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Mapping the Hydrogen Bond Networks in the Catalytic Subunit of Protein Kinase A Using H/D Fractionation Factors

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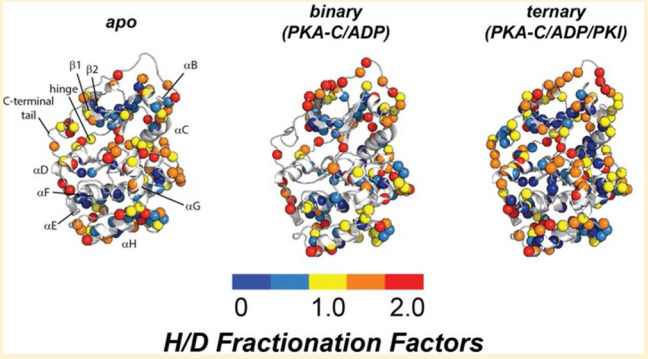
Supporting Information

ABSTRACT: Protein kinase A is a prototypical phosphoryl transferase, sharing its catalytic core (PKA-C) with the entire kinase family. PKA-C substrate recognition, active site organization, and product release depend on the enzyme's conformational transitions from the open to the closed state, which regulate its allosteric cooperativity. Here, we used equilibrium nuclear magnetic resonance hydrogen/deuterium (H/D) fractionation factors (φ) to probe the changes in the strength of hydrogen bonds within the kinase upon binding the nucleotide and a pseudosubstrate peptide (PKI_{5-24}). We found that the φ values decrease upon binding both ligands, suggesting that the overall hydrogen bond networks in both the small and large lobes of PKA-C become stronger. However, we observed several important exceptions, with residues displaying higher φ values upon ligand binding. Notably, the changes in φ values are not localized near the ligand binding pockets; rather, they are radiated throughout the entire enzyme. We conclude that, upon ligand and pseudosubstrate binding, the hydrogen bond networks undergo extensive reorganization, revealing that the open-to-closed transitions require global rearrangements of the internal forces that stabilize the enzyme's fold.



Cyclic AMP-dependent protein kinase A (PKA) is a ubiquitous signaling enzyme that catalyzes the transfer of the phosphoryl group from ATP to the serine or threonine residues of their protein substrates. First crystallized in 1991,[1,2] it served as a prototype for the kinase family. The catalytic subunit of PKA (PKA-C) is a 350-residue protein consisting of a conserved bilobal core flanked by an N-terminal helical segment, known as the A-helix, and a 50-residue C-terminal tail that wraps around the kinase (Figure 1A).[2] The small lobe of the kinase is rich in β-strands with only two short helical segments, while the large lobe is mostly helical. The active site cleft is positioned at the interface of the two lobes and harbors the nucleotide binding site. A glycine-rich loop acts as a lid, protecting the nucleotide's phosphates that protrude from the binding cleft toward the substrate from hydrolysis. A short helix (C-helix) positioned by the activation loop contributes to the organization of the active site. The peptide-positioning loop registers the substrate with respect to the nucleotide, aligning the P site toward the γ-phosphate, with two Mg^{2+} ions participating in the active site organization for phosphoryl transfer. While the catalytic regions of PKA-C are responsible for the chemistry at the active site, signaling involves the core of the protein,[3] with two hydrophobic spines (C- and R-spines) that define active and inactive states of the enzyme. The C-spine is assembled upon nucleotide binding, while the R-spine is assembled upon phosphorylation of the activation loop.[4,5] Structural studies of other protein kinases suggest that the spines are responsible for both allosteric signaling[6] and regulation.[7,8]

During the catalytic cycle, the kinase is thought to undergo significant structural rearrangements. High-resolution crystal structures revealed at least three main conformational states: apo, intermediate (nucleotide-bound), and closed (ternary complex).[9] Nuclear magnetic resonance (NMR) relaxation experiments have shown that the conformational dynamics of the enzyme drive the structural transitions from the open to closed conformation and play a key role in substrate recognition and turnover.[10,11] The nucleotide acts as an allosteric effector, completing the catalytic spines[4,10,12] and shifting the kinase toward a structurally and dynamically committed state.[11] Reaching the committed state may require the rearrangement of the internal forces that stabilize each conformational state, such as hydrophobic, electrostatic, van der Waals, and hydrogen bonding interactions. Although to a lesser

