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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 (PKAc) enabled the atomic characterization of inhibitor and/or substrate peptide analogs 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 enzymatic cycle. NMR 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 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 corresponded to the cytoplasmic regions of the wild-type and lethal mutants of the membrane protein phospholamban, while the fourth peptide corresponded to the binding epitope of the heat-stable protein kinase inhibitor (PKI₅₋₂₄). The target peptides were fused to the maltose binding protein (MBP), which is further purified using a His₆ tag approach. This convenient protocol allows for the purification of milligram amounts of peptides necessary for NMR analysis.

Keywords

Protein kinase A; Phospholamban; protein kinase inhibitor; recombinant peptides; solution NMR; isotopic labeling

Introduction

Phosphorylation of protein substrates containing a serine or threonine residue by the catalytic subunit of cAMP-dependent protein kinase A (PKAc, 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

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hydrophobic residue [2]. Much of our understanding for the atomic details of PKAc-substrate interactions has been guided by crystallographic data of inhibitor or pseudo-substrate bound complexes [3]. These data are focused on analogs of the high affinity binding region (residues 5 to 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 PKAc, 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 PKAc binding region (PKI₁₋₂₄) and the nuclear export factor (PKI₂₆₋₇₅) [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 PKAc. Moreover, the analysis of PKI₁₋₂₅ 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.

The natural PKAc protein substrate, phospholamban (PLN), could provide more biologically relevant information about how structural dynamics of substrates change upon PKAc interaction. PLN is an integral membrane protein in the sarcoplasmic reticulum of cardiomyocytes where it inhibits the Ca²⁺ATPase, SERCA [5]. NMR analyses 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 PKAc-catalyzed phosphorylation which, in turn, relieves the inhibition of SERCA [8]. The cytoplasmic region of PLN was also found to contain two types of mutations which are correlated with dilated cardiomyopathy: 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 how structural dynamics are affected by these mutants during substrate recognition and phosphorylation by PKAc at the atomic level. A major obstacle for such studies is that a suitable membrane mimicking environment must be compatible for both proteins and finding the correct conditions can be quite challenging [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 PKI₅₋₂₄ from crystallography, comparisons could also be made with this peptide based upon further high resolution NMR analyses of isotopically enriched peptides.

Any of these peptides could be obtained by solid-phase peptide synthesis (SPPS), although it does not permit a cost-efficient approach for peptides isotopically enriched with ¹⁵N, ¹³C, or ²H. Our aim here is to devise a recombinant system to express four different peptide constructs: the cytoplasmic region of PLN (PLN₁₋₂₀) and its two mutants, R9C (R9C-PLN₁₋₂₀) and R14Del (R14del-PLN₁₋₂₀), as well as the high affinity region of the PKAc inhibitor, PKI₅₋₂₄. 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 His₆ 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 His₆ tag (TEV) used for enzymatic cleavage of fusion protein was provided as a kind 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. Ni²⁺-NTA resin (HIS-Select™ Nickel Affinity Gel) was obtained from Sigma.

Cloning of peptide inhibitors and substrates

The gene for PLN₁₋₂₀ was designed using the wild type PLN parental template which contained codons optimized for usage in *E. coli* [12]. An *Eco*RI site was encoded at the 5'-end, followed by a His₆-tag and a TEV protease cleavage site and the sequence for the peptide of interest. A *Hind*III site was encoded at the 3'-end. The His₆-TEV-PLN₁₋₂₀ 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 PKI₅₋₂₄ was also designed from oligonucleotides optimized for usage in *E. coli* [4]. Similar upstream elements were introduced to this gene as the PLN peptide constructs. Modifications of the PLN₁₋₂₀ 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 ddH₂O 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 one min, and annealing at 72 °C for one min; and 29 more cycles with melting at 94 °C for one min, elongation at 60 °C for one min, and annealing at 72 °C for one min. The double stranded DNA containing a target peptide construct (His₆-TEV with either PLN₁₋₂₀, R9C-PLN₁₋₂₀, R14del-PLN₁₋₂₀, or PKI₅₋₂₄) and pMal-c2e plasmid were digested with *Eco*RI and *Hind*III 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-His₆-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-His₆-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 OD₆₀₀ of 1.2 (~12 hours), the culture was centrifuged at 3,000g 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 OD₆₀₀ of ~0.9, they were induced with 1 mM IPTG. The cells were allowed to express for 5 hours and were then harvested by centrifugation at 6,000g for 20 min at 4 °C. Approximately 10–14

grams 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 minutes 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 45,000g 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). 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 hours against 3 L of Tris buffer (100 mM Tris, pH 8, 1 M urea, 1 % glycerol), followed by an additional 3 hours at 4°C in the same buffer without urea.

