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MgATP-induced conformational change of the catalytic subunit of cAMP-dependent protein kinase

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Abstract

Conformational changes of the cAMP-dependent protein kinase (PKA) catalytic (C) subunit are critical for the catalysis of γ-phosphate transfer from adenosine 5′-triphosphate (ATP) to target proteins. Time-resolved fluorescence anisotropy (TRFA) was used to investigate the respective roles of Mg^{2+}, ATP, MgATP, and the inhibitor peptide (IP20) in the conformational changes of a 5,6-carboxyfluorescein succinimidyl ester (CF) labeled C subunit (^{CF}C). TRFA decays were fit to a biexponential equation incorporating the fast and slow rotational correlation times ϕ_F and ϕ_S. The ^{CF}C apoenzyme exhibited the rotational correlation times ϕ_f=1.8±0.3 ns and ϕ_S=20.1±0.6 ns which were reduced to ϕ_f=1.1±0.2 ns and ϕ_S=13.3±0.9 ns in the presence of MgATP. The reduction in rotational correlation times indicated that the ^{CF}C subunit adopted a more compact shape upon formation of a ^{CF}C · MgATP binary complex. Neither Mg^{2+} (1–3 mM) nor ATP (0.4 mM) alone induced changes in the ^{CF}C subunit conformation equivalent to those induced by MgATP. The effect of MgATP was removed in the presence of ethylenediaminetetraacetic acid (EDTA). The addition of IP20 and MgATP to form the ^{CF}C · MgATP · IP20 ternary complex produced rotational correlation times similar to those of the ^{CF}C · MgATP binary complex. However, IP20 alone did not elicit an equivalent reduction in rotational correlation times. The results indicate that binding of MgATP to the C subunit may induce conformation changes in the C subunit necessary for the proper stereochemical alignment of substrates in the subsequent phosphorylation.

Keywords: Time-resolved fluorescence anisotropy; cAMP-dependent protein kinase; MgATP; Conformational change

1. Introduction

Protein kinases comprise a diverse family characterized by highly conserved catalytic cores [1,2]. These enzymes play a central role in the regulation of cellular processes through the phosphorylation of diverse protein targets. Cyclic AMP-dependent protein kinase (PKA) catalyzes the transfer of the γ-phosphate from adenosine 5′-triphosphate (ATP) to serine and threonine residues. PKA is a multifunctional enzyme involved in the regulation of many cellular processes, including cell signaling, glycogen metabolism, and transcriptional control [3–6]. The isozymes of PKA are tetrameric enzymes consisting of a regulatory subunit dimer and two catalytic (C) subunits. It is through interactions with regulatory subunits or with the heat stable protein kinase inhibitor (PKI) that PKA is inhibited. It is generally thought that elevated intracellular adenosine cyclic 3′,5′-monophosphate (cAMP) causes the release of the C subunits from the inhibitory action of the regulatory dimer. The fact that the free C subunit is one of the simplest protein kinases, lacking regulatory elements, makes it an ideal representative of the protein kinase enzyme family.

Abbreviations: cAMP, adenosine cyclic 3′,5′-monophosphate; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; PKA, cAMP-dependent protein kinase; EDTA, ethylenediaminetetraacetic acid; C, catalytic subunit of PKA; CF, 5,6-carboxyfluorescein succinimidyl ester; ^{CF}C, carboxyfluorescein-labeled catalytic subunit of PKA; TRFA, time-resolved fluorescence anisotropy; PKI, heat stable inhibitor protein of PKA; $PKI_{[5–24]}$ or IP20, a pseudosubstrate inhibitor peptide containing residues 5–24 of PKI; AMP-PMP, 5′-adenylyl imidodiphosphate.

