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Role of Conformational Entropy in the Activity and Regulation of the catalytic subunit of Protein Kinase A

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Abstract

Protein kinase A (PKA) is the archetypical phosphokinase, sharing a catalytic core with the entire protein kinase superfamily. In eukaryotes, the ubiquitous location of PKA makes it one of the most important cellular signaling molecules, involved in a myriad of events. The catalytic-subunit of PKA (PKA-C) is one of the most studied enzymes and was the first kinase to be crystallized; however, the effects of ligand binding, post-translational modifications, and mutations on the activity of the kinase have been difficult to understand with only structural data. Here, we review our latest NMR studies on PKA-C, the results of which underscore the role of fast and slow conformational dynamics in the activation and inhibition of the kinase.

Keywords

Protein Kinase A; Allostery; Conformational Selection; Structural Dynamics; Conformational Entropy; NMR relaxation

PKA-C: a dynamic signaling molecule

Protein kinases populate approximately 2% of the human genome, with 500 genes identified[1]. In eukaryotic cells, kinases mediate a myriad of cellular signaling events, and dysfunctions in these processes result in a variety of diseases and disorders[1]. Our group has focused on the structural and dynamic signaling of the cAMP-dependent protein kinase A (PKA, EC 2.7.11.11) in cardiac muscle. In the inactive state, this prototypical kinase assembles in a macromolecular complex (holoenzyme) formed by two regulatory subunits (R-subunits) and two catalytic subunits (PKA-C, Figure 1 *top*). As the cellular concentrations of cAMP are increased, active PKA-Cs are released [2]. NMR studies[3-7] carried out on the kinase's regulatory process along with the recent X-ray structure of the holoenzyme reveal the molecular details of the inhibited state, as well as the molecular determinants of its activation by cAMP[8]. Once unleashed from the holoenzyme, PKA-C is localized in the proximity of its substrates by ancillary proteins (A-kinase anchoring proteins) that provide spatiotemporal control on its enzymatic activity[9]. PKA-C, however, is only transiently activated and de-activated[10]. Endogenous inhibitors, co- and post-translational modifications (phosphorylation, myristoylation, and de-amidation), and other binding partners contribute to its regulation[11].

PKA-C was the first kinase to be crystallized, thereby unveiling the molecular organization of its catalytic core[12]. Several other structures of PKA-C trapped in well-defined states or

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in putative intermediate conformations have offered valuable insights as to how substrate phosphorylation may occur[2]. In spite of the plethora of functional and structural studies, however, there are several outstanding questions that remain unanswered: How is PKA-C activated by the nucleotide? How does PKA-C bind and recognize the different substrates? How is PKA-C inhibited? What is the role of post-translational modification in PKA-C activation and de-activation? Answering these questions will lead to a deeper understanding of the cellular signaling processes involving PKA-C.

In the laboratory, we use NMR spectroscopy as our main tool to correlate protein structure and conformational dynamics to function. Several recent studies have clearly demonstrated that the function of biological macromolecules can be understood only by correlating structural features to conformational dynamics (motions)[13-15]. In light of this new understanding, NMR is emerging as the method of choice to quantify motions in proteins at the atomic level[16, 17].

