Structural Characterization of Protein Kinase A as a Function of Nucleotide Binding

HYDROGEN-DEUTERIUM EXCHANGE STUDIES USING MATRIX-ASSISTED LASER DESORPTION IONIZATION-TIME OF FLIGHT MASS SPECTROMETRY DETECTION*

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Transient state kinetic studies indicate that substrate phosphorylation in protein kinase A is partially rate-limited by conformational changes, some of which may be associated with nucleotide binding (Shaffer, J., and Adams, J. A. (1999) Biochemistry 38, 12072–12079). To assess whether specific structural changes are associated with the binding of nucleotides, hydrogen-deuterium exchange experiments were performed on the enzyme in the absence and presence of ADP. Four regions of the protein are protected from exchange in the presence of ADP. Two regions encompass the catalytic and glycine-rich loops and are integral parts of the active site. Conversely, protection of probes in the C terminus is consistent with nucleotide-induced domain closure. One protected probe encompasses a portion of helix C, a secondary structural element that does not make any direct contacts with the nucleotide but has been reported to undergo segmental motion upon the activation of some protein kinases. The combined data suggest that binding of the nucleotide has distal structural effects that may include stabilizing the closed state of the enzyme and altering the position of a critical helix outside the active site. The latter represents the first evidence that the nucleotide alone can induce changes in helix C in solution.

Protein kinases are the essential enzymes that direct protein phosphorylation in the cell. The results of this posttranslational modification on protein structure and function can have extraordinary effects ranging from changes in carbohydrate and neurotransmitter metabolism to organelle trafficking and cell division. Given the general role that protein phosphorylation plays in these and many other signal transduction pathways, understanding how these enzymes process substrates has become key to understanding cell function. The insights derived from biophysical studies will support the intense consideration that this enzyme family is now being given as chemotherapeutic targets (1). As essential components for normal cell function, protein kinases are tightly regulated through a broad host of processes including phosphorylation (2), second messengers such as cAMP and Ca2+, fatty acylation, protein-protein and domain-domain interactions, and localization through scaffolding and adaptor proteins (3, 4). These processes ensure that the correct protein kinase is activated or repressed at the appropriate time and at the correct location in the cell. Indeed, mutations in protein kinases that alter their regulation are frequently linked to disease (5–10).

Protein kinases possess a well conserved core composed of a small ATP binding domain and a larger substrate binding domain as exemplified in Fig. 1 for the catalytic subunit (C-subunit)1 of protein kinase A (PKA) (11, 12). The active site lies between these two domains with ATP embedded deep within the pocket and the substrate fixed toward the periphery. Protein kinases are conformationally dynamic, and several movements within the core kinase structure have been observed. For example, PKA has been crystallized in both open and closed (Fig. 1) forms that differ primarily by domain rotation (13). Small angle x-ray scattering methods suggest these conformational dynamics may also occur in solution (14). In addition, phosphorylation of the activation loop segment in Cdk2 and the kinase domain of insulin receptor kinase lowers B factors and causes an ordering of this region (15–17). While it is unlikely that the activation loop serves the universal function of an autoinhibitor (18, 19), loop motion has been linked to other interesting changes in structure that may have a general role in regulation. For protein kinase structures that have been solved in both phosphorylated (active) and dephosphorylated (inactive) states, the ordering of the activation loop upon phosphorylation results in a notable shift in helix C (16, 20, 21). This is thought to be a key element in kinase activation, since this movement places a conserved glutamate in this helix (Glu93 in PKA) within hydrogen bonding distance of a conserved lysine (Lys72 in PKA) residue in the active site. This lysine chelates either the β or the α/β phosphates of ATP and upon mutation results in a low activity mutant (22–24). Based upon these findings, it is thought that protein kinase regulation through activation loop phosphorylation is linked to the formation of this essential glutamate-lysine dyad.