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Several X-ray crystal structures of the C subunit, including both the native form and the recombinant form lacking in posttranslational modification, have been solved [7–15]. The enzyme is bilobate, composed of a small nucleotide-binding lobe, and a large substrate-binding lobe. The active site is located in the cleft between these two lobes. The C subunit is known to exhibit remarkable conformational flexibility, and two main structural types designated as "open" and "closed" conformations have been identified. In comparison to the open form, the closed conformation involves a hinge-like closure of the two lobes as well as differences in secondary structure of the small lobe [15]. In order to facilitate phosphorylation, it is necessary for the free C subunit to correctly position substrates through the adoption of a closed conformation [16]. Although it is clear that the C subunit in complex with MgATP and peptide substrate/pseudosubstrate exists in a closed conformation, it is currently unclear which ligand triggers this closure. Attempts to address this issue through crystallography, thermostability, fluorescence resonance energy transfer, small angle X-ray scattering, and chemical footprinting techniques have produced varied results [7–15,17–21].

Time-resolved fluorescence anisotropy (TRFA) is capable of monitoring the rotational mobility of fluorophore-labeled proteins. Proteins of smaller volume exhibit greater rotational mobility in solution. Therefore, TRFA is an ideal technique for the direct measurement of volume changes in proteins that exist in multiple conformations of varying size. In light of the known C subunit open and closed conformations, in the present study, TRFA was used to investigate the effects of Mg^{2+}, ATP, MgATP, and a 20-amino acid segment of PKI (IP20) on native C subunit isolated from bovine heart and labeled with a 5,6-carboxyfluorescein succinimidyl ester (CF) fluorophore. Previous studies demonstrated that the C subunit labeled with CF fluorophore (^{CF}C) exhibited unaltered phosphotransferase activity and unaltered PKI inhibition [22,23]. We found that closure of the ^{CF}C subunit decreased the slow rotational correlation time (ϕ_S) associated with whole body rotations.

2. Materials and methods

2.1. Materials

5,6-Carboxyfluorescein succinimidyl ester (CF) was obtained from Molecular Probes (Eugene, OR). All other reagents were from Sigma (St. Louis, MO) and reagent grade or better. The inhibitor peptide (Thr–Thr–Tyr–Ala–Asp–Phe–Ile–Ala–Ser–Gly–Arg–Thr–Gly–Arg–Arg–Asn–Ala–Ile–His–Asp) was purchased from Sigma and resuspended in a 50-mM Tris buffer (pH 7.4) before use. The peptide constitutes the major inhibitory segment of PKI and has an apparent Ki of 2.3 nM [24,25].

2.2. Proteins

Catalytic subunits of PKA were isolated and purified from bovine hearts, and were labeled with CF and characterized as described previously [23]. The stoichiometry of labeling for the various preparations ranged between 0.5 and 0.8 mol of CF/mol of C subunit. The concentration of catalytic subunits ^{CF}C used for anisotropy measurement was approximately 0.2 μM in 20 mM KH2PO4 buffer, 5 mM β-mercaptoethanol, pH 6.7.

2.3. Nanosecond time-resolved fluorescence anisotropy decay

The time dependence of fluorescence anisotropy was determined as detailed previously [23]. The orthogonally polarized fluorescence decay I_{VV} and I_{VH} were collected by exciting a sample with vertically polarized light while orienting the emission polarizer (polaroid HNP'B dichroic film) in a vertical and horizontal direction, respectively. The emission polarizer was rotated at 5-min intervals while alternately recording I_{VV} or I_{VH}. The ratio of I_{VV}/I_{VH} and G-factor (I_{VH}/I_{HH}) were determined by average of the counts collected in the four combination of vertically (V) and horizontally (H) polarized excitation and emission beams. Excitation and emission bands were selected with Oriel 500-nm short-pass (no. 59876) and 3–68 interference filters, respectively.

2.4. Analysis of fluorescence anisotropy decay data

The fluorescence anisotropy $r(t)$ is defined as:

$$r(t) = [i_{VV}(t) - i_{VH}(t)]/[i_{VV}(t) + 2i_{VH}(t)] \quad (1)$$

and

$$I_{VV}(t) = i_{VV}(t) \otimes l_{VV}(t) \quad (2)$$

$$I_{VH}(t) = i_{VH}(t) \otimes l_{VH}(t) \quad (3)$$

where $I_{VV}(t)$ and $I_{VH}(t)$ are the orthogonally polarized fluorescence decays measured in above experiments; $l_{VV}(t)$ and $l_{VH}(t)$ are the measured lamp pulse. The symbol $\otimes$ denotes the convolution product. Deconvolution from the finite-width lamp pulse was done with the Globals Unlimited computer program [26]. Goodness of fit was evaluated from the value of χ^2 and visual inspection of the difference between experimental and theoretical curves.