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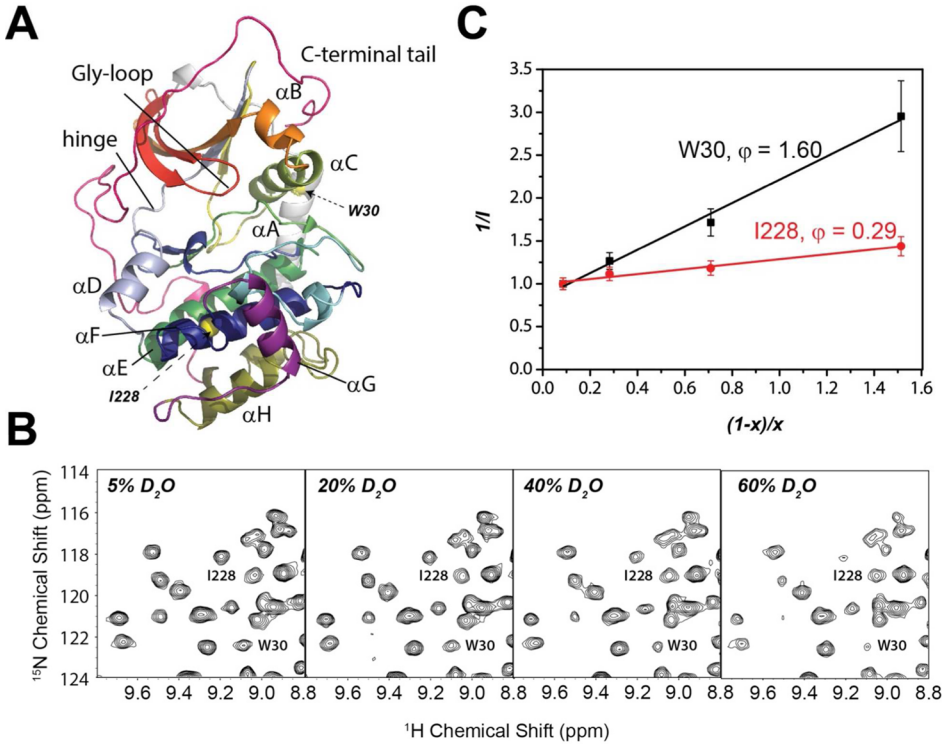

Figure 1. Determination of fractionation factors. (A) Structural motifs mapped onto the ternary complex of PKA-C (Protein Data Bank entry 1ATP). (B) Representative portions of the $^{1}H-^{15}N$ TROSY-HSQC spectra for apo PKA-C at various D_2O concentrations, highlighting W30 and I228 located in helical domains of the kinase. (C) Linear least-squares fit of the inverse of intensities for W30 and I228 vs $(1 - x)/x$, where x is the mole fraction of H_2O.

extent, hydrogen bonds are thought to play an important role in protein stability and in fine-tuning their tertiary structure.[13]

In this paper, we studied the changes in the kinase hydrogen bond strengths across the three major conformational states. To obtain residue-specific information about the hydrogen bond strengths, we measured the hydrogen/deuterium (H/D) fractionation factors (φ) using solution NMR spectroscopy.[14,15] The φ values report on the preference of each amide site for exchanging deuterium over protium from a mixed H_2O/D_2O solution. Initially developed to probe hydrogen bond strengths in dicarboxylic acids,[16] φ values have been widely utilized to investigate low-barrier hydrogen bonds in enzymes[17] and to evaluate the contribution of hydrogen bonds to protein stability.[18,19] Recently, Cao and Bowie revisited the issue of hydrogen/deuterium fractionation, providing a revised energetic scale for the φ values and underscoring the importance of analyzing φ values to determine the changes in hydrogen bond strength for ligand binding to proteins.[20]

Generally, a φ value of unity indicates an equal distribution of deuterium and protium between protein and bulk solvent. The amide sites involved in strong hydrogen bonds prefer protium over deuterium with φ values of <1, whereas amides involved in weak hydrogen bonds have φ values of >1.[18] In proteins, amide hydrogens have φ values ranging from 0.3 to 2.0, with the latter measured for very weak hydrogen bonds.[18] We measured the φ values for the backbone amides of PKA-C in three different forms: apo, ADP-bound (binary complex), and ADP/PKI peptide-bound (ternary complex). Our results show that the overall φ values decrease as the kinase binds ADP and a pseudosubstrate peptide (PKI_{5-24}), indicating that the strength of hydrogen bonds gradually increases from the free to the ternary forms. Importantly, the changes in hydrogen bond strengths are not localized to the binding site but are pervasive throughout the entire protein. Whereas the strengths of the majority of the hydrogen bonds increase, several other hydrogen bonds away from the ligand binding sites become weaker, suggesting a redistribution of the protein free energy. From this study, φ measurements emerge as an important complement to other NMR parameters such as chemical shifts,[21−24] H/D protection factors,[25,26] residual dipolar couplings,[27,28] and nuclear spin relaxation measurements[29−33] to characterize protein conformational dynamics and to identify long-range allosteric changes upon ligand binding or mutations.