Detailed kinetic studies of the paradigm protein kinase, PKA, reveal that conformational changes not only are part of normal catalysis but also may be slow relative to turnover and provide a means of regulating enzyme function. Two conformational changes (one before and one after the phosphoryl transfer step) partially control kcat in wild-type PKA under physiological concentrations of magnesium (25, 26). At least one of these steps appears to be linked to nucleotide binding. Stopped-flow experiments performed on a fluorescently labeled mutant of PKA dem-

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1 The abbreviations used are: C-subunit, catalytic subunit of PKA; PKA, protein kinase A; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; MS, mass spectrometry; H-D, hydrogen-deuterium; mnt, N-methylanthraniloyl; Mops, 3-(N-morpholino)propanesulfonic acid; HPLC, high pressure liquid chromatography; pH*, pH values uncorrected for the isotope effect.
-exchange of deuterium under conditions that favor the re-equilibration of the protein prior to mass spectrometric analysis. The measured deuterium content was corrected for remaining procedure as normal. This sample corresponds to time point 0. The back-exchange occurring during the procedure was measured using a spectrophotometric coupled enzyme assay (31). The C-subunit was preequilibrated with 1 mM ATP and 11 mM MgCl2 in 100 mM Mops (pH 7), and the reaction was initiated with 0.5 mM Kemptide.

**Deuterium Exchange Experiments**—All exchange mixtures for the C-subunit contained the following: 16 mM C-subunit, 40 mM KPi, 50 mM KCl, and 10 mM MgCl2. Final pH* was 6.9, and final percentage of D2O was 87.5%. Exchange experiments performed in the presence of nucleotide included 1 mM ADP and 11 mM MgCl2. The C-subunit was pre-equilibrated with ADP in H2O before starting the deuterium exchange by diluting into D2O. The D2O mixtures were prepared as follows (numbers are per 12-ml aliquot). The amounts of 100 mM KPi and 4 mM KCl needed were mixed, dried in a Speedvac, dissolved in D2O, and mixed with D2O solutions of ADP and MgCl2. The final volume was 10.5 μl, and it contained 36 mM KPi, 57 mM KCl, and either 10 mM MgCl2 or 1 mM ADP and 11 mM MgCl2. The H2O mixtures (1.5 μl per 12-ml aliquot) contained 128 μM C-subunit, 67 mM KPi, and either 10 mM MgCl2 or 1 mM ADP and 11 mM MgCl2. The deuterium exchange was initiated by combining the H2O and D2O solutions. The solutions were incubated at 20 °C. At various times a 12-μl aliquot was added to an ice-cold tube containing 36 μl of 0.19% trifluoroacetic acid and 25 μl of pepsin bead slurry (previously washed twice in 1 ml of cold 0.05% trifluoroacetic acid). This brought the pH* of the C-subunit solution down to 2.5 and quenched the deuterium exchange. The mixture was incubated on ice with occasional mixing for 5 min to facilitate pepsin proteolysis of the C-subunit.

**In- and Back-exchange Controls**—In-exchange and quench conditions was measured by adding the protein solution directly into D2O. The D2O mixtures were prepared as follows (numbers are per 12-ml aliquot). The amounts of 100 mM KPi, and 4 mM KCl needed were mixed, dried in a Speedvac, dissolved in D2O, and mixed with D2O solutions of ADP and MgCl2. The final volume was 10.5 μl, and it contained 36 mM KPi, 57 mM KCl, and either 10 mM MgCl2 or 1 mM ADP and 11 mM MgCl2. The H2O mixtures (1.5 μl per 12-ml aliquot) contained 128 μM C-subunit, 67 mM KPi, and either 10 mM MgCl2 or 1 mM ADP and 11 mM MgCl2. The deuterium exchange was initiated by combining the H2O and D2O solutions. The solutions were incubated at 20 °C. At various times a 12-μl aliquot was added to an ice-cold tube containing 36 μl of 0.19% trifluoroacetic acid and 25 μl of pepsin bead slurry (previously washed twice in 1 ml of cold 0.05% trifluoroacetic acid). This brought the pH* of the C-subunit solution down to 2.5 and quenched the deuterium exchange. The mixture was incubated on ice with occasional mixing for 5 min to facilitate pepsin proteolysis of the C-subunit. The mixture was then centrifuged for 20 s at 12,000 × g at 4 °C to remove the pepsin beads, and the solution was divided in aliquots and frozen in liquid N2. The samples were stored at −80 °C until MALDI-TOF MS analysis.