The fluorescence anisotropy data were best fitted by a sum of two exponentials,

$$r(t) = r_0[f_S \exp(-t/\phi_S) + f_F \exp(-t/\phi_F)] \quad (4)$$

$$f_S + f_F = 1 \quad (5)$$

where r_0 is the limiting anisotropy, f_S and f_F are the preexponential weighting factors, and ϕ_S and ϕ_F are the

slow and fast rotational correlation times, respectively. The fast exponential is attributed to the fast internal rotations of the protein, i.e., the localized rotations of the label; the slow one is attributed to the whole-body rotations of the entire protein molecule.

3. Results

The isolated and labeled ^{CF}C subunits were examined by TRFA and decays were fit to a biexponential equation (best-fit parameters summarized in Table 1). The fast and slow rotational correlation times for the ^{CF}C apoenzyme were ϕ_f=1.8±0.3 ns and ϕ_S=20.1±0.6 ns, respectively. This ϕ_S value is in reasonable agreement with a theoretical value of ϕ_S=21.3 ns derived from the Stokes–Einstein equation and the recombinant C subunit hydrodynamic radius [27]. Furthermore, both the fast and slow correlation time values are consistent with our previously reported ^{CF}C subunit TRFA values of ϕ_S=1.7±0.3 ns and ϕ_S=18±1 ns [23]. Anisotropy decay events are best detected when fluorescence lifetimes are within an order of magnitude of the rotational correlation time [28]. We have previously determined ^{CF}C fluorescence lifetimes of 1.4±0.1 ns [23], making the CF label ideal for detection of slow rotational correlation time changes observed in this study.

Neither 1 mM Mg^{2+} nor 0.4 mM ATP elicited a significant alteration of the ^{CF}C subunit ϕ_S values (Fig. 1). However, the addition of both 1 mM Mg^{2+} and 0.4 mM ATP to form MgATP produced a significant reduction of slow rotational correlation time to ϕ_S= 13.3±0.9 ns (Fig. 2). This reduction in ϕ_S indicated that MgATP was capable of decreasing ^{CF}C subunit size through conformational changes. The presence of the chelation agent ethylenediaminetetraacetic acid (EDTA) at a 10-mM concentration eliminated the MgATP-induced reduction in ϕ_S, resulting in rotational correlation values similar to those obtained for the ^{CF}C apoenzyme (Fig. 2).

