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cAMP-DEPENDENT PROTEIN KINASE A SELECTS THE EXCITED STATE OF THE MEMBRANE SUBSTRATE PHOSPHOLAMBAN

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Abstract

Phosphorylation of membrane proteins is a central regulatory and signaling mechanism across cell compartments. However, the recognition process and phosphorylation mechanism of membrane-bound substrates by kinases are virtually unknown. cAMP-dependent protein kinase A (PKA) is a ubiquitous enzyme that phosphorylates several soluble and membrane-bound substrates. In cardiomyocytes, PKA targets phospholamban (PLN), a membrane protein that inhibits the sarcoplasmic reticulum Ca^{2+} -ATPase (SERCA). In the unphosphorylated state, PLN binds SERCA, reducing the calcium uptake and generating muscle contraction. PKA phosphorylation of PLN at S16 in the cytoplasmic helix relieves SERCA inhibition, initiating muscle relaxation. Using steady state kinetic assays, NMR spectroscopy, and molecular modeling we show that PKA recognizes and phosphorylates the excited, membrane detached R state of PLN. By promoting PLN from a ground state to excited state, we obtained a linear relationship between rate of phosphorylation and population of the excited state of PLN. The conformational equilibrium of PLN is crucial to regulate the extent of PLN phosphorylation as well as SERCA inhibition.

INTRODUCTION

Protein phosphorylation is a central mechanism in cell signaling and regulation. Inhibitory proteins are reversibly phosphorylated to cycle between active and inactive states¹. These different states are characterized by conformational and dynamic transitions that are essential for stability and function^{2, 3}. Muscle proteins are salient examples of these structure-dynamics-function correlations. The rhythmic contraction and relaxation of the heart muscle, for instance, are orchestrated by protein complexes that regulate calcium homeostasis, and phosphorylation is the driving force to control these cyclic events⁴.

In muscle cell membranes, protein kinases target peripheral membrane proteins or small membrane proteins involved in regulatory complexes, initiating signaling events that cross the membrane bilayer⁴. This is the case of phospholamban (PLN), a reversible inhibitor of the sarcoplasmic reticulum calcium ATPase (SERCA)^{5, 6}. SERCA is a 110 kDa membrane-

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embedded ATP-driven calcium pump and is regulated by intramembrane interaction with PLN. In its storage form, PLN exists in a pinwheel pentameric assembly that de-oligomerizes upon interaction with SERCA^{5, 7}. PLN comprises multiple dynamic domains: the inhibitory domains include the transmembrane segment (domain II) and the juxtamembrane domain Ib^{8–12}, while the regulatory elements include a short dynamic loop and an amphipathic helix (domain Ia) that interacts reversibly with the membrane and contains the PKA recognition sequence^{13, 14}. Domain Ia of PLN exists in at least four conformational states: T-state (membrane attached and helical), T'-state (membrane attached, partially helical), R'-state (membrane attached, unfolded) and R-state (membrane detached, unfolded)^{6, 11, 15}. PLN phosphorylation at Ser 16 by PKA in domain Ia is sufficient to reverse SERCA inhibition and increase the enzyme affinity for calcium that is pumped into the sarcoplasmic reticulum, initiating muscle relaxation¹⁶. Disruptions in this phosphorylation/dephosphorylation cycle lead to pathologies such as dilated cardiomyopathy (DCM)¹⁷.

Once unleashed from its tetrameric assembly by cyclic-adenosine monophosphate¹⁸, the monomeric catalytic subunit of PKA (PKA-C) recognizes, binds, and phosphorylates PLN. The molecular mechanisms of PLN recognition by PKA are still elusive. Recent NMR and X-ray data on PKA-C in a ternary complex with nucleotide and the cytoplasmic domain of PLN suggest that binding by PKA-C may involve an unfolding of PLN domain Ia (Figure 1A)². This result is striking given that in several membrane mimics the most populated state for the full-length PLN is folded, with the majority of the recognition sequence in close proximity with the lipid membrane^{7–9, 12, 19, 20}.