The mixtures for deuterium exchanging C-subunit at pH* 5.9 were prepared similarly to the pH* 6.9 samples. A predetermined amount of phosphoric acid that would bring the solution to pH* 5.9 was added together with KCl and KP, before drying in the Speedvac. The concentration of trifluoroacetic acid used to quench the reaction was adjusted accordingly to reach pH* 2.5.

**Experimental Procedures**

**Materials—**PD10 columns for buffer exchange were obtained from Amersham Pharmacia Biotech. Dipotassium-ADP was obtained from ICN Biomedicals Inc. D2O (99.9% deuterium) was obtained from ISO-TEC Inc. Pepsin immobilized on 6% beaded agarose was obtained from Pierce. Trifluoroacetic acid and acetonitrile were obtained from Fisher and were of peptide synthesis grade and optima grade, respectively. α-Cyano-4-hydroxycinnamic acid was obtained from Aldrich and recrystallized once from ethanol. ATP, Mops, lactate dehydrogenase, pyruvate kinase, reduced nicotinamide adenine dinucleotide (NADH), and phosphoenolpyruvate were purchased from Sigma. The substrate peptide, LRRASLG (Kemptide), was synthesized at the USC Microchemical Core Facility using Fmoc (N-(9-fluorenylethoxycarbonyl) chemistry and purified by C-18 reverse phase HPLC.

**Protein Preparation and Activity Assays**—The C-subunit of murine PKA was expressed in E. coli and purified as previously described (30). Isozyme I, the first isof orm eluting from the cation exchange column, was used for all experiments. The buffer was changed to 100 mM KP, pH 7.0, 5 mM 2-mercaptoethanol on a PD10 column, and the protein was concentrated to 192 μM. The protein was stored at 4 °C and used in the experiments without lyophilizing. The activity of the C-subunit was measured using a spectro photometric coupled enzyme assay (31). The C-subunit was preequilibrated with 1 mM ATP and 11 mM MgCl2 in 100 mM Mops (pH 7), and the reaction was initiated with 0.5 mM Kemptide.

The data suggest that when nucleotides bind to PKA, helix C changes conformation, perhaps causing formation or strengthening of the dyad, and the closed form of the enzyme is stabilized.

**FIG. 1. Closed form of the C-subunit of PKA.** A, the standard representation of the C-subunit derived from the ternary complex with ATP and a peptide inhibitor (not shown). The arrow indicates the direction of domain movement observed in the open conformation derived from a binary complex with a peptide inhibitor (13). The open and closed forms are related by a 15° rotation of the β-sheet in the small domain relative to the larger domain. The activation loop is shown in magenta, and helix C is shown in blue. Co-crystallized ATP and phosphorylated Thr197 in the activation loop are shown in ball and stick representations. B, rotation of the closed C-subunit structure in A by 90 °C in the z axis. The figure was prepared using Protein Data Bank accession number 1ATP (Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ).
bone amide protons for deuterium. To assess the amount of label lost during sample workup (back-exchange) the deuterated samples were treated to quench and MALDI-TOF MS analysis as described above. This control measures, for each peptide, the maximal experimental mass that corresponds to a fully exchanged peptide.

**MALDI-TOF MS—** MALDI-TOF MS was performed essentially as described previously (29), under which conditions the H-D exchange is kept at a minimal rate. Samples were kept on finely crushed dry ice, and target plates were kept at 4 °C. The matrix solution consisted of 5 mg/ml 2-cyano-4-hydroxycinnamic acid in 1:1:1 acetonitrile, ethanol, 0.5% trifluoroacetic acid (final pH 2.0). The samples were thawed quickly, and 5 μl were mixed with a 5-μl aliquot of 4 °C matrix solution. One μl was spotted on the target plate at 4 °C and dried in 1.5 min under moderate vacuum. Mass spectra were acquired on a PerSeptive Biosystems Voyager DE STR MALDI-TOF. Data were acquired at a 2-GHz sampling rate, 100,000 data channels, with a 20,000-V accelerating voltage, 78% grid voltage, and 0.012% guide wire voltage and using delayed extraction with a 100-ns pulse delay. 256 scans were averaged in ~3 min.