Protein structures fluctuate in conformational basins whose minima are very close to the X-ray structures. These fluctuations make up the conformational entropy, which contributes to the overall free energy of ligand binding [16]. While conformational entropy is dominated by the fast motions in the sub-ns time scale of the atomic groups (such as amides and methyl groups) in proteins[18], slower motions in the μs to ms time scale are correlated to large structural interconversions[17]. Both fast (ps to ns) and slow motions (μs to ms) are detectable by NMR relaxation experiments. The timescales of these motions can be decomposed and analyzed separately. In favorable cases, fast motions on the order of sub-nss can be converted into site-specific order parameters[19]. Because the order parameter defines the degree of order of a particular internuclear vector, it could, in principle, be linked to conformational entropy. However, correlating the motions to conformational entropy remains challenging and several approaches have been proposed. A model-dependent approach was first introduced by Akke *et al.* [20]. This work inspired both Li *et al.* [21] and Yang & Kay [22] who adopted model-dependent formalisms to quantify the contributions of bond vector fluctuations to conformational entropy. More recently, a model-independent approach has been proposed by Frederick *et al.* [23], who found that for calmodulin substrate binding the apparent changes in conformational entropy are linearly correlated with the overall binding entropy as measured by thermocalorimetry. The ‘entropy meter’ concept[24], which follows the latter observations, introduced a more quantitative interpretation of the conformational entropy without major assumptions on the nature of the internuclear vectors motions. The linear relationship between changes in the overall binding entropy and the conformational entropy measured was recently compounded by Tzeng & Kalodimos [25], who found a similar situation for the catabolite activator protein. Although with different slopes, both the calmodulin and catabolite activator protein data underscore the importance of conformational entropy in the regulation of protein function[26]. However, it should be noted that the decomposition of the fast and slow timescale motions for complex systems may be challenging[27]. Under these circumstances, model free analysis fails and the raw NMR relaxation data (R_1, R_2, and heteronuclear [^{1}H and ^{15}N]-NOE) are used to probe fast dynamics time scale[14]. Slow motions, on the other hand, are correlated with the existence of energetically excited conformational states often implicated in substrate recognition and product release[28, 29] and have been proven to be functionally critical and rate-limiting[30-32]. It should be noted that these slow motions, however, do not affect the chemical step of enzymatic catalysis[33], which often occurs in much faster time scales.

Using nuclear spin relaxation measurements, we monitored the motions of PKA-C in different states (apo, intermediate, and closed) and found that conformational entropy plays a critical role in nucleotide and substrate recognition[34]. We discovered that the nucleotide

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activates the internal fast conformational dynamics, which persist in the Michaelis complex. By contrast, inhibitor binding drastically reduces the internal conformational dynamics. Moreover, analysis of the slow structural fluctuations of PKA-C helped us to draw a qualitative free energy landscape for this important kinase, connecting thermodynamic and kinetic data to its structural fluctuations. This work provides a new framework for the design of new molecules that fine-tune the function of PKA-C.

Allosteric Cooperativity: nucleotide as an allosteric effector

Phosphoryl transfer between kinase and substrate is mediated by the nucleotide ATP, which binds the enzyme in a well-defined pocket[12]. Crystal structures revealed that the nucleotide binding pocket is rather complex and is organized in such a way that multiple tasks are accomplished. First, the nucleotide provides the phosphoryl group necessary for chemistry at the active site. Second, the nucleotide provides a structural support to connect both the small and large lobe via a hydrophobic array of residues (i.e., catalytic spine or C-spine, Figure 1 *bottom*). Third, it shifts the timescale of the atomic fluctuations of the kinase to a range synchronous with catalysis. ATP binds the kinase in a well-defined orientation, with the adenine ring sandwiched between V57 and L173 to complete the C-spine of the enzyme [35]. This motif crosses the enzyme and, with the regulatory spine (R-spine, Figure 1)[35], constitutes a well-conserved motif in catalytic cores. While the structural features of the nucleotide bound to the kinase were recapitulated by the crystal structures of the apo[36], binary, and ternary forms (PDB codes 1J3H, 1BKX, and 1ATP, respectively), its allosteric effector was revealed only by analyzing the chemical shift perturbation in the enzyme amide fingerprint. The NMR data revealed that the changes affecting the kinase are not limited to the active site and flanking elements such as the glycine rich loop and DFG loop; rather, they encompass peripheral and remote regions of the molecule that are not directly connected to the active site where phosphoryl transfer takes place[37]. In particular, residues involved in substrate binding (i.e., peptide positioning loop) show chemical shift perturbations indicating that nucleotide binding primes the binding pocket for substrate binding. NMR titrations also indicate that the *K-type cooperativity* identified between nucleotide and substrate is preserved under NMR conditions [37]. Binding of the substrate in the absence of nucleotide produces similar long-range chemical shift perturbations that involve the nucleotide binding pocket. This two-way correlation underlies our conclusion that long-range changes probed by chemical shift perturbations are responsible for the allosteric cooperativity of binding.