To gain insights into the recognition mechanism of PKA-C for PLN, we used a combination of steady state kinetic assays, NMR, and molecular modeling. We show that PKA-C preferentially binds and phosphorylates a membrane detached and unfolded conformation of PLN, corresponding to the excited R state of this protein¹⁵. Using a series of PLN variants in different lipid environments, we tuned the extent that domain Ia of PLN interacts with the membrane. We found a linear correlation between the catalytic efficiency of phosphorylation and the population of membrane detached and unfolded domain Ia of PLN. Molecular modeling from NMR data shows that the kinase binds an extended conformation of PLN in solution. Taken together, these data demonstrate that the conformational equilibrium of PLN is an important requirement for efficient phosphorylation by PKA and activation of SERCA.

RESULTS

PKA-C selects an extended conformation of PLN

To understand if PKA-C has a preference for the different conformational states of PLN, we prepared several PLN analogs which mimicked different populations of the ground and excited states of the cytoplasmic domain Ia (Figure 1B,C). The first PLN analog was obtained by synthetic incorporation of dialkyl 1', 3'-dioctadecyl-N-succinyl-L-glutamate (Lip) to the N-terminus of full-length PLN. This analog has been shown to skew the equilibrium toward the well-folded, membrane associated T-state, anchoring domain Ia to the lipid membrane²¹. On the other hand, to mimic the membrane detached, unfolded R-state, we synthesized a peptide corresponding to the cytoplasmic domain Ia (PLN_{1–20}) and studied it in the absence of any membrane mimicking environment. Under these conditions, PLN_{1–20} is an intrinsically disordered polypeptide as probed by circular dichroism, NMR chemical shifts, and nuclear spin relaxation measurements² (Figure S1). To mimic the gradual progression from a full T-state to the R-state, synthetic and recombinant versions of full-length PLN with no lipidation, as well as a lipidated peptide corresponding to domain Ia of PLN (Lip-PLN_{1–20}) were used. Finally, since full-length PLN reconstituted in

dodecylphosphocholine micelles has been well characterized by our laboratory, we also included this form in our studies.

For each of the PLN analogs, PKA-C catalyzed steady-state phosphorylation assays were performed and the Michaelis-Menten constants were measured. Although all reactions eventually reached completion and displayed similar values for k_{cat} (Table S1), the K_{m} values for the reactions differed significantly. Specifically, PLN₁₋₂₀ in the absence of membranes was phosphorylated with a high catalytic efficiency ($k_{\text{cat}}/K_{\text{m}} \sim 0.6 \mu\text{M}^{-1}\text{s}^{-1}$), while full-length PLN in DPC micelles was phosphorylated with a low catalytic efficiency ($k_{\text{cat}}/K_{\text{m}} \sim 0.06 \mu\text{M}^{-1}\text{s}^{-1}$). A variety of intermediate catalytic efficiencies were measured for the phosphorylation of PLN analogs in different conditions as reported in Figure 2. The trend in catalytic efficiencies agrees qualitatively to an increasing population of R-state in PLN.

To provide a more quantitative analysis for the T-state population of the PLN analogs, the degree of folding for each was characterized using NMR. As reported previously², the PLN peptide constitutes a good mimic of the cytoplasmic domain of full-length PLN as verified by chemical shift indices (CSI) and secondary structure propensity (SSP) values. Our laboratory has shown that the cytoplasmic domain of PLN is mainly α -helical ($\sim 4\%$ R-state)²² in DPC micelles¹⁵. CSI and secondary structure propensity scores for PLN₁₋₂₀ in the presence of DPC micelles agreed well with those findings, indicating that the peptide forms a stable α -helix (Figure S1). In fact, only small differences were detected between the cytoplasmic regions of both constructs. The latter demonstrates that PLN₁₋₂₀ closely mimics the cytoplasmic region of the full-length protein.

Our laboratory has shown that the chemical shifts of methyl-bearing sidechains of the PLN cytoplasmic domain provide excellent probes for the T- and R-states of the protein¹⁵. Using these probes, we measured the chemical shift positions of the methyl groups for each of the PLN analogs under similar conditions as the steady-state assays. The methyl groups showed a linear trend between the PLN₁₋₂₀ free in solution (100% R-state¹⁵) and PLN in DPC micelles ($\sim 4\%$ R-state²², (Figure 3A). The other analogs represented different intermediates between these two extremes, illustrating that these species gradually shift the population of the R-state toward the T-state (Figure 3A). Validating this trend in folding, CD spectroscopy measurements on PLN₁₋₂₀ based analogs revealed a gradual increase in α -helical content in the presence of bicelles and, in particular, when lipidated. We therefore used the position of the methyl resonances as reporters for the extent of unfolding to quantify the R-state for these analogs (Figure 3B). As expected, the comparison of PLN and Lip-PLN in isotropic bicelles showed a decrease in the R-state for Lip-PLN, consistent with the trends observed by CD spectroscopy.