**Data Analysis—** The mass spectra were calibrated in the software GRAMS using the 1194.6485 and 1793.9704 mass peptides. The spectra were then base line-corrected, and centroids of each peak were determined using in-house software (29). The number of deuteriums in-exchanged at time t was calculated as in Ref. 33 using Equation 1,

\[ D(t) = \frac{m(t) - m(0)}{m(100) - m(0)} \times N \]  

(Eq. 1)

where \( m(t) \) is the observed centroid mass of a peptide at time point t, \( m(0) \) is the observed mass at time point 0 (in-exchange control), \( m(100) \) is the observed mass of a fully exchanged digest (with consideration to back-exchange; see above), and N is the total number of peptide amide protons in the peptide. The data presented here originate from a single incubation experiment. Similar results have been obtained in four previous experiments.

**RESULTS**

**Experimental Design—** H-D exchange reactions are exquisitely sensitive to changes in structure and dynamics that accompany protein folding, ligand binding, or formation of protein-protein interactions. H-D exchange in small proteins can be monitored at the residue-specific level by combining H-D exchange with NMR detection. In the case of PKA, a 40-kDa protein, we utilize a medium resolution method that gives region-specific information by combining H-D exchange, pepsin fragmentation, and MALDI-TOF MS detection. A schematic for this technique is displayed in Fig. 2. The C-subunit, prequillibrated with or without the nucleotide, is incubated in D₂O up to 3 h. After designated time periods, the exchange is quenched at pH* 2.5, and the protein is digested with pepsin. During the exchange reaction, the nucleotide concentration is ~100-fold higher than the \( K_i \) for ADP (25), assuring more than 99% binding at all times. In addition, the observed association rate for ADP is estimated to be 2000 s⁻¹ (34), whereas the average intrinsic H-D exchange rate is slower (3.4 and 0.34 s⁻¹ at 20 °C and pH* 6.9 and 5.9, respectively (35)), ensuring that H-D exchange does not outcompete the ligand binding reaction.

The peptides appearing in the mass spectrum after pepsin fragmentation (Fig. 3) have been assigned to the amino acid sequence of the C-subunit. They cover ~65% of the primary structure (29). Due to the nature of our exchange protocol, we monitored a subset of these peptides (Table I). The incorporation of deuterium necessarily broadens the peaks (Fig. 3, lower panel) with resulting lower signal-to-noise ratio. Three sets of peaks separated by 3–6 mass units in the H₂O samples became, upon deuteration, predominantly overlapping and not suitable for analysis (data not shown).

**Data Analysis and In- and Back-exchange Controls—** The average mass of each peptide was determined by integrating over the full envelope of peaks (29), and the mass was converted into number of in-exchanged deuteriums using Equation 1. The in- and back-exchange controls set the zero and infinite time points for \( D(t) \). The in-exchange controls ranged from 13–20% (16% average), whereas the back-exchange controls ranged from 73 to 91% (83% average) for the specific peptides. Since each peptide fragment can contain a number of ligand-sensitive exchangeable protons, with different intrinsic exchange rates, a visual inspection of the curves was used to evaluate the data. The C-subunit is not stable for more than a day under our experimental conditions, which excludes measurement of the slowest exchanging protons that are only exposed with the global unfolding of the protein. For deuterium in-exchange experiments (Fig. 2), an apparent equilibrium is generally observed after 100–200 min (Fig. 4), as has also been reported for other systems (32, 33, 36, 37). Thus, the focus of this study is the specific identification of structural elements that display differences in exchange when the C-subunit has ADP bound.

**Time-dependent Deuterium Incorporation at pH* 6.9—** The extent of deuteration of each peptide probe was followed as a function of time at pH* 6.9. Fig. 4 is a presentation of several probes typical of the results obtained. Over the time course of the ex-
change experiments, the masses of the probes tend to increase in a biphasic manner. The presence of ADP either has no effect or protects amide protons from H-D exchange compared with the apoenzyme, as is evident in the decreased level of label incorporation. The amide protons within probes covering residues 92–100 and 164–174 are clearly protected from H-D exchange over a 3-h period in the presence of ADP (Fig. 4, upper and lower panels). The results for all probes are summarized in Table I. Given the experimental error in the technique, we designate a probe as protected if the mass is 1 or more units lower compared with the free C-subunit after 3 h of exchange.