Conformational entropy, substrate recognition, and inhibition

Ligand binding often reduces the conformational dynamics of the receptor, as in a classical induced fit binding model[38]. The latter is consistent with an increase in intermolecular interactions that add a negative enthalpic contribution to the free energy of binding. There is growing evidence that ligand binding can increase the mobility of both backbone and side chain groups in regions peripheral to the binding site[39, 40], indicating that an entropic compensation takes place upon establishing new intermolecular interactions in the binding pocket. Alternatively, ligand binding to hydrophobic pockets can result in an increase in the conformational dynamics of the binding pocket as well as residues not directly involved in binding, suggesting that the binding is entropically-driven[18, 39, 41].

Using NMR nuclear relaxation experiments (R_1, R_2, and heteronuclear [^{1}H,^{15}N]-NOE)[14], we probed the fluctuations of the amide groups in the apo and nucleotide bound form (intermediate state) of the kinase. We found that upon nucleotide binding, the conformational dynamics of the kinase in the proximity of the binding site increase. In particular, the glycine rich loop and the DFG loop in the proximity of the C-helix increase

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their intrinsic dynamics[42]. Other regions in the binding pocket show a reduction of the conformational dynamics on the fast time scale. Concomitant with the rigidification of residues in the binding pocket, we observed an increase in conformational dynamics in regions far from the binding pocket, suggesting a compensatory increase of conformational entropy in response to a partial rigidification of the residues in the binding pocket. Overall, nucleotide binding increases the fast dynamics, which underscore its role as an allosteric effector, activating the motions in the kinase and priming it for the interactions with the substrate[42, 43].

X-ray structures of the complexes of PKA-C with the peptide inhibitor PKI (PDB code: 1ATP) and with inhibitory regions of RIα and RIβ (PDB codes: 2QCS and 4DIN, respectively) show that the recognition sequence binds within the active cleft in an extended configuration[10] (Figure 2). Interestingly, the two substrate (or inhibitor) arginine residues are arranged in a *trans* configuration, with the first arginine pointing toward the small lobe and forming a hydrogen bond with the backbone carbonyl group of Thr51 in the glycine-rich loop, while the second argi-nine points toward the large lobe and forms a salt bridge with Glu230 (Figure 2). Both arginines are crucial for positioning substrates and pseudo-substrate within the binding cleft by pinning down the glycine-rich loop and screening the nucleotide from the bulk water. Because the free inhibitor peptide is disordered in the free-state[44], the kinase may select and bind its extended configuration, locking it in the active site and maximizing intermolecular contacts. Isothermal titration calorimetry shows that the overall free energy of binding for PKI is enthalpically driven[43]. The latter is in qualitative agreement with the nuclear spin relaxation studies. In fact, the NMR relaxation data show that the conformational entropy is drastically reduced upon binding, demonstrated by an overall attenuation of conformational dynamics in the fast NMR time-scale both in the proximity of the ligands (nucleotide and pseudo-substrate) and at long-range [43].

As a substrate mimic, we chose the cytoplasmic domain of the membrane protein phospholamban (PLN). PLN is a single pass membrane protein that downregulates SERCA function, modulating Ca^{2+} reuptake in the sarco(endo)plasmic reticulum[45, 46]. Phosphorylation of PLN at Ser16 upregulates SERCA by increasing its apparent affinity for Ca^{2+}, thereby leading to an increase in Ca^{2+} flux from the cytoplasm to the lumen and a subsequent augmentation of cardiac diastole. The PLN cytoplasmic domain comprises a canonical recognition sequence for PKA-C ($R_{13}RAST_{17}$) located between domain Ia and a dynamic loop, preceding a short juxtamembrane region and the hydrophobic transmembrane domain domains Ib and II, respectively. The protein's recognition sequence adopts a helical conformation, which populates the ground state (membrane absorbed, T state)[46]. This ground state is in equilibrium with a small population in a membrane detached and unfolded conformation, representing the excited state (membrane desorbed, R state)[47]. The kinase selects and binds from the ensemble of conformations that constitutes the R state, or extended conformation of PLN[48]. This recognition process takes place via a conformational selection mechanism determined by steady state kinetic measurements, which indicate that phosphorylation efficiency of the R state is three times greater than the T state. Moreover, calorimetric titrations show that the binding of PLN to PKA-C is entropically-driven. Remarkably, nuclear spin relaxation data demonstrate that the residual conformational entropy of the Michaelis complex (PKA-C/AMP-PNP/PLN_{1-20}) is substantially higher than the corresponding fully inhibited complex (PKA-C/AMP-PNP/PKI)[42, 43]. Together, these results show that the residual entropy plays a central role in substrate recognition, while inhibition is achieved by quenching the internal conformational dynamics.

