies in the range of 0.0 – 0.60, whereas when the D occupancy is refined to be -0.56 – 0.0 the main chain amide is considered non-exchanged. Because the neutron scattering length of H is -0.56 times the scattering length of D, the amount of D (%D) at each position can be calculated according to the following equation: %D = [(Doccup. + 0.56)/1.56]*100. Thus, the amount of D is > 3/4 for fully exchanged, between 1/3 and 3/4 for partially exchanged and < 1/3 for non-exchanged amide sites. dPKAc has ~ 53% of the amide protons fully exchanged, ~ 20% of the amide protons partially exchanged, and ~ 27 % sites with no exchange (excluding proline residues that lack exchangeable protons) (Figure 7). Clearly, only about half of the main chain amides underwent complete H/D exchange, whereas the other half exchanged poorly, indicating that the enzyme is rather dynamically rigid. This is not surprising taking into account that dPKAc:Sr_2ADP:pPKS is a stable ternary complex with the high-affinity peptide substrate pPKS. The main-chain amides in the large C-lobe appear to be H/D exchanged to a high degree, with the helices and loops containing mostly fully exchanged sites. But, in the small N-lobe the extended β-sheet and the αC-helix pushing at it from below are mostly not exchanged, suggesting that they are less dynamic. The low level of H/D exchange in the β-sheet is not surprising, because this secondary structure element is greatly stabilized by strong hydrogen bonds between amides of one β-strand and carbonyls of the opposite β-strand. Helices, however, frequently contain well-exchanged amides. Therefore, it appears that αC-helix is less dynamic than other helices in dPKAc, probably because it is tightly packed against the β-sheet.

It is also instructive to compare the H/D exchange of the main-chain amides obtained from our neutron structure of dPKAc:Sr_2ADP:pPKS complex with H/D fractionation factors, φ, measured by the NMR for PKAc:Mg_2ADP:PKI complex (where PKI is an inhibitor peptide identical to PKS, except for a single Ser-to-Ala substitution at the phosphorylation site 21 (Li et al., 2015). φ values determine the strength of hydrogen bonds, with φ < 0.5 indicative of stronger hydrogen bonds and higher φ values are associated with weaker hydrogen bonds. The φ values should correlate with H/D exchange levels because stronger hydrogen bonds are less accessible

and therefore accumulate less deuterium compared to the weaker and more accessible ones. To perform this analysis, we grouped the main-chain amides into two groups: a) slow-exchanging amides with D occupancies below 0.5 ($\varphi < 0.5$), and b) fast-exchanging amides with D occupancies above 0.5 ($\varphi > 0.5$). We then focused on the slow-exchanging sites, because they indicate reduced dynamics of the specific regions. In the NMR dataset, there are 55 residues that belong to the category of slow-exchanging amides for the PKAc:Mg_2ADP:PKI complex, with most of them confined to the small N-lobe, in striking agreement with the H/D exchange pattern observed in the neutron structure. Interestingly, though, about 1/3 of these amides are found fully exchanged in our neutron structure of the dPKAc:Sr_2ADP:pPKS complex. The φ values for the same amides had increased values in the NMR datasets for apo-PKAc or Mg_2ADP binary complex, which correlates with the PKAc transitioning from closed (ternary) to semiopen (binary) and open (apo) conformations, with weakened hydrogen bonds and increased dynamics (Masterson et al., 2010; Li et al., 2015). We concluded that the differences in the H/D exchange pattern, indicative of altered dynamics, observed in the neutron structure arise from our complex representing products of the phosphoryl transfer reaction rather than reactants.

3.3. Analysis of D incorporation into C-H bonds

An interesting aspect for further analysis is how much D is incorporated into the non-exchangeable C-H bonds of dPKAc by the *E. coli* metabolic pathways. This is especially interesting given the fact that the enzyme was expressed in 80% D_2O, with fully hydrogenous glycerol as a sole carbon source. Table 1 presents this analysis. To make this analysis manageable and comprehensible we first averaged occupancies of all D atoms of the C-D bonds for each amino acid residue and treated the whole residue as a single entity. Thus, we identified each residue as having no D incorporation, partial D incorporation or full D incorporation. On average, ~ 70% of all amino acid residues in dPKAc have partial incorporation of D into their C-H bonds, ~ 18% with full D incorporation and ~ 13% with no D incorporation. Interestingly, ¾ of hydrophobic and aromatic residues have partial D incorporation, whereas hydrophilic residues have higher levels of D incorporation. All leucine, proline and methionine residues at least partially incorporate D, with no methionine residues having either no or full D incorporation. On the other hand, none of the tyrosine and trypotophan residues have full D incorporation. In fact, aromatic residues show the poorest levels of D incorporation into the C-H bonds. Hydrophilic residues have most residues

with full D incorporation (~30%) compared to 16% of hydrophobic and only 8% of aromatic residues. It is quite remarkable that, in spite of expressing dPKAc in a solution containing 20% H_2O and 100% hydrogenous glycerol, nearly one-fifth of amino acid residues have full incorporation of D into the non-exchangeable C-H bonds, with five residues containing ≥ 94% of D (one each of glycine, proline, threonine, asparagine and glutamine), reflecting the complexity of metabolic processes in the bacterial cell.