Following dialysis, the fusion protein was pooled and enzymatic cleavage was initiated by addition of 1 mg TEV protease/40 mg fusion and stirred by shaking at 30°C for 6 hours. The reaction was stopped by fast unfolding with the addition of guanidinium hydrochloride to a final concentration of 3.0 M and the solution was bound batch-wise to a slurry containing 50 ml of Ni^{2+} resin at room temperature for 20 min with stirring. The protein/resin mixture was loaded onto a column and the flow through was collected along with a single 25 ml wash. The solution was filtered through a $0.2\text{ }\mu\text{m}$ membrane and loaded onto a semi-preparative reverse phase C-18 Vydac HPLC cartridge ($2.5 \times 10\text{ cm}$, $15\text{ }\mu\text{m}$, $300\text{ }\text{\AA}$). Loading was ~30 mg total protein per injection based on $\epsilon_{\text{MBP}} = 1.6\text{ mg}^{-1}\text{cm}^{-1}$ at 280 nm. Peptides were eluted using a linear gradient from Buffer A (0.1% TFA in H_2O) to 40% Buffer B (0.1% TFA in CH_3CN) and detected at 220 nm. Purities of the peptides exceeded 95% as determined by analytical HPLC using a Waters C18 column ($0.46 \times 25\text{ cm}$) of the collected peptide peak. Pooled fractions containing peptide were lyophilized immediately after purification.

Quantification and protein identification

Protein concentrations prior to TEV cleavage was determined by absorbance at 280 nm or via gel densitometry using a BioRad Molecular Imager FX using BioRad Quantity One software. Final concentrations of pure peptides were determined using analytical HPLC and standard addition of synthetic peptides which were quantified by amino acid analysis. Pure recombinant peptides were confirmed by ESI-TOF: PKI₅₋₂₄ calculated 2279.4 m/z , found 2278.6 m/z ; PLN₁₋₂₀ calculated 2440.8 m/z , found 2440.1 m/z ; R9C-PLN₁₋₂₀ calculated 2387.9 m/z , found 2387.1 m/z ; R14del-PLN₁₋₂₀ calculated 2284.4 m/z , found 2283.7 m/z .

NMR spectroscopy

All peptides were dissolved to a concentration of 1 mM in 20 mM phosphate buffer (pH 6.5), 150 mM KCl, 1 mM NaN_3 , 5 mM DTT, and 5% D_2O . $^1\text{H}/^{15}\text{N}$ heteronuclear single quantum coherence (HSQC) spectra were acquired at 300 K on a Varian Inova 600 MHz spectrometer equipped with a triple resonance probe with z-axis gradients. Spectra were processed using NMRPipe [15] and visualized with the software SPARKY [16].

RESULTS AND DISCUSSION

Plasmid construct and protein expression

The general plasmid construct and primary sequences of the target peptides studied here are shown in Fig. 1. Previous results published by our laboratory for the production of PLN and sarcolipin showed that the selection of MBP as a soluble fusion partner results in milligram quantities of these proteins [12]. We extended that approach to obtain these short recombinant peptide analogues of the PLN cytoplasmic region, and the high affinity binding epitope of PKI. One difference of the construct used here is that a modification of the MBP primary sequence was done by introducing a C-terminal His₆ tag (Fig. 1) before the TEV cleavage site. This provided a robust approach to isolate the fusion protein from the cell lysate via Ni²⁺-NTA chromatography. The introduction of this tag was also useful in latter purification steps to allow removal of MBP and TEV protease (which also contained a His₆ tag) after fusion protein cleavage. Yields for MBP fusion constructs were typically 80–125 mg/L in LB or 60–90 mg/L in M9 minimal media with >90% purity by SDS-PAGE (Table 1). A similar approach has been successfully applied to the expression and purification of isotopically labeled transmembrane domains by Cross and co-workers [13]