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Slow structural fluctuations link substrate recognition to catalysis

The challenges we have encountered in the model-free analysis of PKA-C relaxation data suggest that the enzyme undergoes complex motional modes. As a first approach, we utilized classical MD simulations, starting from the X-ray coordinate, to interpret nuclear spin relaxation data and correlate them to the physical motions of the enzyme. The MD trajectory thus obtained revealed that the apo enzyme undergoes local and long-range dynamic changes[43]. Upon binding the nucleotide (intermediate state or nucleotide bound form), the motions can be described as an asymmetric opening and closing of the active site, with a shearing motion between the two lobes. With these results, we proceeded by analyzing the slow conformational dynamics. Given the relatively large size of the kinase, we probed the slow conformational interconversions on the order of μs to ms time scales using an NMR relaxation method developed by Wang et al.[49] This experiment enables one to extract the rates of exchange in large perdeuterated proteins. We found that although dynamic, the apo form of the enzyme did not manifest conformational dynamics in the observation window for the TROSY Hahn-echo experiment[49]. By contrast, nucleotide binding activated the slow motions, the rates of which are synchronous with the catalytic turnover. These motions are persistent in the Mi-chaelis complex that PKA-C forms with PLN, present in all of the catalytic elements, and pervasive of the entire enzyme. These motions are also correlated with the opening and closing of the conserved glycine-rich loop and are synchronous with the slow step of catalysis as determined by kinetic measurements[42]. Unlike metabolic enzymes, which are optimized for turnover, PKA-C has evolved as a signaling enzyme with relatively slow kinetics. Whereas the chemical step is relatively fast (k_{chem} ~500 s^{-1}), the maximum turnover is slow (k_{cat} ~20 s^{-1})[50]. The latter has been attributed to the product release that represents the rate-limiting step of the catalytic process. The slow turnover suggests a direct involvement of the conformational transitions of catalytically important structural elements in the kinetics.

By contrast, binding the pseudo-substrate inhibitor (PKI) locks the enzyme in an inert state, where the conformational dynamics of the enzyme are quenched. Note that the enzyme fingerprint among the different forms of the kinase changes only minimally, indicating that on average there are not dramatic changes in the overall structure.

Energy landscape of the kinase: dynamically committed, uncommitted and quenched states

Taken with the thermocalorimetric and kinetic measurements, the NMR data make it possible to picture a qualitative free energy landscape (Figure 3)[43]. The apo state of PKA-C exists in an ensemble of conformational states that are in fast equilibrium. These states are populated according to the Boltzmann distribution and possess relatively low energy barriers. A small population of these states possesses high complementarity to the conformation of the nucleotide and binds the latter with high affinity, shifting the conformational equilibrium toward the bound form (intermediate state). This process involves small conformational changes and a significant contribution from small amplitude atomic fluctuations (conformational entropy). Nucleotide binding also activates the slow conformational dynamics. PKA-C changes from a *dynamically uncommitted* state (apo form) to *dynamically committed* state (intermediate state or nucleotide-bound form), where both structure and dynamics are committed to catalysis. The dynamically committed state is primed for substrate binding, which redistributes both fast and slow motions throughout the entire enzyme. Importantly, relaxation measurements carried out on the nucleotide and substrate bound form revealed the presence of motions in the ternary complex (i.e., Mi-chaelis complex), suggesting that these motions may drive product-release. Upon binding of the endogenous inhibitor of the kinase (PKI) we found a totally different scenario. NMR

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relaxation parameters show that PKI binding freezes the enzyme motions both in the ps to ns and the μs to ms timescales. We defined this state as the *dynamically quenched state*, where the residual conformational entropy and slow dynamics are not competent for catalysis[43].