A quantitative relationship between the efficiency of phosphorylation and the population of the membrane detached state (full R-state) of PLN was identified when we plotted $k_{\text{cat}}/K_{\text{M}}$ against the population of R-state (Figure 3B). Notably, the extent of unfolded R-state is linearly correlated with increased phosphorylation efficiency. These results demonstrate that PKA-C preferentially binds and phosphorylates the membrane detached conformation of PLN.

Molecular modeling of PLN₁₋₂₀ bound to PKA-C

To investigate how PKA-C recognizes and binds PLN and to characterize the protein-protein interface, we used a combination of NMR, mutagenesis, and sequence homology data to model the complex between enzyme and substrate. Chemical shift perturbation profiles in both the substrate and enzyme were analyzed to provide harmonic distance restraints for the molecular model of the complex. Perturbations in PLN₁₋₂₀ resonances

were almost exclusively localized to the recognition region for PKA-C upon titration with the enzyme. These perturbations were used as restraints for modeling the molecular complex. As for our previous titrations with Kemptide²³, residues perturbed in PKA-C upon titration with PLN_{1–20} are located in the immediate vicinity of the substrate binding groove as well as residues in remote regions of the enzyme. Substantial chemical shift perturbations are observed in the glycine-rich (residues 50–55), DFG (residues 185–187), activation (residues 190–197), and P + 1 (residues 198–205) loops. Distal sites are also affected: residues 30, 31, and 36 which lie toward the N-terminus, and residues 145, 146, 148, and 156 of the E Helix. Perturbations are also apparent for residues 163 and 164 of β 6, 271 in the loop connecting H and I helices as well as residue 315 near the C-terminus.

In the membrane-free conformation, the PLN_{1–20} peptide is mostly unstructured, with chemical shifts displaying characteristics of an intrinsically disordered polypeptide^{24–26}. The negative [¹H, ¹⁵N]-heteronuclear nuclear Overhauser effect (H-X NOE) values and the uniformity of the R_2/R_1 ratios indicate that in solution PLN_{1–20} is flexible, monomeric, and reorients relatively fast²⁷. When bound to PKA-C, the substrate does not show a defined secondary structure (α -helical or β -sheet conformations), as demonstrated by the relatively small changes in the chemical shift perturbation plot as well as the chemical shift indices for the ¹³C $^\alpha$ /¹³C $^\beta$ resonances (Fig. S2), supporting the previously published crystallographic structure².

In addition to the chemical shift perturbation-derived restraints, we used the coordinates from crystal structure of PKA-C in the closed conformation to model the complex. Since PLN_{1–20} has sequence similarity with protein kinase inhibitor (PKI), the endogenous PKA-C inhibitor, we aligned the PLN recognition sequence in the PKA-C binding groove as defined by the crystal structure (1ATP) bound to PKI_{5–24} and ATP²⁸. We oriented the side chain of the phosphorylation site (S16) of PLN_{1–20} toward the γ -phosphate of the ATP molecule. Chemical shift perturbations for PKA-C indicated that many areas distal to the binding site were perturbed by allosteric effects. Therefore, the crystal structure of PKA-C bound to PKI_{5–24} was used to filter out these long range effects, considering only those residues in PKA-C with large chemical shifts (greater than the median value) and within 10 Å radius in the binding pocket defined by PKI_{5–24} (Figure 4B). The PKA-C crystal structure depleted of PKI_{5–24}²⁹ was used as the initial structure and held fixed during simulated annealing calculations. The NMR data were implemented as harmonic distance restraints³⁰. PLN_{1–20} was modeled starting from a helical conformation^{8, 12} and was allowed to be fully flexible during the docking protocol. The final model of the complex is reported in Fig. 4C. The ensemble of the PLN_{1–20} conformers is in an extended conformation within the enzyme binding pocket. Taken with the kinetics experiments, this model supports the hypothesis that the enzyme selects an extended conformation from the ensemble of states sampled by PLN. This extended conformation has its recognition sequence fully accessible and detached from the membrane surface.