EXPERIMENTAL PROCEDURES

NMR Sample Preparation. Uniformly ^{15}N-labeled PKA-C was expressed in *Escherichia coli* bacteria and purified as reported previously.[34] A single protein stock solution was used for each set of experiments for each form of the kinase. Each NMR sample contained ~200 μM PKA, 20 mM KH_2PO_4, 90 mM KCl, 10 mM $MgCl_2$, 10 mM MEGA-8 (octanoyl *N*-methylglucamide), 5% glycerol, 20 mM dithiothreitol (DTT), and 1 mM NaN_3. The pH of the buffer solutions in both protonated and deuterated water was adjusted to 6.5 (without correction for the isotope effect). For the nucleotide-bound form, PKA-C was saturated with 12 mM ADP, while for the ternary complex, the enzyme was saturated with 12 mM ADP and a peptide (350 μM) corresponding to the recognition sequence of the protein kinase inhibitor (PKI_{5-24}) and 60 mM $MgCl_2$ was used. The samples containing varying concentrations of D_2O (5, 20, 40, and 60% by volume for the apo and ternary forms; 5, 20, 40, 50, and 60% by volume for the binary form) were prepared by mixing buffer solutions of protonated and deuterated water. A maximal D_2O concentration of 60%

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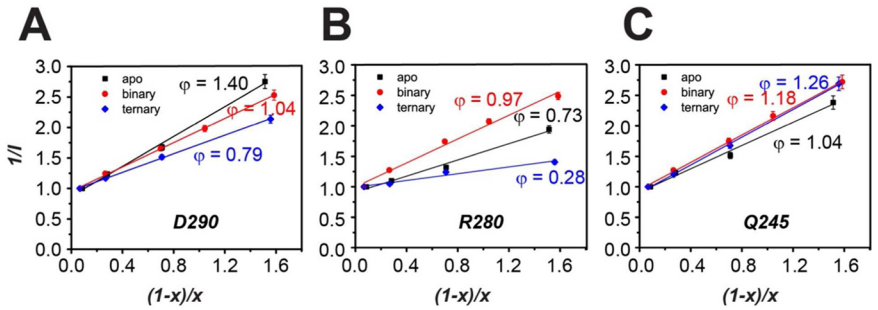

Figure 2. Representative least-squares fitting of residues showing different types of changes in hydrogen bond strengths upon binding nucleotide and pseudosubstrate.

was chosen to minimize errors caused by the increase in T_1 values at high deuteration levels.

Determination of H/D Fractionation Factors. The H/D equilibrium fractionation factor (φ) is defined as the equilibrium constant of the following reaction:

$$\mathrm{NH{:}B} + \frac{1}{2}\mathrm{D_2O} \Leftrightarrow \mathrm{ND{:}B} + \frac{1}{2}\mathrm{H_2O} \quad (1)$$

$$\varphi = \frac{([\mathrm{D}]/[\mathrm{H}])_{\mathrm{protein}}}{([\mathrm{D}]/[\mathrm{H}])_{\mathrm{water}}} \quad (2)$$

where NH represents the weak acid and B the weak base.

To determine the fractionation factors, a series of ^{1}H−^{15}N TROSY-HSQC[35] spectra were acquired using the CLEAN-TROSY version of the original pulse sequence[36] and recorded for all of the forms of the kinase for all of the D_2O concentrations. The spectra were acquired on an 850 MHz Bruker spectrometer equipped with a triple-resonance probe at 27 °C with either 128 scans (for the binary and ternary forms) or 64 scans (for the apo form) and 60 increments and a recycle delay of 1.5 s. The spectra were processed using NMRPipe.[37] The peak intensities were determined using Sparky[38] with assignments previously determined for PKA-C.[39] Fractionation factors are obtained by the linear least-squares analysis of

$$\frac{1}{I} = C\left(\varphi\frac{1-x}{x} + 1\right) \quad (3)$$

where I is the peak intensity, x is the mole fraction of water, and C is the normalization parameter, which is the inverse peak intensity at 100% H_2O. Note that for high D_2O concentrations, it is advisable to use nonlinear fitting as linear fitting amplifies the errors.[40]