Concluding Remarks

Our NMR studies aim to correlate thermodynamic and kinetic parameters with structure and conformational dynamics to understand PKA-C signaling in the cell. Our data fit with an ensemble view model of allostery, where ligands cause a conformational selection and population shift of the kinase upon binding[51, 52]. The latter is a common mechanism for several signal transduction processes[53]. The nucleotide acts as the allosteric effector, binding and shifting the pre-existing conformational equilibrium of the kinase toward the committed state. Small structural changes are also accompanied by conformational entropy changes that pervade the entire enzyme, priming it for substrate binding. This view of allostery has always been present in the literature, but binding studies carried out at high concentrations of ligands have biased the data interpretation in favor of the more popular induced fit mechanism. Kinetic measurements have clarified that these mechanisms are concurrent, though ligand concentration may make one more likely than the other. In the case of the kinase, where the ratio of enzyme to nucleotide in the cellular compartment is similar to those utilized in our research, conformational selection may be the most plausible mechanism. PKA-C offers a unique opportunity to understand allosteric phenomena and to trace signaling pathways at the atomic level. These NMR results are highly complementary to the X-ray studies and promise to have significant implications for drug design targeting kinases, especially considering that competitive inhibitors of the kinase targeting the ATP binding site often lead to undesirable side effects, since the nucleotide active-site is conserved in the family. By contrast, the identification of the allosteric pathways provides the opportunity to act on less conserved sites to modulate kinase function. As we continue our investigation, we plan to follow-up with the analysis of the allosteric communication via side chains and extend the analysis of the conformational entropy to the binding partners.

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ABBREVIATIONS

PKA	protein kinase A
PKA-C	C-subunit of PKA
PKI	protein kinase inhibitor
ATP	adeno-sine triphosphate
AMP-PNP	Adenylyl-imidodiphosphate

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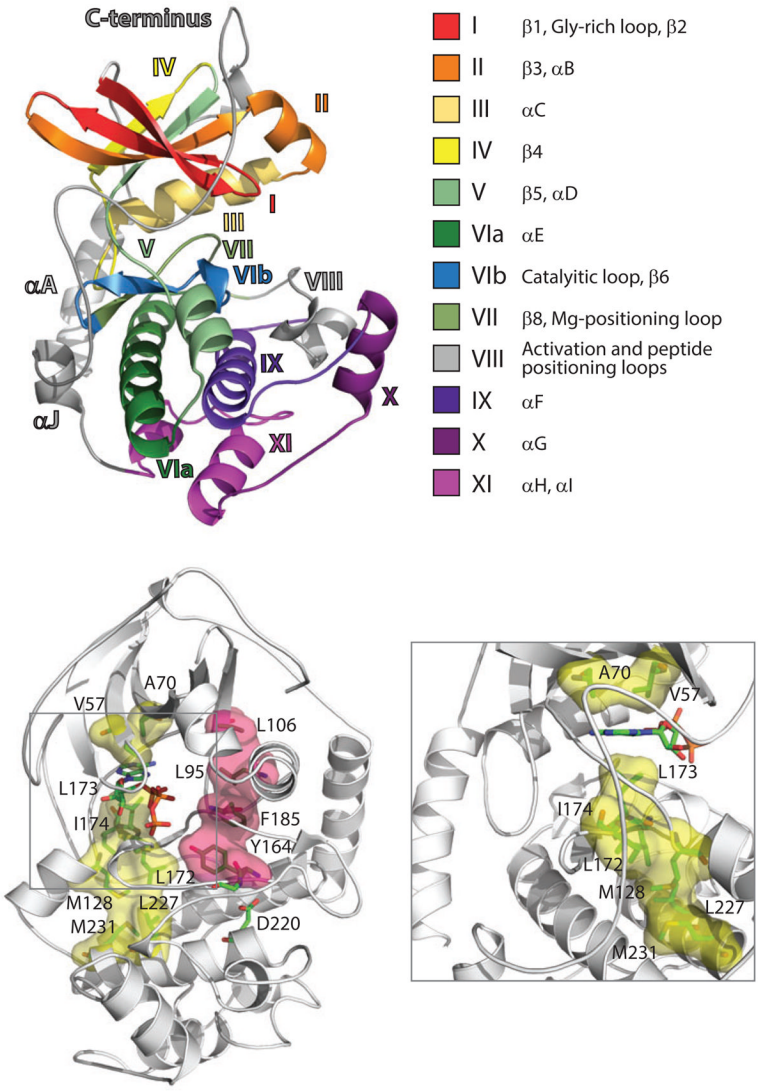

