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Expression and purification of isotopically labeled peptide inhibitors and substrates of cAMP-dependant protein kinase A for NMR analysis

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ABSTRACT

Extensive X-ray crystallographic studies carried out on the catalytic-subunit of protein kinase A (PKA-C) enabled the atomic characterization of inhibitor and/or substrate peptide analogues trapped at its active site. Yet, the structural and dynamic transitions of these peptides from the free to the bound state are missing. These conformational transitions are central to understanding molecular recognition and the enzymatic cycle. NMR spectroscopy allows one to study these phenomena under functionally relevant conditions. However, the amounts of isotopically labeled peptides required for this technique present prohibitive costs for solid-phase peptide synthesis. To enable NMR studies, we have optimized both expression and purification of isotopically enriched substrate/inhibitor peptides using a recombinant fusion protein system. Three of these peptides correspond to the cytoplasmic regions of the wild-type and lethal mutants of the membrane protein phospholamban, while the fourth peptide correspond to the binding epitope of the heat-stable protein kinase inhibitor (PKI_{5-24}). The target peptides were fused to the maltose binding protein (MBP), which is further purified using a His_6 tag approach. This convenient protocol allows for the purification of milligram amounts of peptides necessary for NMR analysis.

Introduction

Phosphorylation of protein substrates containing a serine or threonine residue by the catalytic-subunit of cAMP-dependent protein kinase A (PKA-C,[1] EC 2.7.11.11) is a ubiquitous step in the regulation of many metabolic pathways [1]. Substrates for this enzyme contain the consensus sequence Arg-Arg-X-Ser/Thr-Y, where X can be any residue and Y is typically a large hydrophobic residue [2]. Much of our understanding for the atomic details of PKA-C-substrate interactions has been guided by crystallographic data of inhibitor or pseudo-substrate bound complexes [3]. These data are focused on analogues of the high affinity binding region (residues 5–24) of the heat-stable protein kinase inhibitor (PKI). Although these crystallographic snapshots have provided a wealth of information, the biological relevance obtained is somewhat limited and dynamic information is absent. Obtaining both high resolution structure and dynamic information for this system is important since structure and dynamics are hypothesized to play fundamental roles in enzymatic function [3], but they are difficult to study under functional conditions. Acquiring this type of data is even more difficult for the case of membrane bound or membrane associated protein substrates.

Nuclear magnetic resonance (NMR) provides an avenue to acquire structural and dynamic information of biologically relevant protein substrates or inhibitors of PKA-C, although such studies would require milligram quantities of isotopically labeled proteins. In this respect, NMR analysis has been limited to a structure determination performed on full-length PKI and two shorter PKI constructs corresponding to the high affinity PKA-C binding region (PKI_{1-24}) and the nuclear export factor (PKI_{26-75}) [4]. This study provided the solution structures of these proteins, but did not provide insight about the changes in dynamics in the absence or presence of PKA-C. Moreover, the analysis of PKI_{1-25} at natural abundance restricted the spectral resolution needed to provide the atomic detail for dynamic changes, echoing the need for a method to isotopically enrich NMR active nuclei for this short peptide.

Also, binding studies on the PKA-C protein substrate, phospholamban (PLN), will provide crucial information on how structural dynamics of substrates change upon PKA-C interaction. PLN is an integral membrane protein in the sarcoplasmic reticulum of cardiomyocytes where it inhibits the Ca^{2+}ATPase, SERCA [5].

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[1] *Abbreviations used:* PKAc, catalytic-subunit of protein kinase A; PKI, protein kinase inhibitor; MBP, maltose binding protein; NMR, nuclear magnetic resonance; PLN, phospholamban; SSPS, solid-phase peptide synthesis; PCR, polymerase chain reaction; HSQC, heteronuclear single quantum coherence; PEG, polyethylene glycol.

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NMR studies of PLN by our laboratory have shown that the structure of this protein contains both a transmembrane and a cytoplasmic helix linked by a short, flexible loop [6,7]. The cytoplasmic region of PLN contains a consensus sequence for PKA-C-catalyzed phosphorylation which, in turn, relieves the inhibition of SERCA [8]. In patients affected by dilated cardiomyopathy, the cytoplasmic region of PLN was found to contain two types of mutations: an arginine to cysteine mutation at position 9 (PLN-R9C) [9] and a deletion of arginine 14 (PLN-R14Del) [10]. Since both mutations are in the vicinity of the PKAc binding region, they could result in a change in interaction with PKAc.

NMR will be the method of choice to understand at the atomic level how structural dynamics are affected by these mutants during substrate recognition and phosphorylation by PKAc. A major obstacle for such studies is that a suitable membrane mimicking environment must be compatible with both proteins [11]. However, model peptides corresponding to the cytoplasmic residues 1–20 of PLN, PLN-R9C, and PLN-R14Del could be used instead to provide such insight. Since much more is known about PKI_{5-24} from crystallography, comparisons between PLN and PKI peptides could also be based on further high resolution NMR analyses of isotopically enriched peptides.

Any of these peptides could be obtained by solid-phase peptide synthesis (SSPS), although this is not a cost-efficient approach for peptides isotopically enriched with ^{15}N, ^{13}C, or ^{2}H. Our aim here is to devise a recombinant system to express four different peptide constructs: the cytoplasmic region of PLN (PLN_{1-20}) and its two mutants, R9C (R9C-PLN_{1-20}) and R14Del (R14del-PLN_{1-20}), as well as the high affinity region of the PKAc inhibitor, PKI_{5-24}. We used *Escherichia coli (E. coli)* BL21(DE3) cells to overexpress fusion proteins composed of maltose-binding protein (MBP) and a target peptide, separated by a TEV cleavable linker region. As noted for other difficult recombinant proteins [12,13], this fusion system combined increased resistance to proteolytic degradation of the peptide in the host cell with the ability to obtain milligram quantities of isotopically labeled peptide per liter of media. The introduction of a His_6 tag and a TEV cleavage site to the C-terminus of MBP also allowed a convenient approach to obtain these peptides at purities which exceed 95%.