4. Summary

Macromolecular neutron crystallography is a powerful technique capable of providing mechanistic details of enzyme function that are hard, or impossible, to obtain with other biophysical methods. Some challenges, including large crystal growth, protein deuteration, *etc.* mostly stemming from the weak neutron beams, need to be overcome to elevate neutron crystallography to a level of a mainstream structural biology technique. It is nevertheless advisable to consider neutron crystallography in many cases because, if successful, it can answer long-standing biochemical questions. By exploring different protein deuteration and crystal growth methodologies and by careful manipulation of the complex chemical composition, we were able to obtain a neutron structure of PKAc trapped in a product complex with bound Sr_2ADP and phosphorylated PKS peptide. We directly observed protonation of the phosphate group transferred to the serine residue of PKS and determined that the catalytic Asp166 is deprotonated. We also visualized numerous hydrogen bonding interactions by correctly orienting O-D and N-D groups and D_2O molecules, and by determining the protonation states of ionizable residues, such as histidine. Analysis of the main-chain amide H/D exchange levels gave us an opportunity to look into the dynamics of PKAc. Comparison of the H/D exchange with the previous NMR measurements provided further understanding of how dynamics might be altered in the product complex relative to the substrate complex.

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Table 1. Incorporation of D into the C-H bonds. Percentage values indicate how many residues of the same type relative to their total number have a particular level of D incorporation. The level of D incorporation for each residue is calculated by averaging the occupancies of D atoms bound to C atoms.

Amino Acid	% no incorporation	% partial incorporation	% full incorporation	Number of residues
hydrophobic				
Gly	20	50	30	20
Ala	26	64	10	19
Val	11	84	5	19
Ile	5	76	19	21
Leu	0	81	19	32
Pro	0	69	31	13
Met	0	100	0	6
Average	*9*	*75*	*16*	
hydrophilic				
Ser	47	27	26	15
Cys	0	50	50	2
Thr	13	53	34	15
Asp	21	53	26	19
Asn	7	53	40	15
Glu	0	75	25	24
Gln	7	50	43	14
Arg	7	73	20	15
Lys	13	78	9	32
Average	*13*	*57*	*30*	
aromatic				
His	13	63	24	8
Tyr	23	77	0	13
Phe	19	73	8	26
Trp	17	83	0	6
Average	*18*	*74*	*8*	
Overall Average	*13*	*69*	*18*	

Figure Legends.

Figure 1. Overall structure of PKAc:Sr2ADP:pPKS complex in cartoon representation. pPKS peptide product is colored blue. $pP21_{PKS}$ and ADP are shown as sticks, and two Sr^{2+} ions are pink spheres.

Figure 2. Crystal of deuterated PKAc:Sr2ADP:pPKS complex of ~ 0.1 mm^3 volume mounted in a 2 mm inner diameter quartz capillary and used for neutron diffraction data collection. Notice that the crystal is several millimeters long, but small in the other two dimensions.

Figure 3. (**A**) Possible hydrogen bond interactions made by the phosphate of $pS21_{PKS}$ and Asp166 based on the O…O distances. (**B and C**) Chemical diagram of the same part of the active site depicted in (**A**) demonstrating differences in hydrogen bonding caused by lack or presence of a proton on Asp166. Importantly, several additional chemical diagrams are possible if the phosphate of $pS21_{PKS}$ is protonated because the proton can be on any of the three oxygen atoms.

Figure 4. Neutron scattering length density maps, protonation states and hydrogen bonding interactions visualized for $pS21_{PKS}$, Asp166, other active site residues and the associated water molecules. The $2F_O$-F_C map is contoured at 1.5 σ level, and the omit difference F_O-F_C map directly showing protonation of the phosphate of $P21_{PKS}$ is contoured at 3 σ level. Hydrogen bonds are depicted as blue dashed lines and the D…O distances are in Å.

Figure 5. Neutron scattering length density map, protonation states and hydrogen bonding interactions visualized for ADP, other active site residues and the associated water molecules. The $2F_O$-F_C map is contoured at 1.5 σ level. Hydrogen bonds are depicted as blue dashed lines and the D…O distances are in Å.

Figure 6. Neutron scattering length density map, protonation states and hydrogen bonding interactions visualized for pThr197, His87 and other nearby residues. The $2F_O$-F_C map is contoured at 1.5 σ level. Hydrogen bonds are depicted as blue dashed lines and the D…O distances are in Å.

Figure 7. H/D exchange pattern for the main-chain amide protons in the deuterated PKAc. Fully exchanged sites are colored cyan green, partially exchanged amides are purple and non-exchanged amides are red. The pPKS and Sr_2ADP are omitted for clarity.

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