**Time-dependent Deuterium Incorporation at pH* 5.9**—The previously described experiments were performed at pH* 6.9, where the average half-life for exchange of an amide proton is ~200 ms (35). We repeated the experiments with the pH* of the H-D exchange reaction lowered by 1 unit, which decreases the intrinsic H-D exchange rate by 10-fold. Thus, more subtle differences in amide exchange protection can be detected over the same time period. Lowering the pH value further is not possible due to instability of the C-subunit at pH* values below 5.5.

Fig. 5 displays several exchange studies at pH* 5.9. As shown in Fig. 5E and summarized in Table I, exchange protection by ADP is observed at pH* 5.9 for all probes that are also protected by the nucleotide at pH* 6.9 (i.e. probes encompassing residues 92–100, 163–172, and 164–174 are protected from exchange by ADP at pH* 5.9 and 6.9). Most, but not all, other regions displayed no difference in H-D exchange in the presence or absence of nucleotide at pH* 5.9 (Fig. 5F, Table I). However, at the lower pH, additional nucleotide-protected regions are apparent. As shown in Fig. 5, A–D, two new regions of the protein, covered by a total of four probes, are protected from exchange by ADP. These probes include residues 41–54, 44–54, 303–326, and 303–327 (Table I). These additional regions displaying exchange protection with nucleotide at pH* 5.9 are not likely to be due to changes in structure at low pH for several reasons. The steady-state kinetic parameters, $k_{on}$ for ATP and $k_{off}$, are identical over a wide pH range, which includes the two pH values of this study (38). The rate of phosphoryl transfer, measured in pre-steady-state kinetic studies, is constant from pH 6 to 9 (39). Finally, PKA does not undergo a time-dependent inactivation within the time frame of our experiments. The specific activity of the C-subunit was followed at pH* 5.9 and 6.9 over 3 h, and it was found that the C-subunit did not lose more than 5% of its original activity (data not shown).

**DISCUSSION**

Probing hydrogen bonding and structural dynamics in proteins with hydrogen exchange was initiated by Linderstrøm-Lang nearly 50 years ago (40). In recent years, the approach of H-D exchange coupled with NMR analysis has revealed detailed site-specific information on bond formation and changes in bond energetics as a function of folding (41–51), conformational change (52), or ligand binding (53–60). However, these methods are confined to a small (yet growing) subset of proteins that are amenable to NMR or crystallization and neutron diffraction analysis (61, 62). While site-specific information is highly desirable, for the majority of proteins, this approach is not feasible at the present time. Insights into larger protein systems are facilitated by region specific analysis first introduced by Rosa and Richards in which H-D (H-T) exchange is followed by a quench/protease fragmentation methodology and subsequent analysis by HPLC (63, 64) or more recently mass spectrometric techniques (29, 33). The functional labeling techniques of Englander et al. (65, 66) allowed assessment of allosterically active segments of human hemoglobin and elucidated key regulatory structural changes that are coupled to energy transduction. In a similar vein, structural characterization of the conformations of apomyoglobin and a key partially folded form thereof were facilitated by H-D exchange studies of the respective states at equilibrium (41).

In the present study, a variation of the techniques of Englander et al. (65, 66) and Hughson et al. (41) were employed to assess structural variations as a function of nucleotide binding on the solution conformation dynamics of PKA. In this case, we have applied H-D exchange methodology in combination with MALDI-TOF MS detection to understand the nature of conformational changes in solution in the apoC subunit (where no structural data are available to date) of PKA. The goal is to determine whether nucleotide binding alters the conformational dynamics of specific regions of PKA. We selected ADP to reflect the vialible enzyme complex that occurs subsequent to the phosphoryl transfer step. The transition from ADP-bound C-subunit to free C-subunit represents a rate-limiting step in catalysis at 10 mM free Mg$^{2+}$ and a partially rate-limiting step at physiological Mg$^{2+}$ (25). The focus of our studies is to observe regions displaying different behavior in the presence of ADP, and the time scale of hours has previously proven adequate for the detection of exchange differences in other proteins (32, 36, 37). By following the mass of the probes as a function of incubation time in D$_2$O, we are able to identify regions of the polypeptide chain that are either sensitive or insensitive to ADP (Table I, Figs. 4 and 5). The results obtained were mapped onto the crystal structure of the
C-subunit (Fig. 6). Many regions showed similar H-D exchange behavior independent of the presence or absence of nucleotide. Four regions, however, displayed exchange protection in the presence of ADP. Regions 41–54 and 163–174 covering the glycine-rich and catalytic loops, respectively, and regions 92–100 and 303–327, covering the helix C and the C-terminal tail, respectively, were protected from exchange by ADP. Thus, both regions in the active site and distal to the active site were affected by the presence of ADP.