Experimental procedures

Materials

All oligonucleotide synthesis and DNA sequencing were performed at the BioMedical Genomics Center of the University of Minnesota. All fusion constructs were cloned using the expression plasmid pMal-c2e (New England Biolabs). The plasmid encoding the tobacco etch virus protease containing a His_6 tag (TEV) used for enzymatic cleavage of fusion protein was kindly provided as a gift from Dr. Robert Gorelick at the National Institute of Health. Expression and purification of TEV was performed as previously described [14]. The *E. coli* strain XL1-Blue (Stratagene) was used for plasmid cloning, while strain BL21(DE3) (Novagen) was used for protein expression. Ni^{2+}-NTA resin (HIS-Select™ Nickel Affinity Gel) was obtained from Sigma.

Cloning of peptide inhibitors and substrates

The gene for PLN_{1-20} was designed using the wild-type PLN parental template which contained codons optimized for usage in *E. coli* [12]. An EcoRI site was encoded at the 5′-end, followed by a His_6-tag and a TEV protease cleavage site and the sequence for the peptide of interest. A HindIII site was encoded at the 3′-end. The His_6-TEV-PLN_{1-20} gene was amplified using the forward primer 5′-CCG GAA TTC CAT CAT CAT CAT CAT CAT GAA AAC CTG TAT TTT CAG GGC ATG GAA AAA GTG-3′. The gene for PKI_{5-24} was also designed from oligonucleotides optimized for usage in *E. coli* [12]. Similar upstream elements in the PKI peptide construct were introduced to this gene as the PLN peptide constructs. Modifications of the PLN_{1-20} sequence to produce the mutant analogue peptides were performed using the Stratagene Quickchange Kit.

Polymerase chain reaction (PCR) was performed using 1X buffer, forward and reverse primers (0.5 μM), dNTP mix (200 μM), Pfu Turbo polymerase (Stratagene; 5 U), and ddH_2O to final volume of 100 μl. The double-stranded DNA was amplified with a first cycle of melting at 94 °C for 2.5 min, elongation at 55 °C for 1 min, and annealing at 72 °C for 1 min; and 29 more cycles with melting at 94 °C for 1 min, elongation at 60 °C for 1 min, and annealing at 72 °C for 1 min. The double stranded DNA containing a target peptide construct (His_6-TEV with either PLN_{1-20}, R9C-PLN_{1-20}, R14del-PLN_{1-20}, or PKI_{5-24}) and pMal-c2e plasmid were digested with EcoRI and HindIII restriction enzymes, and purified by agarose gel. The digested products were ligated back into the pMal-c2e vector using T4 DNA ligase (Invitrogen) and transformed into XL1-Blue *E. coli* competent cloning cells. DNA purification was performed with the Quick-Spin Miniprep kit (Qiagen) and quantitated by measuring UV absorption at 260 nm. Correct PCR products were confirmed with DNA sequencing. Plasmids encoding for MBP-His_6-TEV-peptide were then transformed into *E. coli* strain BL21(DE3) competent cells.

Expression of fusion proteins

A single colony containing the plasmid for MBP-His_6-TEV-peptide was inoculated into 1 L of sterile Luria–Bertani (LB) medium with 1 mM ampicillin and incubated with shaking at 30 °C. After reaching an OD_{600} of 1.2 (∼12 h), the culture was centrifuged at 3000*g* for 10 min at room temperature. The pellet was resuspended in 2.5 L of M9 minimal media containing 1 mM ampicillin and incubated with shaking at 37 °C. Once the cells reached an OD_{600} of ∼0.9, protein expression was induced with 1 mM IPTG. The cells were allowed to express for 5 h and were then harvested by centrifugation at 6000*g* for 20 min at 4 °C. Approximately 10–14 g of cells (weight mass) were typically obtained from 2.5 L of media. The cell pellet was collected, flash frozen in liquid nitrogen, and stored at −20 °C.

Cell lysis

Frozen cell pellets were resuspended in 100 ml lysis buffer (0.1 M sodium phosphate, pH 8.0, 6 M guanidinium hydrochloride, 10 mM imidazole), and homogenized in a blender for 10 min on ice. The lysis mixture was sonicated on ice with a probe sonicator (Branson Sonifier 450) at 40% duty cycle and output control of 4 for 10 min. Cell debris was cleared by centrifugation at 45000*g* for 20 min at 4 °C. The supernatant containing fusion protein was collected for purification.

Protein purification

The supernatant was bound batch-wise to a slurry containing 50 ml of Ni^{2+}-NTA resin at room temperature for 20 min with stirring. The protein/resin mixture was loaded onto a column and the flow through was collected. The resin was subsequently washed twice with 50 ml of lysis buffer. The fusion protein was then isolated by washing the resin with lysis buffer containing 50 mM imidazole until UV absorption at 280 nm was less than 0.1 (∼150 ml lysis buffer). Fractions containing isolated fusion protein were confirmed via 16% SDS–PAGE and Coomassie staining. The fractions were collected and dialyzed using a 10 kDa molecular weight cut-off membrane at 4 °C for 2 h against 3 L of Tris buffer