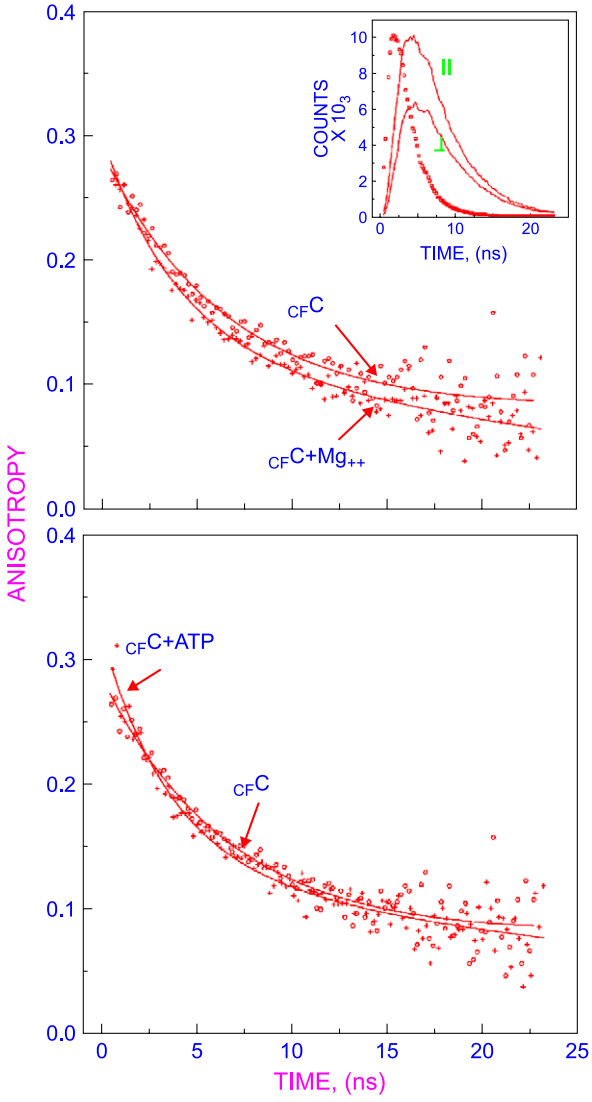

Fig. 1. Time-resolved fluorescence anisotropy decay of ^{CF}C subunits. The time courses of the experimental anisotropy decays (○ and + symbols) and the theoretical decays (solid lines) derived from the best-fit parameters are shown. The Globals Unlimited program was used to fit the decays to Eq. (4) as described in Materials and methods. The inset shows the time course of the excitation lamp pulse (dotted lines) and the time decay of emission (solid lines) with the emission polarizer oriented either vertically (||) or horizontally (⊥) relative to the vertically polarized excitation beam.

Table 1
Effect of Mg^{2+}, ATP, IP20 and EDTA on the fluorescence anisotropy decay of PKA[a]

PKA complex	f_F	ϕ_F (ns)	ϕ_S (ns)
^{CF}C (n=4)	0.43±0.05	1.8±0.3	20.1±0.6
^{CF}C+Mg^{2+} (n=3)	0.51±0.01	1.6±0.1	19.4±1.6
^{CF}C+ATP (n=3)	0.43±0.04	1.8±0.4	20.5±2.1
^{CF}C+MgATP (n=7)	0.37±0.06	1.1±0.2	13.3±0.9
^{CF}C+MgATP+EDTA (n=5)	0.39±0.04	1.9±0.4	19.9±0.3
^{CF}C+IP20 (n=7)	0.42±0.05	1.1±0.3	17.7±3.8
^{CF}C+MgATP+IP20 (n=6)	0.37±0.05	0.7±0.5	14.3±0.8

[a] Shown are the mean±S.D. of the fractions of fast rotation (f_F) and rotational correlation time (ϕ_i) of fluorescence anisotropy decay fitted with a sum of two exponentials. n represents the number of the replications. The concentration of C subunit, Mg^{2+}, ATP, EDTA and IP20 were 0.2 μM, 1 mM, 0.4 mM, 10 mM and 100 μM, respectively.

A limited reduction of mean ϕ_S was apparent in the presence of 100 μM IP20 with rotational correlation times of ϕ_S=17.7±3.8 ns (Fig. 3). However, this effect was not significant due to a high standard deviation. Furthermore, the addition of IP20 and MgATP together produced slow rotational correlation times of ϕ_S=14.3±0.8 ns; this is similar to the ϕ_S values induced by MgATP alone (Fig. 3). Thus, although IP20 did not induce a decrease in ^{CF}C subunit size, it did not affect the contraction induced by MgATP.

The fast rotational correlation times were significantly reduced from ^{CF}C apoenzyme values in the presence of MgATP, IP20, or MgATP+IP20 (Figs. 2 and 3). Fast

rotational correlation times represent local motion of the fluorophore label and have been utilized in the determination of local backbone flexibility in the C subunit [19,29]. The molar fluorophore/protein ratios ranging from 0.5 to 0.8 suggest a preponderance of singly labeled ^{CF}C subunit. However, while the labeling procedure primarily labels aliphatic amino groups, it does not discriminate between possible fluorophore conjugation sites on the protein surface. Multiple reactive lysine residues have been identified at both amino- and carboxy-terminal regions of the C subunit even when MgATP is used to protect the active site [30]. Therefore, the fast correlation times do not reflect the local motion of any given site, and only reflect a composite ordering of the ^{CF}C subunit surface.