RESULTS

H/D Fractionation Factors and Structural Transitions of PKA-C. Fractionation factors (φ) were measured by incubating PKA-C in aqueous solutions with D_2O concentrations ranging from 5 to 60%. Under these conditions, the intensities of the amide resonances in the ^{1}H−^{15}N TROSY-HSQC spectrum decrease according to their relative propensity to incorporate deuterium (Figure 1B), reflecting the strength of the hydrogen bonds in which they are involved.[14,18] To ensure that the exchange reached equilibrium, we incubated the samples and kept them at 25 °C for 24 h prior to acquiring the ^{1}H−^{15}N TROSY-HSQC spectra (Figure S1 of the Supporting Information).[35] Indeed, we found that the amide resonance intensities were unchanged after a 7 day incubation period (Figure S2 of the Supporting Information). To calculate the φ values, we fit the trend of the amide peak intensities as a function of the mole ratio of D_2O. To mimic the three major conformational states of PKA-C, we performed our experiments on the apo, ADP-bound, and ADP/PKI$_{5-24}$-bound kinase. ADP was used in place of the common ATP mimic, ATPγN, because of the very slow hydrolysis on the time scale of weeks that was observed with ATPγN.[41] The ^{1}H−^{15}N TROSY-HSQC spectra of PKA-C saturated with either ADP or ATPγN are almost superimposable (Figure S1 of the Supporting Information), indicating that the average conformations of the kinase are very similar. As for the binary complexes, the spectra of the ternary complexes formed with either ADP/PKI$_{5-24}$ or ATPγN/PKI$_{5-24}$ bound are similar. Only small chemical shift perturbations are observed near the active site, reflecting the effect of the γ-phosphate group (Figure S3A,B of the Supporting Information). When ligand binds, PKA-C undergoes linear chemical shift changes between the apo and closed forms of the enzyme,[39,42,43] indicating that ligand binding shifts a preexisting conformational equilibrium from the open and closed state in the fast exchange regime on the chemical shift time scale.[42] To estimate the average conformational state of the kinase free and bound undergoing fast exchange equilibrium, we used CONCISE (Coordinated Chemical Shifts Behavior).[42] As reported in Figure S3C of the Supporting Information, the probability densities of the complexes with ADP and ATPγN are essentially overlapped. A small but significant difference is observed for the probability densities for the ternary complexes, which reflect the differences in the observed chemical shifts (Figure S3A of the Supporting Information). Nonetheless, the average positions of the distributions on the average principal component (PC) score indicate that these complexes recapitulate both the intermediate and the closed states (Figure S3C of the Supporting Information).

When ADP is bound, the enzyme's resonances undergo significant line broadening originating from conformational dynamics on the microsecond to millisecond time scale similar to those experienced with ATPγN or ATP (Figure S4 of the Supporting Information).[10,43] Therefore, several resonances in the intermediate states are not available for analysis. Overall, 211, 201, and 243 resonances for the apo, binary, and ternary forms of PKA-C, respectively, were available for the calculations of the φ values (Figure S5 of the Supporting Information). Of the calculated φ values, we eliminated those exceeding the value of 2.0, which are affected by rapid exchange and T_1 values[18] as well as those residues showing poor correlation coefficients (R^2 < 0.70). Note that in a few instances we measured φ values slightly lower than 0.20. Although included in the plots, those residues are considered to be not exchanging with the solvent

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Table 1. Average φ and $\Delta\varphi$ Values for the Various Structural Motifs of PKA-C in the Different Conformational States