Protein purification

The scheme used to obtain high purity recombinant peptides is shown in Fig. 2. One key procedure in this scheme is the unfolding step to fully expose the C-terminal His₆-tag between MBP and the peptide to bind Ni²⁺-NTA resin, followed by a refolding step to allow TEV cleavage to occur efficiently. Isolation of the fusion protein from the cell lysate was done in a batch-wise manner under denaturing conditions. Initial attempts to unfold MBP in the presence of 8 M urea were successful, but a small amount of peptide contaminant 44 amu larger as determined by ESI-TOF mass spectroscopy was consistently observed, regardless of the construct purified. This increased mass corresponds to a carbamylated amine group (either at the N-terminus or at an amine bearing sidechain, such as lysine) via isocyanate [17]. Urea is in equilibrium with ammonium and isocyanate and was the likely source for this amine modification. We decided to use 6 M guanidine-hydrochloride to unfold the fusion protein. After isolation of the fusion protein, protein mis-folding and precipitation tended to occur when guanidine-HCl was dialyzed from the solution. To prevent this from occurring, a short exposure to 1 M urea (two hours) in the presence of one percent glycerol at 4 °C was performed during a first refolding step, followed by a second exposure to one percent glycerol in Tris buffer. The short exposure to urea did not result in any detectable levels of carbamylated product after HPLC purification.

TEV cleavage of these fusion proteins was optimal with ~1 mg of TEV protease per 40 mg of fusion protein at 30 °C. In contrast to previously published conditions for this step, use of Triton X-100 to increase protein solubility during cleavage was avoided. Triton X-100 contains trace amounts of ethylene oxide [18] which can form polyethylene glycol (PEG) in water. Trace amounts of PEG co-eluted with these peptides during HPLC purification and obscured proper identification of the target peptides by mass spectrometry analysis, showing a distinct polymeric pattern separated by 44 amu (data not shown). Therefore, the concentration of the fusion protein was kept below 1 mg/ml with one percent glycerol to increase solubility of all cleavage products which resulted during reactions. Fusion protein cleavage was >90% complete in 6 hours for all constructs as determined by the differential migration of fusion protein and MBP-His₆-TEV cleavage product (~2.5 kDa smaller) using a 16% SDS-PAGE gel (Fig. 3, indicated by the arrows). Under the dilute conditions used at this step, the cleaved peptides could not be visualized on SDS-PAGE gels using either Coomassie blue dye or silver stain. Cleavage reactions were stopped by rapid unfolding of the solution using guanidine-HCl to a final concentration of 3 M. This allowed efficient extraction of the bulk of MBP and TEV

from the reaction solution via Ni^{2+} -NTA chromatography (Fig. 3) and well resolved peptide elution during reverse-phase HPLC (Fig. 4). Typically after the cleavage reactions, the cleaved fusion protein did not bind completely to Ni^{2+} -NTA resin, which resulted in small amounts carried to HPLC purification. However, as shown in the representative chromatogram of R14del-PLN₁₋₂₀ in Fig. 4, all recombinant peptides were well resolved from any other contaminant from the fusion cleavage step reverse-phase chromatography. A representative ESI-TOF spectrum of the pooled fractions containing R14del-PLN₁₋₂₀ obtained from HPLC purification is shown in Fig. 4.

NMR Spectroscopy

The amide fingerprint of ^{15}N -labeled recombinant peptides acquired using $^1\text{H}/^{15}\text{N}$ HSQC NMR spectroscopy under similar conditions to those used to study PKAc by our laboratory [19] is shown in Fig. 5. The amide resonance linewidths displayed by all of these spectra were indicative of peptides which are not aggregated, while the lack of dispersion in the ^{15}N and ^1H dimensions for the PLN peptide analogues suggested that these peptides adopt predominant random coil conformations in aqueous buffer. On the other hand, the more dispersed $^1\text{H}/^{15}\text{N}$ HSQC spectrum for PKI₅₋₂₅ may be indicative that this peptide assumes a more defined secondary structure. The latter agrees well with homonuclear solution NMR data previously published [4]. From these HSQC spectra, PKI₅₋₂₄ displayed 18 of 20 expected peaks (two peaks contained overlapping resonances, Fig. 5A), while PLN₁₋₂₀ and R9C-PLN₁₋₂₀ showed 17 of 20 expected peaks (each had three peaks containing overlapping resonances, Fig. 5B and 5C), and R14del-PLN₁₋₂₀ displayed 18 of 19 expected peaks (one peak contained overlapping resonances, Fig. 5D).

CONCLUSIONS

We report a robust method to express and isolate high purity recombinant peptides (~20 amino acids), corresponding to the PKAc binding regions of PKI, PLN and two mutants of PLN. Since our method uses *E. coli* as the host cell for recombinant expression, it permits all of the widely utilized and cost-efficient isotope labeling approaches in this host system for NMR spectroscopic analysis [20]. By using MBP-fusion constructs, we were also able to limit the amount of proteolytic degradation of these peptides in *E. coli*. From one liter of media, our approach results in the production of 3 mg of PKI₅₋₂₄ (0.5 mg peptide per gram wet cell mass), 2 mg of PLN₁₋₂₀ or R14del-PLN₁₋₂₀ (0.3 mg peptide per gram wet cell mass), and 1 mg R9C-PLN₁₋₂₀ (0.2 mg peptide per gram wet cell mass). Thus, quantities sufficient for NMR structural and dynamic studies can be obtained in as little as one liter of media.