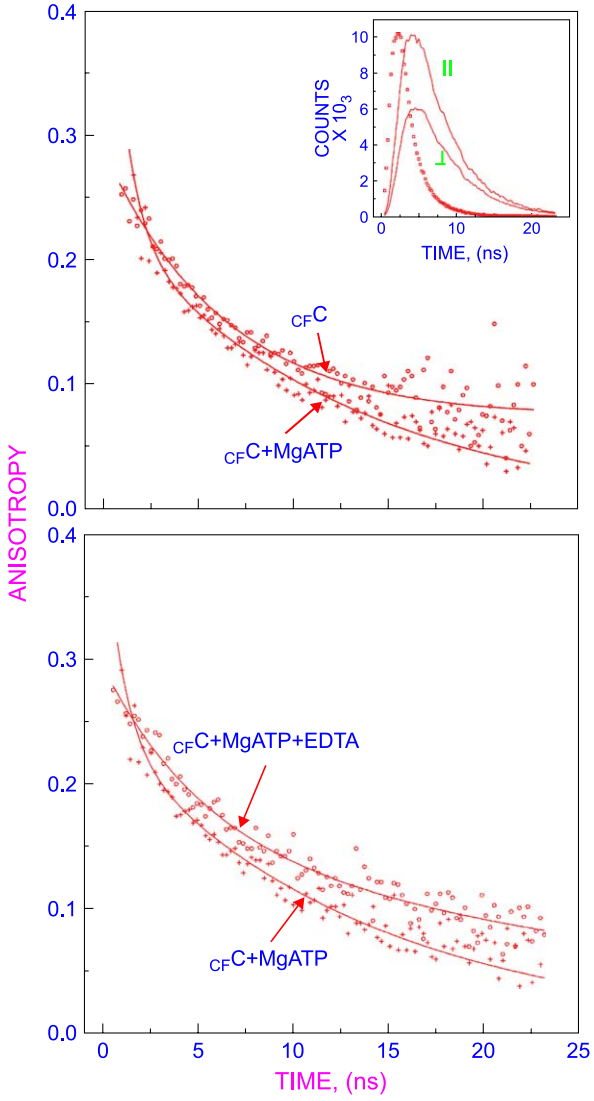

Fig. 2. The effect of MgATP complex on the rotational mobility of ^{CF}C subunits. The time courses of the experimental anisotropy decays (○ and + symbols) and the theoretical decays (solid lines) derived from the best-fit parameters are shown. The Globals Unlimited program was used to fit the decays to Eq. (4) as described in Materials and methods. The inset shows the time course of the excitation lamp pulse (dotted lines) and the time decay of emission (solid lines) with the emission polarizer oriented either vertically (||) or horizontally (⊥) relative to the vertically polarized excitation beam.