	$\langle\varphi\rangle$			$\langle\Delta\varphi\rangle$	
motif	apo	binary	ternary	$\varphi^{binary} - \varphi^{apo}$	$\varphi^{ternary} - \varphi^{binary}$
A-helix	1.30 ± 0.27	1.12 ± 0.34	0.98 ± 0.23	−0.16 ± 0.37	−0.08 ± 0.21
αA−β linker	1.52 ± 0.16	1.32 ± 0.39	1.26 ± 0.25	−0.23 ± 0.24	−0.13 ± 0.20
hinge (residues 120−127)	1.03 ± 0.48	1.33 ± 0.65	1.23 ± 0.47	−0.03 ± 0.06	0.35 ± 0.30
E-helix	0.49 ± 0.13	0.41 ± 0.09	0.51 ± 0.57	−0.03 ± 0.07	−0.16 ± 0.23
F-helix	0.40 ± 0.12	0.56 ± 0.27	0.51 ± 0.33	0.12 ± 0.34	−0.19 ± 0.32
G-helix	1.39 ± 0.28	1.15 ± 0.46	1.31 ± 0.40	0.14 ± 0.002	−0.08 ± 0.84
H-helix	0.86 ± 0.60	1.36 ± 0.57	1.00 ± 0.88	0.06 ± 0.05	−0.10 ± 0.49
C-terminal tail					
residues 298−314	1.44 ± 0.63	1.10 ± 0.55	1.03 ± 0.59	−0.34 ± 0.22	−0.11 ± 0.33
residues 315−345	1.51 ± 0.39	1.65 ± 0.27	1.32 ± 0.37	0.28 ± 0.08	−0.25 ± 0.47
residues 346−350	1.18 ± 0.48	1.19 ± 0.18	1.07 ± 0.35	−0.18 ± 0.34	−0.13 ± 0.38

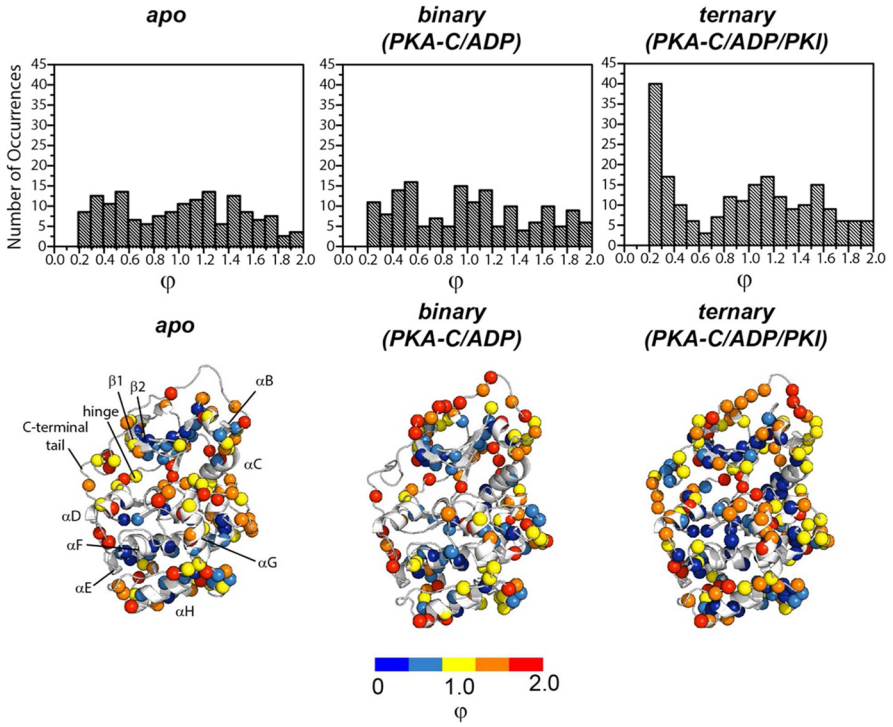

Figure 3. Distribution of amide fractionation factors in three major states of PKA-C. Histogram of the occurrences vs fractionation factor values (top). Mapping of the fractionation factors on the PKA-C structure (bottom, Protein Data Bank entry 1ATP). The amide groups are represented as spheres and color-coded according to the spectrum bar (blue for strong hydrogen bonds and high φ values and red for weak hydrogen bonds and low φ values).

on the time scale we analyzed. These observations are in agreement with previous work by Taylor and co-workers, who found similar nonexchanging residues in the hydrophobic core of PKA-C using deuterium exchange mass spectrometry (DXMS).[44] The overall mean fractionation factor values for the apo, binary, and ternary complexes were 1.06 ± 0.52, 1.04 ± 0.52, and 0.96 ± 0.55, respectively.