DISCUSSION

Based on phosphorylation kinetic assays, NMR data, and molecular modeling, we propose a recognition model for PLN as reported in Figure 5. Since both PLN and PKA-C undergo conformational equilibria between ground and excited states^{2, 3, 11, 15, 31}, the recognition model exemplifies the recently theorized *extended conformational selection model*, where the two binding partners affect each other, with a mutual adjustment process and changing both energy landscapes³². In fact, PLN exists in equilibrium between four major conformational states, driven by the reversible binding of the cytoplasmic domain Ia to the membrane. The T-state (L-shaped configuration), with domain Ia adsorbed on the membrane surface is the most populated and represents the ground state of PLN, while the remaining

three states (T', partially unfolded in domain Ib, R' partially unfolded at the N-terminus and in the C-terminal part of domain Ia, and an R-state, which has domain Ia and Ib unfolded) are less populated and represent the excited states. In the ground state, PLN is less accessible to the protein kinase and is poorly phosphorylated, whereas the excited R-state comprises an ensemble of conformations from which PKA-C selects the extended conformation that molds into the substrate binding site. On the other hand, PLN binding drives PKA-C conformational equilibrium toward the closed state of the enzyme as probed by both X-ray and NMR spectroscopy^{2, 3}.

The folding/unfolding transitions of PLN are relatively fast¹¹ and the phosphorylation efficiency is proportional to the population of the most excited state (R), in which the helix of domain Ia is melted. In contrast, for the most helical construct studied, (PLN in DPC micelles), the conformational equilibrium is skewed toward the full T-state and the phosphorylation efficiency is drastically lower.

The overall phosphorylation efficiency trends for both the peptide and full-length protein mirror one another, demonstrating that the cytoplasmic portion of the protein is a good model for the full protein. This latter result supports the selection of the peptide to model the molecular complex of PKA-C/ATP/PLN₁₋₂₀. Using chemical shift changes which occurred upon formation of the Michaelis complex for both PKA-C and PLN₁₋₂₀, sequence homology with the high affinity inhibitor peptide of PKA-C, and results from mutations, we modeled the PKA-C/PLN₁₋₂₀ complex. This complex indicates that PKA-C binds an unfolded conformation of PLN. Initial molecular dynamics simulations (data not shown) indicate that this complex is stable, with the recognition sequence of PLN₁₋₂₀ confined to the active site of the enzyme and regions outside of this remaining relatively mobile. This matches well with our previous nuclear spin relaxation measurements of the bound peptide, which indicated high mobility across the substrate backbone, with the exception of the recognition sequence which was more ordered².

The conformational dynamics of the cytoplasmic domain of PLN is reminiscent of the binding-and-folding model proposed for amphipathic helices of membrane proteins^{33, 34}. According to this model, interactions with the membrane bilayer surface (likely driven by electrostatic residues) causes the amphipathic peptide to insert and fold onto the membrane with the hydrophobic face pointing toward the hydrocarbon region of the membrane bilayer and the hydrophilic face toward the lipid headgroup/water interface. In addition to regulating the extent of PLN interaction with the membrane bilayer, a similar binding-and-folding mechanism is probably present between PLN and SERCA in the inhibitory complex⁸. As suggested by NMR and electron paramagnetic resonance studies, phosphorylation probably shifts the conformational equilibrium toward a non-inhibitory state of PLN, affecting the stability of domains Ia and Ib. We are currently investigating this hypothesis.

The reversible folding of domain Ia is likely part of a more general class of regulation mechanisms that are mediated by amphipathic domains^{35, 36}. For instance, a parallel can be made with the autoinhibitory process that takes place in Vav, a multidomain protein that is negatively regulated by its DH domain³⁷. In Vav, autoinhibition is reversibly modulated by a folding/unfolding equilibrium of the amphipathic DH-helix, which fluctuates between a protein free and bound conformation. This equilibrium is shifted toward the unfolded state upon phosphorylation at Tyr174. Phosphorylation efficiency was correlated with unfolding of this amphipathic domain. Another example is LOV-2^{38, 39}. As for Vav, LOV also contains a region which can switch between an amphipathic α -helix and an unfolded conformational state. The conformational equilibrium of this region dictates the inhibitory strength of the protein. These results mirror the current study with PLN, where the catalytic

efficiency of the kinase is linearly correlated with the population of the unfolded excited state, which exposes the phosphorylation site to the protein kinase.