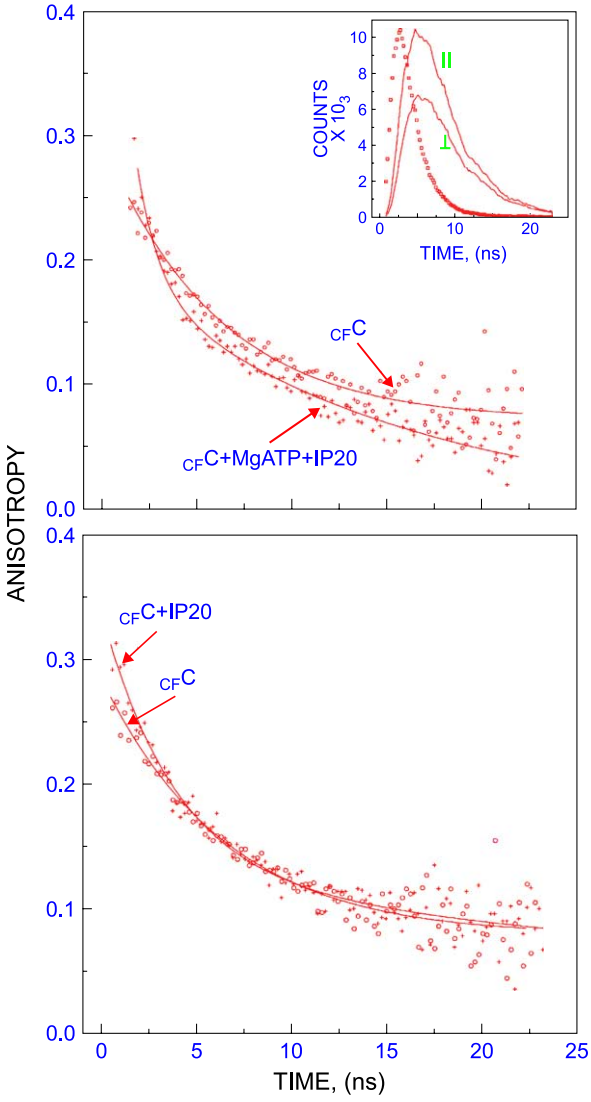

Fig. 3. The effect of IP20 on the rotational mobility of ^{CF}C subunits. The time courses of the experimental anisotropy decays (○ and + symbols) and the theoretical decays (solid lines) derived from the best-fit parameters are shown. The Globals Unlimited program was used to fit the decays to Eq. (4) as described in Materials and methods. The inset shows the time course of the excitation lamp pulse (dotted lines) and the time decay of emission (solid lines) with the emission polarizer oriented either vertically (||) or horizontally (⊥) relative to the vertically polarized excitation beam.

4. Discussion

The present study demonstrates the first clear detection of C subunit domain closure by the TRFA method, and that MgATP is sufficient to induce this conformational change in the ^{CF}C subunit of PKA. The presence of MgATP caused a significant reduction in the slow rotational correlation time of ^{CF}C, attributable to a decrease in ^{CF}C size. This reduction was reversed through chelation of

Mg^{2+} by EDTA. Furthermore, addition of Mg^{2+}, ATP or IP20 alone did not initiate significant decreases in ^{CF}C size. Presumably, decreases in size may be attributed to domain movement in a transition from an open state to the closed state necessary for catalysis. The ^{CF}C apoenzyme slow correlation times decreased by approximately 35% upon formation of the closed C·MgATP·IP20 ternary complex. While the magnitude of such a change is not intuitive, it is possible to use the slow correlation times reported in the present study and the Stokes–Einstein equation to calculate the hydrodynamic radii for the various complexes. The calculated ^{CF}C apoenzyme hydrodynamic radius was 26.9 Å, decreasing by approximately 11% to 24.0 Å in the C·MgATP·IP20 complex. This represents a greater decrease than the 5% decrease in radius of gyration calculated from open and closed X-ray crystal structures [8]. However, crystallography is limited to single snapshots of the conformational dynamics and may be an unreliable indicator of the solution equilibrium. An analysis of intermolecular crystal structure contacts and molecular dynamics simulations based on known crystal structure have indicated that multiple open conformations may exist [31,32]. Furthermore, solution X-ray scattering studies have demonstrated an approximate 9% decrease in the radius of gyration [20], which is consistent with the present findings.

Crystallography data indicate that the ternary complexes of the recombinant mouse catalytic (rC) subunit rC·MgATP·$PKI_{[5–24]}$ [12], and rC·MnATP·$PKI_{[5–24]}$ [10], and the native porcine C·MnAMP-PMP·$PKI_{[5–24]}$ complex [11] exhibit persistent closed conformations, while the native porcine C apoenzyme exhibits an open conformation [15]. However, binary complexes of rC with either adenosine [8], or the ATP analogue balanol [13], have been crystallized in intermediate conformations between open and closed states. In the present study, we have demonstrated that the decrease in slow rotational correlation time caused by MgATP is similar to that observed in response to addition of MgATP+IP20. This is a clear indication that the conformational change resulting from MgATP binding is the result of a transition to a ternary structure equivalent, but not a transition to an intermediate conformation. Crystal structures of C·PKI binary complexes have been solved in either an open conformation for the native porcine heart C·$PKI_{[5–24,\ Diiodinated]}$ binary complex [7], or closed conformations for both rC·$PKI_{[5–24]}$ [14] and rC·$PKI_{[5–24]}$ with detergent [9] complexes. Such seeming discrepancies raise the possibility that the crystallized conformation may be determined in part by stabilizing crystal contacts and lattice forces within a given crystal form [31,7]. Furthermore, it is unknown what effect the lack of posttranslational N-terminal myrstylation may have on the intrinsic flexibility of the C subunit. However, the myrstylic acid group is known to provide some protection against heat denaturation [33] and therefore may affect flexibility.