Although the overall strength of the hydrogen bonds increases in agreement with the thermostability of the protein,[9] there is variability in the φ values across the enzyme, reflecting local changes in hydrogen bond strength upon ligand binding (Figure 2). In fact, the plots describing the number of occurrences versus φ values for all three forms of PKA-C show broad distributions of φ values (Figure 3). This contrasts with the tight bell-shaped distribution of φ in staphylococcal nuclease that was centered at 0.85.[18] The variability of φ is apparent in all of the structural motifs for all of the states (Table 1). In the apo state, high φ values ($\langle\varphi\rangle$ > 1.2) were measured for the N-terminus (αA and β1) and C-terminal tail, while the helices in the large lobe, namely along αE and αF, display very low φ values [$\langle\varphi\rangle$ < 0.5 (Table 1)]. These results, in qualitative agreement with the DXMS experiments, suggest that these hydrogen bonds make a significant contribution to the stability of the large lobe.[44] Unlike previous fractionation studies,[18,45−47] there is no correlation between φ values and secondary structure in PKA-C. For instance, W30 and I228, both located in α-helices, exhibit rather different hydrogen bond strengths (Figure 1). We also observed that most residues with φ values of ≫2 (Figure S6A of the Supporting Information) are located in the solvent-exposed, unstructured regions of the enzyme undergoing significant motions.[10,11,39,43] Because of exchange broadening on the microsecond to millisecond time scale upon ADP binding, we were unable to measure φ in the active site, including the Gly-rich, DFG,

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activation, and peptide-positioning loops. However, we observed several changes in φ values radiating away from the nucleotide binding pocket to more peripheral domains of the enzyme (Figures 3 and 4 and S7 of the Supporting Information). In the small lobe, these changes include β-strands 1, 4, and 5 ($\langle\Delta\varphi^{\text{binary-apo}}\rangle = -0.27$), the αA–β linker ($\langle\Delta\varphi^{\text{binary-apo}}\rangle = -0.23$), and αA ($\langle\Delta\varphi^{\text{binary-apo}}\rangle = -0.16$) (Figure 4). Interestingly, the loops connecting helices αE, αF, and αH in the large lobe display φ values lower than those of loops in the apo state [$\langle\Delta\varphi^{\text{binary-apo}}\rangle = -0.25$]. Previous research has shown that the nucleotide in PKA-C functions as an allosteric effector.[10,12] These results suggest that nucleotide binding may affect short- and long-range intermolecular forces by altering in part the hydrogen bonding network.

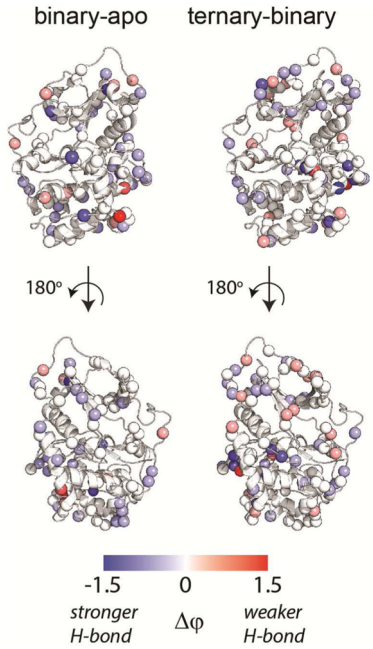

Figure 4. Differences in the fractionation factor between apo and binary complexes (left) and between binary and ternary complexes (right) of PKA-C mapped onto the PKA-C structure.

Binding of PKI_{5-24} increases the compactness of the enzyme as it adopts a closed, solvent-protected conformation,[9] with 54 of 231 resonances displaying φ values around 0.3 (Figure 3), including residues in β-strands 2 and 3, the C-helix, the αC–β4 loop, hinge, αD, αE, the segment from the P+1 loop to the F-helix, αF, αH, and the αH–αI loop. Most of these residues are buried in the interior of the kinase (Figure S6B of the Supporting Information). Overall, we observed a decrease in the average φ values ($\langle\Delta\varphi^{\text{ternary-binary}}\rangle = -0.10 \pm 0.38$), particularly for residues localized in the large lobe and C-terminal tail (Figure 4), except for a few residues in helix αA ($\langle\Delta\varphi^{\text{ternary-binary}}\rangle = -0.08 \pm 0.21$) and the small lobe ($\langle\Delta\varphi^{\text{ternary-binary}}\rangle = -0.09 \pm 0.24$) (Figure 4). In contrast, the hinge region shows a positive change in $\langle\Delta\varphi\rangle$ [0.35 ± 0.30 (Figure 4 and Table 1)], which may result from a reorientation of the hinge region that allows the active site cleft to close. Additionally, PKI_{5-24} binding increases the hydrogen bond strength in the inner core of the kinase for helices αE, αF, and αH (Table 1) as well as for several residues in the C-terminal tail (Figure 4), consistent with the crystallographic data.[9,48,49] Specifically, the acidic patch (residues 330–334) has been hypothesized to recruit the basic substrate into the active site cleft, where Y330 is poised to interact with the substrate's P-3 arginine residue.[39,50] Substrate binding primarily strengthens the hydrogen bonds in the large lobe of the enzyme, and also those in the C-terminal tail, recruited to assemble the active site for catalysis.