This current study demonstrates that the modulation of the cytoplasmic domain to different states affects the ability of PLN to adapt to its binding partner, PKA-C. The ability to interact with other proteins through a population shift highlights the scaffolding capability PLN has for a number of known binding partners: PKA-C, protein phosphatase-1, Ca^{2+} /calmodulin-dependent kinase II, SERCA, HS1-associated protein X-1, and A-kinase anchoring protein 188. The coupling of conformational plasticity of the PLN cytoplasmic domain and its interactions with cytosolic proteins (for example, PKA-C) also demonstrates the importance of dynamics for signaling processes to occur efficiently and transiently to facilitate multiple functional interactions^{40–44}. This appears to be particularly important for the case studied here, where a protein links signaling between the cytosolic and membranous environments of the cell.

Finally, this work underscores the role of the complex conformational fluctuations that PLN undergoes to cater to the phosphorylation process by PKA-C and ultimately SERCA regulation. Future studies on the localization of PKA (i.e., role of N-terminal myristoylation and AKAP binding) will elucidate how regulatory domains such as domain Ia of PLN are integrated into macromolecular complexes to achieve signal specificity.

MATERIAL AND METHODS

Materials

Lactic dehydrogenase (LDH) from bovine heart and pyruvate kinase (PK) from rabbit muscle were purchased from Sigma (Solon, OH, USA). Adenosine 5'-triphosphate (ATP), phosphoenolpyruvate (PEP), magnesium chloride, and reduced nicotinamide adenine dinucleotide (NADH), were purchased from Sigma Aldrich (St. Louis, MO, USA).

Expression and purification of PLN and PLN_{1–20}

PLN_{1–20} was expressed in *E. coli* cells as a fusion system with maltose binding protein (MBP) and a 6-histidine tag to facilitate purification^{45, 46}. The cells were grown overnight at 30 °C in LB (Luria Broth) rich media. At OD₆₀₀ ~ 1.0, cells were transferred into M9 minimum media, containing ¹⁵N-labeled NH₄Cl or ¹³C-labeled glucose for uniformly ¹⁵N or ¹³C labeled NMR samples. IPTG (Isopropyl-β-D-thiogalactopyranoside) (0.5 mM) was added to induce fusion protein expression at 37 °C for 5 hours. The pelleted cells were lysed in phosphate buffer (pH = 8.0) in the presence of 6 M guanidine. The cell lysate was separated by centrifugation (18,000 rpm at 4 °C for 40 minutes). The purification of the fusion protein was carried out using Ni²⁺-NTA resin. The eluted fusion protein was then dialyzed and cleaved with Tobacco Etch Virus (TEV) protease, which separated the PLN_{1–20} from MBP and the 6-his tag. The final purification was achieved by reversed-phase HPLC using a C-18 column (Vydac). After HPLC purification, approximately 2 mg of PLN_{1–20} were obtained from 150 mg of fusion protein. The mass of the purified PLN_{1–20} (2472.8 Da) was confirmed by electrospray ionization time-of-flight (ESI-TOF) mass spectroscopy. The full length PLN was expressed and purified as reported previously⁴⁶.

Peptide synthesis

The peptide synthesis was performed using standard Fmoc chemistry on an Applied Biosystems Pioneer or CEM Liberty/Discover microwave synthesizer, starting with Fmoc-Glu(O^{*t*}Bu)-PEG-PS resin (0.4 g, 0.5 mmol/g). Side chain protecting groups were 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for arginine, N^ω-triphenylmethyl (Trt) for asparagine and glutamine, *tert*-butyl ester (O^{*t*}Bu) for glutamic acid, and *tert*-butyl ethers (tBu) for