FIGURE 1.

Top: Architecture of PKA-C. The backbone cartoon is colored according to Johnson *et al.* [2]. Bottom: catalytic and regulatory spines on the kinase.

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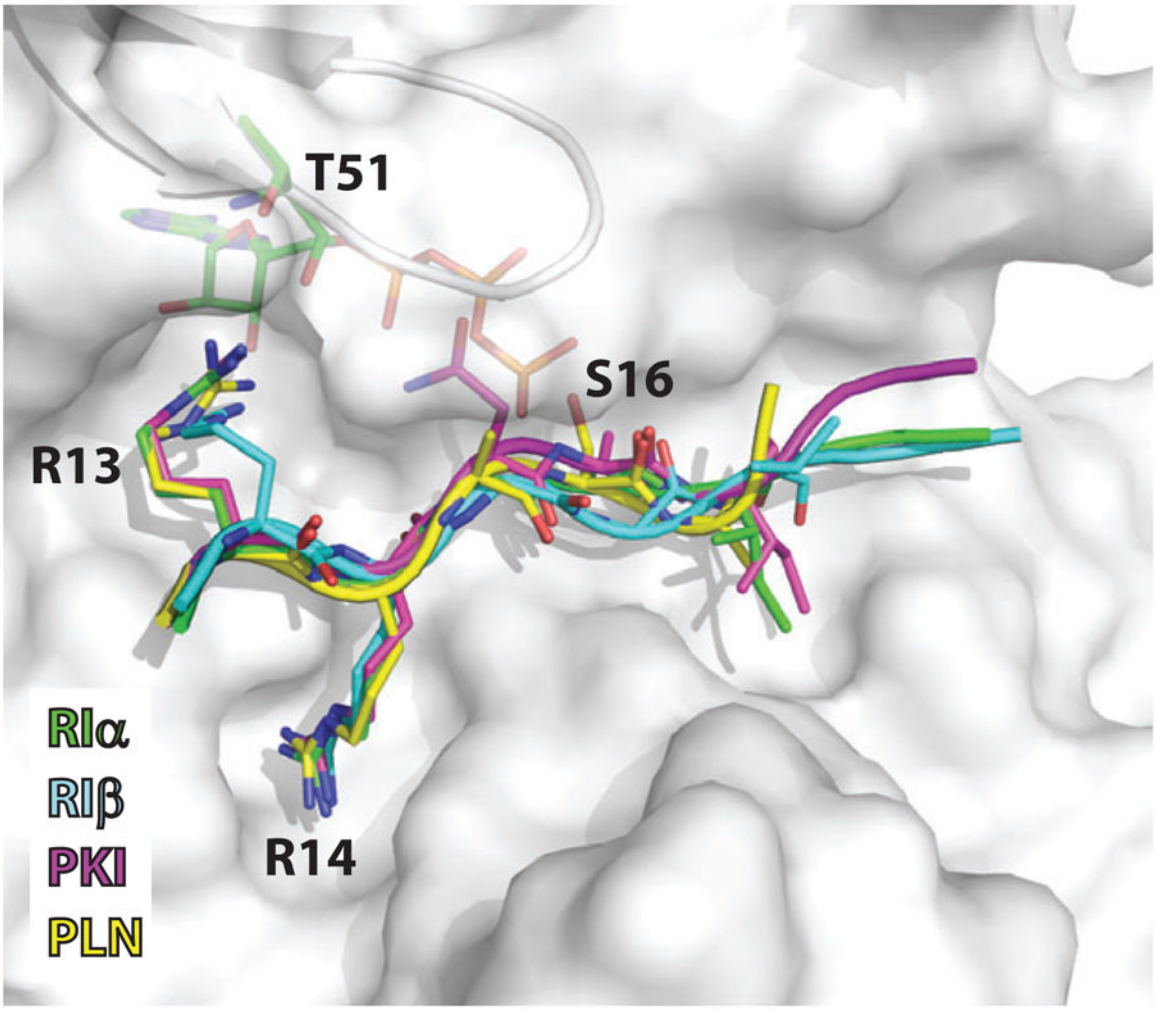