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Fig. 1.

A) Plasmid constructs used for the production of recombinant peptides expressed as a fusion protein with MBP. The *EcoRI* and *HindIII* endonuclease sites were used to introduce a DNA insert containing a His₆-(TEV cleavage site)-peptide codon sequence which was optimized for expression in *E. coli*. B) The primary amino acid sequences which were fused to the C-terminus of MBP. Due to the introduction of a TEV protease site (shown simply as TEV above the six residue sequence), each construct is expressed with an additional N-terminal glycine.

Fig. 2.
Flowchart for the expression and purification of the recombinant peptides studied here.

Fig. 3.

Sixteen percent SDS-PAGE gel depicting the purification of the R14del-PLN₁₋₂₀ peptide starting from selection via Ni²⁺-NTA chromatography and ending with TEV protease cleavage of the fusion protein. Due to the lack of ability to bind coomassie blue dye or silver stain, the peptide was not detected. The arrow on the left corresponds to the full fusion protein, MBP-His₆-TEV-R14del-PLN₁₋₂₀, while the arrows at the right correspond to cleaved fusion (MBP-His₆-TEV) and TEV protease. The small difference in migration (indicated by the dashed line) before and after cleavage reactions was used to monitor the extent of the cleavage reactions. After the cleavage reaction, residual cleaved MBP which did not bind to the Ni²⁺-NTA resin was typically found in the 2nd flow through. These results also reflect those obtained for the three other recombinant peptides.

Fig. 4.

Isolation and identification of cleaved peptides. A) Chromatogram of the semi-preparative reverse-phase HPLC of R14del-PLN₁₋₂₀ peptide after TEV cleavage from the MBP fusion partner. The peak shown at the arrow corresponds to pure R14del-PLN₁₋₂₀. B) ESI-TOF mass spectroscopy of HPLC purified R14del-PLN₁₋₂₀. The two major peaks which were detected correspond to M+3 and M+2 positive ionization states of the peptide and respective patterns are shown in the insets.

Fig. 5. $^1\text{H}/^{15}\text{N}$ HSQC NMR spectra of ^{15}N labeled A) PKI₅₋₂₄, B) PLN₁₋₂₀, C) R9C-PLN₁₋₂₀, and D) R14del-PLN₁₋₂₀ at 1 mM total peptide concentration. The conditions used to obtain these spectra reflect the buffer and temperature in which PKAc has been previously studied [19]. Arrows indicate unresolved peaks which were detected. Side chain amide peaks are indicated in the boxed regions.

Table 1

Protein yields at various purification steps for PKI₅₋₂₄, PLN₁₋₂₀, R14del-PLN₁₋₂₀, and R9C-PLN₁₋₂₀ recombinant peptides.

Protein	Amount (mg/L)	Purity (%)	Fold enrichment ^a
<i>E. coli</i> soluble fraction (MBP/PKI ₅₋₂₄ fusion) ^b	90	6	-
Ni ²⁺ -NTA chromatography (MBP/PKI ₅₋₂₄ fusion) ^b	90	>90	15
HPLC chromatography (isolated PKI ₅₋₂₄) ^c	3	>95	1.1
<i>E. coli</i> soluble fraction (MBP/PLN ₁₋₂₀ or R14del-PLN ₁₋₂₀)	75	4	-
Ni ²⁺ -NTA chromatography (MBP/PLN ₁₋₂₀ or R14del-PLN ₁₋₂₀)	75	>90	23
HPLC chromatography (isolated PLN ₁₋₂₀ or R14del-PLN ₁₋₂₀)	2	>95	1.1
<i>E. coli</i> soluble fraction (MBP/R9C-PLN ₁₋₂₀ fusion) ^b	60	2	-
Ni ²⁺ -NTA chromatography (MBP/R9C-PLN ₁₋₂₀ fusion) ^b	60	>90	45
HPLC chromatography (isolated R9C-PLN ₁₋₂₀) ^c	1	>95	1.1

^a Fold enrichment is calculated as the percent purity of purification step divided by percent purity of preceding step.

^b Gel densitometric measurements of 14% SDS-PAGE were used to estimate protein quantities and purity.

^c Analytical HPLC of the isolated peptide was used to calculate final purity.