serine, threonine, and tyrosine. Addition of the lipid moiety was performed using standard coupling reagents and dialkyl 1', 3'-dioctadecyl-N-succinyl-L-glutamate to the unprotected N-terminal methionine residue⁴⁷. Cleavage of the resin-bound peptide was done using Reagent K (82.5% trifluoroacetic acid, 5% phenol, 5% thioanisole, 2.5% 1,2-ethandiol, and 5% water) for 3 hours at 298 K. The resin mixture was washed three times (2 ml each) using the same cocktail and filtrate was collected. The peptide was precipitated overnight at 273 K in 80 ml of diethyl ether, then collected by centrifugation and washing the pellet 3 times with 30 ml of diethyl ether. The crude peptide was then purified by preparative HPLC using a Waters C18 reversed-phase cartridge (2.5 × 10 cm, 15 μm 300 Å) with 0.1% TFA and CH₃CN as eluents and detection at 220 nm. A linear gradient of 100:0 to 70:30 over 30 minutes at 5 ml/min was used. The purity of pooled fractions was >97% as determined by analytical HPLC using a Vydac C18 column (0.46 × 25 cm) and confirmed by ESI-TOF; for the analog PLN₁₋₂₀, calculated 2252.65 *m/z*, found 2252.73 *m/z*. Stock solutions were prepared in water/acetonitrile and lyophilized. The concentrations of these solutions were verified by amino acid analysis.

Kinetic Assays

The steady-state enzymatic rates of phosphorylation for PLN analogs were measured spectrophotometrically at 299 K following the procedure by Cook et al⁴⁸. Briefly, the oxidation of NADH by pyruvate kinase and lactate dehydrogenase is coupled to the production of ADP, which occurs during the PKA-C catalyzed phosphoryl transfer. The reaction rates were monitored at 340 nm using a Molecular Devices SpectraMax Plus³⁸⁴ equipped with a 96-well microplate reader. The reaction solutions contained 50 mM MOPS (pH 7.0), 20 mM DTT, 10 mM MgCl₂, 5.0 mM ATP, 1.0 mM PEP, 0.2 mM NADH, 12 units LDH, and 4 units PK. For the kinetic assays with lipid bicelles, PEP, NADH, LDH and PK were added after bicelle formation. Stock solutions of 2 mM PLN₁₋₂₀ were prepared fresh and used immediately. To determine the linear region of PKA-C concentration, PLN₁₋₂₀ was kept at 300 μM, while PKA-C was varied between 2–728 nM. The reactions were initiated by the addition of PKA-C, which was equilibrated in a solution containing 50 mM MOPS (pH 7.0), 20 mM DTT, and 10 mM MgCl₂. No differences in the reaction rates were observed when the substrate was used to initiate these reactions. The measurements were performed in triplicate for each peptide concentration. Rates reflecting the small decrease in absorbance due to intrinsic NADH oxidation were measured and did not exceed ~2% of any reaction studied.

The values of k_{cat} and K_M were determined using nonlinear least-squares fitting of the initial velocities versus substrate concentration according to:

$$v = \frac{V_{max} [S]}{K_M + [S]} \quad (1)$$

Where v is the initial velocity, $[S]$ is the concentration of substrate, V_{max} is the maximum velocity for the reaction, and K_M is the Michaelis-Menten constant. The value of k_{cat} was taken as V_{max} divided by the total PKA-C concentration. Fitting of equation 1 to the kinetics data was performed using the software, Origin 7.

NMR spectroscopy

NMR experiments were carried out on a Varian Inova operating at 800 MHz equipped with an HCN probe. The temperature of the experiments was held constant at 27 °C. Samples were prepared in 20 mM KH₂PO₄, 180 mM KCl, 1 mM NaN₃, 10 mM DTT, and 5% v/v D₂O (pH 6.5) at concentrations of ~0.3–0.8 mM. Data were processed with the software

NMRPIPE⁴⁹ and visualized and analyzed using SPARKY⁵⁰. Populations of the R-state ($p_{R-state}$) were quantified using:

$$p_{R-state} = \frac{\Omega_X - \Omega_{T-state}}{\Omega_{R-state} - \Omega_{T-state}} \quad (2)$$

where Ω_X , $\Omega_{T-state}$ and $\Omega_{R-state}$ are the chemical shift of sample X, PLN in DPC, and PLN₁₋₂₀, in aqueous buffer, respectively. The mean of $p_{R-state}$ was determined from six methyl groups in the protein (originating from V4, L7, and I12) which were observed in [¹H/¹³C]-HMQC spectra. Due to poor sensitivity for the bicelle bound states, amide spectra could not be used to estimate the population.