The Active Site—Two of the regions where exchange protection is observed lie in the active site of the C-subunit. The catalytic loop (residues 165–171, RDLKPEN) has, in the crystal structure (67), several interactions with the bound ATP molecule. The side chain of Lys168 interacts with the γ-phosphate of ATP, the carbonyl oxygen of Glu170 hydrogen bonds to the ribose ring, and the side chain of Asn171 chelates the second Mg2+, which in turn interacts with the α- and γ-phosphates of ATP. The data suggest that approximately two to three deuteriums are protected in the nucleotide-bound form compared with the apoenzyme after 3 h of incubation (Figs. 4B and 5E).

In the absence of site-specific information, we will use a more qualitative analysis and characterize the observed protection.

**TABLE I**

| Peptides and structural elements analyzed by H-D exchange |
|----------------------------------------------------------|
| Structure covered                        | Peptidea | Mass MH b | Nc | Exchange protection with ADP boundd |
|------------------------------------------|-----------|-----------|----|------------------------------------|
|                                            | pH* 6.9   | pH* 5.9   |
| Helix A and loop                         | 27–40     | 1643.8760 | 12 | Yes                                 |
| Helix A and loop, K12                    | 27–40     | 12        | No | No                                 |
| Gly-rich loop                            | 41–54     | 1584.8024 | 13 | No                                 |
| Gly-rich loop                            | 44–54     | 1194.6485 | 10 | Yes                                 |
| Gly-rich loop, helix B                   | 66–83     | 2113.2323 | 17 | No                                 |
| Helix C and loop                         | 92–100    | 1088.6582 | 8  | Yes                                 |
| Helix D, loop, helix E                   | 133–145   | 1628.8886 | 11 | No                                 |
| Catalytic loop                           | 163–172   | 1260.8954 | 8  | Yes                                 |
| Catalytic loop                           | 164–174   | 1373.7795 | 9  | Yes                                 |
| Loop and helix F                         | 212–221   | 1167.5799 | 9  | No                                 |
| Loop and helix F, K12                   | 212–221   | 9         | No | No                                 |
| Loop and helix G                         | 237–250   | 1708.8953 | 12 | No                                 |
| Helix G and loop                         | 246–261   | 1907.0545 | 14 | No                                 |
| Helix G and loop, K12                   | 247–261   | 1783.9704 | 13 | No                                 |
| Catalytic loop                           | 247–264   | 2083.0613 | 16 | No                                 |
| Catalytic loop                           | 247–274   | 2676.4517 | 20 | No                                 |
| Catalytic loop                           | 248–274   | 2823.5201 | 21 | No                                 |
| Catalytic loop, helix E                  | 248–274   | 2492.5305 | 18 | No                                 |

a Peptide sequencing and assignment can be found in Ref. 29. K12 indicates the peptide was observed as a potassium adduct.

b Calculated mass for monoisotopic MH+ peak in H2O.

c N denotes the total number of backbone amides in the peptide not including prolines.
d Regions found to experience exchange protection with ADP after 3 h of exchange in D2O. By definition a protected region displays one mass unit or more difference after this time period.