To account for the changes in catalytically important motifs (i.e., Gly-rich, DFG loop, peptide-positioning loops, etc.), we considered only the φ values for the apo and ternary forms (Figure 5). The majority of the catalytic motifs experienced strengthening of hydrogen bonds, including the Gly-rich loop ($\langle\Delta\varphi^{\text{ternary-apo}}\rangle = -0.61 \pm 0.28$), αC ($\langle\Delta\varphi^{\text{ternary-apo}}\rangle = -0.43 \pm 0.27$), and the peptide-positioning loop ($\langle\Delta\varphi^{\text{ternary-apo}}\rangle = -0.39 \pm 0.24$). In contrast, the only reporter of the DFG loop (Phe185) shows a higher φ value for the ternary complex ($\Delta\varphi^{\text{ternary-apo}} = 0.50$). Its propensity to incorporate deuterium could result from the increased number of conformational fluctuations caused by nucleotide binding. Taken together, these results show that nucleotide and pseudosubstrate binding assemble the active site, strengthening the hydrogen bonds in the active site and coordinating catalysis in PKA-C.

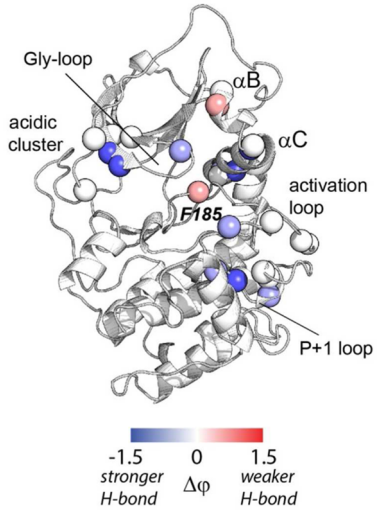

Figure 5. Difference in fractionation factors between apo and ternary complexes for the residues that were exchange-broadened upon ADP binding.

DISCUSSION

In recent years, PKA-C has emerged as a model system for understanding both kinase function and intramolecular allosteric signaling.[4,9] The transitions of PKA-C between different states involve both conformational and dynamic (protein flexibility) changes that propagate from the ligand binding sites to remote parts of the molecule.[10,12] Therefore, a complete understanding of this allosteric signaling necessitates the analysis of intra- and intermolecular interactions that hold the protein together.[51] Among those, hydrogen bonds play a key role in allosteric regulation,[52] and recent reports have demonstrated their importance in enzymatic catalysis and inhibition.[53,54] DXMS techniques have been used to analyze hydrogen bond networks of proteins to identify domains that might be involved in allosteric transitions.[44,55] Unlike DXMS, NMR spectroscopy monitors H/D exchange in a site-specific manner, making it possible to track hydrogen bonds throughout the protein at the atomic level. On the other