Molecular Modeling

The three dimensional model of the PKA-C/ATP/PLN₁₋₂₀ complex was generated using HADDOCK 2.0³⁰. As an initial configuration, the coordinates from the crystallographic ternary complex of PKA-C/ATP/PKI₅₋₂₄ (1ATP) was used and depleted of PKI₅₋₂₄²⁸. The coordinates of the enzyme were kept fixed during the docking of PLN₁₋₂₀ using simulated annealing. Restraints based on NMR chemical shift were implemented as ambiguous interaction restraints (AIR). Specifically, *active residues* were categorized as those which had solvent accessibility >50%, chemical shifts perturbations were greater than the median, and within 10 Å from the binding pocket of the enzyme. Active residues identified were S53, K78, L82, K83, S130, L198, G200, Q245, D328, D329, and Y330. Solvent accessible residues within 3 Å of the active residues were categorized as *passive*. Active and passive residues were defined for the PLN₁₋₂₀ using the same criteria. An AIR was satisfied when any atoms of active residues in one molecule were within 4 Å of an active or passive residue of the docking partner. Also, the canonical recognition sequence of PLN₁₋₂₀ (P-3 to P+1 site) was aligned to match the corresponding positions of PKI₅₋₂₄ in the binding groove of the 1ATP structure²⁸. Based on this alignment, five additional distance restraints were used: three hydrogen bonds from the R14 guanidinium group of PLN₁₋₂₀ to E170, Y204, and E230 of PKA-C, one hydrogen bond from S16 of PLN₁₋₂₀ to S53 of PKA-C, and one hydrogen bond involving the carboxyl group of T17 and the backbone amide of G200 in PKA-C. The hydrogen bond distances (from N or O heteroatoms to hydrogen atoms) were incorporated as NOE distance restraints using a flat well potential with 1.5 (4 Å) as lower (upper) bound distances. PLN₁₋₂₀ was set to be flexible, with the exception of the residues occupying P-3 to P+1 sites of PKA-C, consistent with results based on NMR dynamics measurements². The backbone conformation of PKA-C was kept fixed, while full flexibility was allowed for sidechains. The initial structures of PKA-C and peptide were subjected to rigid-body docking and subsequent structure refinement using the docking protocol from HADDOCK with adjusted weighting factors³⁰. A total of 2000 initial structures of the complex were generated after rigid body docking, and the 200 lowest intermolecular energy structures were optimized by simulated annealing, followed by refinement in explicit water using the Tip3 water model³⁰. These structures were then clustered according to their pairwise root-mean square deviations (*RMSD*) with respect to the conformation of PKI in 1ATP crystal structure. The 20 lowest energy structures were selected for the final analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

A) Crystal structure of PKA-C bound to PLN₁₋₂₀ showing the key contacts between substrate (green) and enzyme (grey) within the binding groove. **B)** Primary structures and **C)** cartoon representations of the different analogs used to mimic the T and R states of PLN.

Fig. 2.

Structure-function correlations for PLN analogs. The catalytic efficiency of the kinase increases with the increase of the R-state population. The errors were propagated from least-squares fitting analysis. Each data point in the Michaelis-Menten plot represented an average of triplicate measurements.

Fig. 3. Quantitative relationship between the extent of R-state and phosphorylation efficiency. **A)** Selected regions of the methyl-TROSY NMR spectra for the different PLN analogs display a linear trend in the chemical shifts from the T to R state. **B)** Linear relationship between the population of Rstate of the PLN analogs and their respective phosphorylation kinetics. Errors in % R-state reflect the spread of populations estimated from the position of 12 chemical shifts. ^{13}C and ^1H chemical shifts from residues V4, L7, and I12 were used as probes of the R-state population.

Fig. 4. Hybrid X-ray and NMR-based molecular model of the complex between PLN₁₋₂₀ and PKA-C. Active (red) and passive (yellow) residues are indicated for **A)** PLN₁₋₂₀ and **B)** PKA-C. **C)** Twenty lowest conformers generated with HADDOCK³⁰. **D)** Atomic details of the interactions observed between PKA-C and PLN₁₋₂₀ based on the molecular model

Fig. 5. Extended conformational selection model for PLN recognition by PKA-C. Both PKA-C and PLN have been shown to undergo conformational exchanges. PKA-C undergoes a global change which opens and closes its active site upon interaction with the nucleotide. This likely facilitates efficient binding of PLN, shown previously². PLN also undergoes conformational fluctuations^{11, 15}. Unfolding of the cytoplasmic portion of PLN and detachment from the membrane facilitates the recognition and subsequent phosphorylation by PKA-C