FIGURE 2.

A. Binding of substrate (PLN), pseudo-substrate (PKI), and inhibitory linkers from R-subunits to the binding pocket.

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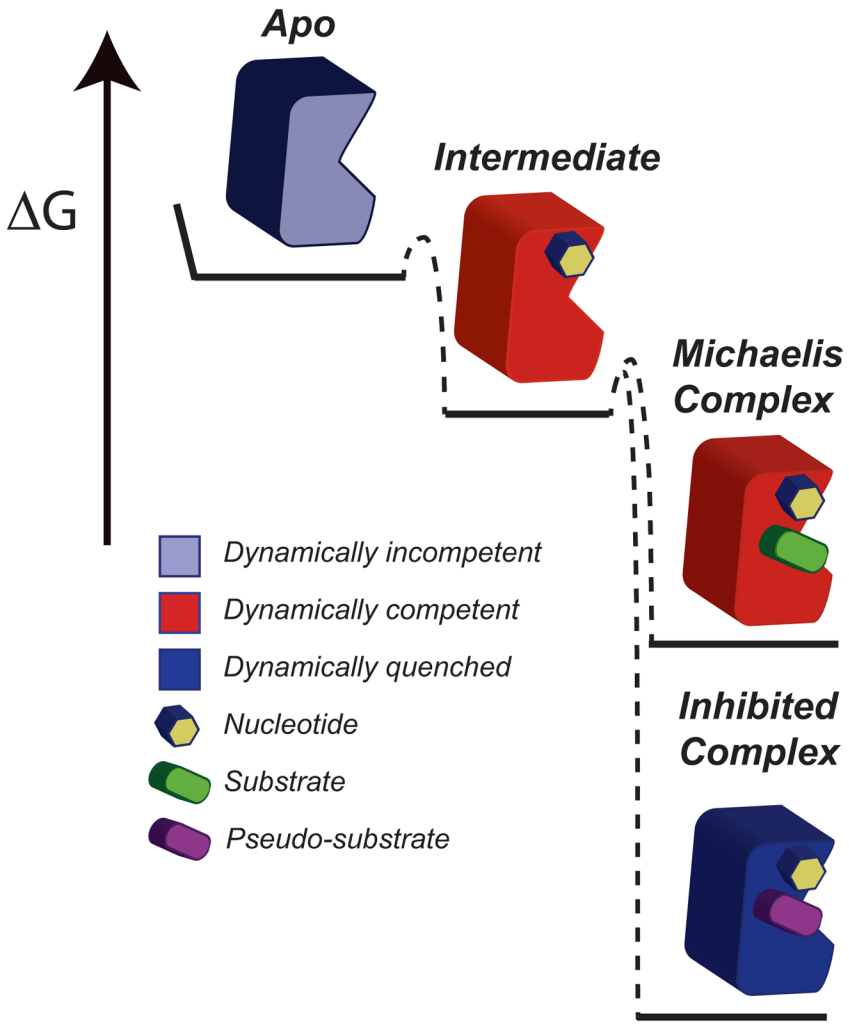

FIGURE 3.
Schematic of PKA-C free energy in the apo (*uncommitted dynamics*), intermediate (*committed dynamics*), Michaelis complex (*committed dynamics*), and inhibited complex (*quenched dynamics*). The structural dynamics depicted in this diagram are in the order of ps to ms timescale, as monitored by NMR relaxation parameters.