**FIG. 4.** Time-dependent in-exchange of deuterium for several probes at pH* 6.9. In-exchange of deuterium is plotted as a function of incubation time in D2O at pH* 6.9 in the absence (A), 164–174 (B), 66–83 (C), and 246–261 (D). The data in all panels are fitted to double exponentials for visualization. A and B show probes that are sensitive to the presence of ADP. The fitted parameters for peptide 92–100 are \(y(t) = 5.5 - 2.9e^{-0.0566t} - 2.6e^{-0.197t} (\text{ADP})\), and for peptide 164–174 the fitted parameters are \(y(t) = 5.3 - 2.7e^{-0.0296t} - 2.5e^{-0.560t} (\text{ADP})\), where \(t\) is time in minutes. The data for peptide 92–100 in the presence of ADP was fitted better using a single exponential. MS-detected H-D exchange data often approximate a three-exponential model (32, 76), and the rates observed here are similar to the previously reported ranges for fast and intermediate exchanging protons, whereas the slow exchanging protons are not detectable in the time scale of the experiment (32). The fitted parameters could suggest that when ADP is bound, three protons go to slow exchange rate for the 92–100 peptide, and one fast and one intermediate proton go to slow exchange rate for the 164–174 peptide.
in terms of regional effects. Using this criterion, the observed lower solvent exchange with ADP in the probes containing the catalytic loop (regions 163–172 and 164–174; Figs. 5E and 4B, respectively) may result from two possible effects: (a) reduced access of solvent to the region as a result of nucleotide binding or (b) stabilization or re-positioning of the catalytic loop.

**Fig. 5.** Time-dependent in-exchange of deuterium for several probes at pH* 5.9. In-exchange of deuterium is plotted as a function of incubation time in D₂O at pH* 5.9 in the absence (■) or presence (○) of ADP (1 mM) for probes corresponding to residues 41–54 (A), 44–54 (B), 303–326 (C), 303–327 (D), 163–172 (E), and 247–264 (F). Only the slower phases of the in-exchange experiment are presented.

**Fig. 6.** Probes from the H-D exchange studies mapped to the x-ray structure of the C-subunit. Regions that experience protection by ADP are shown in red, while regions that are unaffected by ADP are shown in green. The structure on the left represents the standard presentation of the C-subunit, while the structure on the right presents a rotation (~90°) in the z axis. Several key structural elements are designated in both panels. Co-crystallized ATP is shown in blue.
The glycine-rich loop (residues 49–57, LGTGSFGRV) also forms several interactions with the bound ATP. In contrast to the catalytic loop, many of these contacts are backbone amide interactions. The amides of Phe$^{54}$ and Gly$^{55}$ interact with the α- and β-phosphates of ATP, whereas the amide of Ser$^{50}$ interacts with the γ-phosphate of ATP. Furthermore, the side chains of Leu$^{49}$ and Val$^{57}$ are involved in hydrophobic contacts with the adenine and ribose ring (67, 68). The observed exchange protection in peptides 41–54 and 44–54 (Fig. 5, A and B) could have similar origins to that proposed for the catalytic loop, i.e. reduced access of solvent to the region as a result of nucleotide binding or stabilization or repositioning of the loop. The glycine-rich loop is, in the crystal structures, the most mobile region of the protein, but the binding of nucleotides brings the loop into a more stable conformation and lowers the B factors (12, 67–70).

A third region consisting of residues 66–83 covers an important interaction in the active site. The side chain of Lys$^{72}$, which is strictly conserved in all protein kinases, interacts with the α- and β-phosphates of ATP. Further, Ala$^{70}$ is involved in hydrophobic interactions with the adenosine ring (68). Interestingly, we do not observe any exchange protection of backbone amide protons in the 66–83 region with ADP bound. These data are reminiscent of the behavior of calmodulin in complex with the MLCK peptide. Backbone and side chain dynamics studies by Wand and co-workers (71) indicate that only side chains undergo changes in motion upon peptide binding, with no changes in the backbone dynamics. These data are consistent with the idea that the entropy cost of binding is minimized by maintaining motional flexibility in the backbone, a phenomenon that may also be occurring in PKA.