While X-ray crystallography is limited in its application to the dynamics of catalysis, the study of the open–closed transition with solution techniques provides reliable information about the equilibrium state of the C subunit. Protein footprinting through free radical cleavage of the C subunit has indicated that MgATP binding is the main driving force in the adoption of the closed conformation observed in ternary complexes [17]. Free radical cleavage of the C·MgATP·PKI ternary complex resulted in fragmentation patterns similar to those of the C·MgATP binary complex, while those of C·PKI resembled the apoenzyme cleavage patterns. Additionally, both adenosine 5′-diphosphate (ADP) and ATP are capable of increasing C subunit thermostability, presumably by inducing the closed conformation [18]. This increase in thermostability was observed only in the presence of divalent cation. Furthermore, PKI produced minimal stabilization of the C subunit except in the presence of MgATP [18]. These findings indicate that the binding of MgATP but not IP20 is responsible for the transition to a closed conformation, and are in agreement with the present study.

A great deal of evidence indicating that substrate-induced changes occur, including both crystal structures and solution studies, has been previously obtained. However, there is some debate over which ligand or combination of ligands is responsible for these changes. As discussed above, C·PKI binary complex has been crystallized in both open and closed conformations [7,9,14]. Furthermore, small-angle X-ray scattering studies have demonstrated C subunit contraction in the presence of $PKI_{[5–22]}$ [20,21]. Recent TRFA and fluorescence resonance energy transfer (FRET) studies have demonstrated that domain closure occurs when C subunit is in solution with MgADP, MgATP, PKI or MgATP+PKI [19]. It is possible that in solution, the C subunit exists in equilibrium between open and closed states. If this is the case, then it is possible to reconcile findings that indicate that IP20 induces domain closure with the findings of the present study. The IP20 substrate may be capable of initiating ^{CF}C conformational changes that are not stable; perhaps IP20 is only capable of producing minor perturbations of the open–closed equilibrium. We found that IP20 did slightly decrease mean slow rotational correlation time, although this decrease was not significant due to a relatively high standard deviation. It has been suggested that such a difference in conformational dwell time may be due to the greater number of bilobular interactions mediated by MgATP versus peptide substrate [19].

Previous studies have demonstrated that the kinetic mechanism of C subunit phosphoryl transfer is at least partially sequential, with C subunit binding MgATP prior to substrate peptide [34,25]. X-ray crystallography of a closed ternary structure would seem to confirm this as MgATP is buried beneath PKI in the catalytic cleft [12]. Furthermore, the rate-limiting release of the product nucleotide ADP has been assumed to be associated with conformational changes [35]. This conclusion is reasonable given the requirement of

a closed structure for phosphoryl transfer, and the necessity of opening this structure for the subsequent release of the product nucleotide ADP. While kinetic methods do not provide direct evidence of conformational changes, the use of an acrylodan labeled C derivative has led to the determination that conformational changes are indeed linked to the kinetic mechanism [36]. Further investigation has indicated that at physiological Mg^{2+} concentrations, an ATP-linked conformational change is partially rate limiting for kinetic steps prior to phosphoryl transfer [37]. This is consistent with the present finding that MgATP triggers a major conformational change.

In summary, we have demonstrated that TRFA is an effective technique in the detection of ligand-induced domain closure of PKA. TRFA was capable of differentiating between the ^{CF}C subunit open and closed conformations. The rotational correlation time changes observed in the present study support the concept of a dynamic C subunit conformation. The reduction in slow rotational correlation time associated with MgATP binding indicates that formation of the ^{CF}C · MgATP binary complex shifts the conformational equilibrium towards a closed structure. This is consistent with the majority of the available data characterizing the C subunit. However, the lack of a consensus view regarding C subunit catalysis and the open–closed transition will likely necessitate further investigation. Determining the requirements for C subunit conformation change may further a general understanding of the protein kinase family, and is essential for the ultimate understanding of PKA catalysis.

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