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hand, the NMR H/D exchange technique reports on the kinetic protection factors, monitoring the decay of the amide signals versus time.[25,26] However, this technique, with a few exceptions for selected systems,[56−61] usually requires the lyophilization of the protein under examination. In contrast, the equilibrium φ measurements can be accomplished by incubating the proteins in H_2O/D_2O solutions without lyophilization and report directly on the strength of the hydrogen bonds. These measurements are taken under equilibrium conditions, which prevent the freezing−drying procedures required for the H/D protection factor measurements. This is crucial for PKA-C, an enzyme that is partially or completely inactivated upon lyophilization. Previous studies using H/D fractionation factors focused on the enzyme's active sites[19,62] and did not fully exploit the potential of this approach for identifying local and long-range changes in the hydrogen bond network and allosteric communication. In fact, only marginal changes were detected for the φ values upon binding of ligand to the proteins analyzed.[14,18,47,62] These proteins, however, possess relatively rigid and compact structures with uniform φ values. In contrast, the structure of PKA-C is highly dynamic, undergoing substantial conformational changes upon ligand binding.[63,64] As a result, the φ values we measured for PKA-C have a distribution much broader than the distribution of those measured for staphylococcal nuclease,[18] ubiquitin,[45] histidine-containing proteins,[46] and immunoglobulin G binding domain of protein G.[46] As PKA-C undergoes the transition from the open to closed conformation upon binding the nucleotide and the pseudosubstrate inhibitor, the average φ values decrease, suggesting that the intramolecular hydrogen bonds become stronger. Globally, these results correlate well with X-ray crystallography,[9] NMR spectroscopy,[12] fluorescence studies,[65,66] and thermodynamic[67] as well as DXMS analysis[44,55] that show PKA-C's structure becoming more compact and thermostable in the closed conformation. Structurally, nucleotide binding allosterically couples the two lobes of the kinase, causing positive binding cooperativity between the nucleotide and the pseudosubstrate.[12,39] Binding of the pseudosubstrate further stabilizes the complex, as is also evident in the increased strengths of hydrogen bonds in the large lobe as measured by the fractionation factors. Overall, nucleotide binding affects the strength of hydrogen bonds in the small and large lobes, while pseudosubstrate binding affects mostly the residues in the large lobe. This is consistent with the view that the nucleotide is an allosteric effector, organizing the active site, while the large lobe provides a docking surface for the substrate to bind.[9,12] These detailed effects of nucleotide binding went undetected in the previous DXMS measurements[55] because of either a lack of data points or intrinsic limitations of the technique. Recent community map analysis of molecular dynamics simulations[6] predicted that the motions of the different secondary structural elements of the small lobe are highly correlated. β1, β4, β5, and the αA−β linker were attributed to community A (responsible for nucleotide binding), while the A-helix, a portion of the αA−β linker, and the αC−β4 linker were ascribed to community C (Figure S8 of the Supporting Information), a central hub that controls the functions of the other communities.[6] The increase in the strength of the hydrogen bond networks in the small lobe upon nucleotide binding supports the communication between these communities that may act in concert for the enzyme's closure.[6] Moreover, the φ values show that, from the apo to the ternary complex, the hydrogen bonds in the αC helix become stronger, indicating that perhaps strengthening these interactions is essential for proper organization of the active site. From the X-ray structure, the side chains in each turn of helix αC interact with the different domains of the enzyme (Figure S9 of the Supporting Information). Specifically, Arg93 forms a cation−π interaction with the aromatic side chains of Trp30 and Phe26 of helix αA. Glu91 forms a conserved dyad with Lys72 of β-strand 3, while His87 forms an ion pair with the phosphate group of Thr197 in the closed conformation.[9] The H/D fractionation factors also show an extensive rigidification of the hydrophobic core in the enzyme upon ligand binding as previously highlighted by DXMS studies, with helices αE, αF, and αH involved in the intramolecular communication are more resistant to deuterium exchange.[44]

Nucleotide binding also increases the hydrogen bond strengths of residues in the region that caps the bottom of the large lobe. The allosteric coupling between the catalytic loop and the large lobe was also observed using DXMS experiments.[44] In particular, the interactions between conserved residues Glu208 of the APE motif and Arg280 are evident in our data, where these residues show lower φ values upon binding of PKI to the binary form of the enzyme. This ion pair connects the P+1 loop and the loop between helices G and H. The C-terminal tail undergoes significant domain movement when the enzyme undergoes the transition from the open to closed conformation, as observed in the crystal structure (Figure S10 of the Supporting Information).[49] While DXMS data suggest that nucleotide binding increases the amide protection factors for the entire C-terminal tail,[55] our data show that residues 315−345, a region that contains the gate controlling the nucleotide access, display hydrogen bonds weaker than those in the apo form. These weak hydrogen bonds are consistent with the increased flexibility of the intermediate state but eventually become stronger upon formation of the ternary complex, with the interactions between the acidic cluster and the basic residues of the pseudosubstrate.[50]

Overall, this study demonstrates that the changes in the allosteric network of the kinase are manifested through variations in hydrogen bond strengths. These changes are not unidirectional: while several hydrogen bonds become stronger upon ligand binding, other interactions become weaker, underscoring a redistribution of the free energy of binding occurring throughout the whole enzyme. As NMR methodology progresses toward understanding the mechanisms of allosteric transmission at the atomic level, the measurement of equilibrium H/D fractionation factors emerges as a method complementary to other NMR techniques for tracing allosteric communications within proteins and enzymes.

■ ASSOCIATED CONTENT

Supporting Information

Figures S1−S10 and Table S1. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00387.

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Notes

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