**Helix C**—Exchange protection in the presence of ADP is detected in the probe covering part of helix C (positions 92–100) (Fig. 4A). Within 3 h, at least two deuteriums are protected in the nucleotide bound form. Interestingly, helix C has no direct interactions with bound nucleotide, and the backbone amides are more than 12 Å distant from ATP (Fig. 1). Crystal structures of Cdk2, extracellular signal-regulated kinase 2, and insulin receptor kinase show extensive movements in helix C and in the activation loop upon activation of these kinases (16, 17, 20, 21). Cdk2 is activated by binding of cyclin A and activation loop phosphorylation, whereas insulin receptor kinase and extracellular signal-regulated kinase 2 are activated by phosphorylation in the activation loop. In the former case, cyclin A is sufficient to induce these structural changes. In all three examples, the movement of helix C leads to the formation of a conserved electrostatic dyad between a glutamate in the helix and a lysine that interacts with the phosphates of ATP (i.e. glutamate-lysine dyad). The data presented herein demonstrate that the binding of a nucleotide, ADP, to protein kinase A in itself is sufficient to cause an altered environment for helix C. This effect may result in the strengthening of the conserved dyad between Lys$^{72}$ and Glu$^{91}$ (Fig. 7). Interestingly, the constitutively activated mitogen-activated protein kinase kinase mutant displayed increased protection in helix C compared with the repressed form of the kinase (32). For this enzyme, changes in protection result from a glutamate mutation in the activation loop that activates this kinase by mimicking a phosphorylated threonine. The H-D exchange studies on both PKA and mitogen-activated protein kinase kinase attest to the apparent flexibility of this helix depending upon either the enzyme’s phosphorylation state, as in the case of the latter, or the ligand-bound state, as in the case of the former.

**C-terminal Tail**—It has been argued that PKA equilibrates between open and closed forms in solution, but only the open form permits access of ATP (13). Attempts to monitor changes in the structure of the C terminus of PKA upon ligand binding in solution by chemical cleavage or by labeling with fluororescein have failed until now (72, 73). Our studies indicate that ADP protects two probes in the C terminus from H-D exchange (Fig. 5, C and D, Table 1). Phe$^{327}$ moves considerably from its position in the open form and is positioned within 3.5 Å of the adenine ring of ATP in the closed ternary complex and forms a small element of the hydrophobic binding pocket (Fig. 8). Only one of the two probes (residues 303–326 and residues 303–327)
contains Phe²²⁹; therefore, the exchange protection also reflects changes prior to Phe²²⁹. When PKA adopts an open form in the crystalline state, portions of the C terminus, which are within the two probes, become disordered (Fig. 8). Therefore, the H-D exchange experiments are likely to monitor opening and closing of the active site cleft via the C-terminal probes. The exchange protection observed in the C-terminal probes could also suggest an active role for this polypeptide segment in nucleotide binding and possibly catalysis. Consistent with this interpretation, cleavage of the C terminus between residues 332 and 333 by the kinase-splitting memranal proteinase leads to an entirely inactive C-subunit (74). In Sky1p, the C terminus appears to stabilize the activation loop of the catalytic subunit, which plays a different surface on the core than that in PKA (77). In Sky1p, the C terminus may be essential for nucleotide binding. Rotation of the two C-terminal probes, potentially monitored by the C-tail probes, may be required for access to the nucleotide binding pocket. Once this motion occurs, the nucleotide can bind and the closed state may dominate. Further conformational changes may then occur, which include movements of helix C and strengthening of the Glu²³¹–Lys²³² electrostatic dyad. The combined data suggest that the nucleotide has a distal structural effect, including the first evidence that nucleotide alone can induce changes in the C terminus, stabilizing the closed state of the enzyme, and alter the position of a critical helix outside the active site.

**Conclusions**—Transient state kinetic studies indicate that the catalytic cycle in protein kinase A is partially rate-limited by conformational changes, some of which may be associated with nucleotide binding and release (25). The H-D exchange results suggest that two key motions in the C-subunit of PKA may be essential for nucleotide binding. Rotation of the two domains, potentially monitored by the C-tail probes, may be required for access to the nucleotide binding pocket. Once this motion occurs, the nucleotide can bind and the closed state may dominate. Further conformational changes may then occur which include movements of helix C and strengthening of the Glu³¹–Lys³² electrostatic dyad. The combined data suggest that the nucleotide has a distal structural effect, including the first evidence that nucleotide alone can induce changes in the C terminus, stabilizing the closed state of the enzyme, and alter the position of a critical helix outside the active site.

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