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Nucleotide Spin Labeling for ESR Spectroscopy of ATP-Binding Proteins

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Abstract

Site-directed spin labeling of proteins by chemical modification of engineered cysteine residues with the molecule MTSSL (1-Oxyl-2,2,5,5-tetramethylpyrroline-3-methyl methanethiosulfonate) has been an invaluable tool for conducting double electron electron resonance (DEER) spectroscopy experiments. However, this method is generally limited to recombinant proteins with a limited number of reactive Cys residues that when modified will not impair protein function. Here we present a method that allows for spin-labeling of protein nucleotide binding sites by adenosine diphosphate (ADP) modified with a nitroxide moiety on the β -phosphate (ADP- β -S-SL). The synthesis of this ADP analog is straightforward and isolation of pure product is readily achieved on a standard reverse-phase high-performance liquid chromatography (HPLC) system. Furthermore, analyses of isolated ADP- β -S-SL by LC-mass spectrometry confirm that the molecule is extremely stable under ambient conditions. The crystal structure of ADP- β -S-SL bound to the ATP pocket of the histidine kinase CheA reveals specific targeting of the probe, whose nitroxide moiety is mobile on the protein surface. Continuous wave and pulsed ESR measurements demonstrate the capability of ADP- β -S-SL to report on active site environment and provide reliable DEER distance constraints.

1 Introduction

Site-directed spin labeling in concert with electron-spin resonance (ESR) spectroscopy is an invaluable tool for studying the nature of proteins in solution [1–3]. This method has been especially useful for examining proteins that are large, flexible, highly dynamic or membrane associated and hence not easily accessed by techniques such as x-ray crystallography and NMR spectroscopy [1–3]. However, in the majority of cases, spin-labeling is achieved by chemically modifying engineered Cys residues with a nitroxide moiety provided by small molecules such as MTSSL [4]. More recently, additional strategies have been developed, including the targeting of Tyr residues [5] and N-terminal purification tags [6]. The labels are ideally introduced at sites not essential for protein function. Such non-perturbative labeling may not always be possible when the modification sites are near ligand-binding or protein:protein interaction regions. Furthermore, the protein may have many native Cys residues or otherwise be intolerable to residue substitution, genetic manipulation or recombinant expression. To circumvent some of these issues, alternative labeling approaches that do not rely on cysteine labeling have been developed. The use of

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genetically encoded non-natural amino acids has provided a means for spin-labeling proteins without the need for protein chemical modification or mutation of native residues [2, 3]. However, such residue substitutions may again perturb protein function if near active centers or protein interaction regions.

In addition to protein labeling, strategies have been developed to attach spin labels to DNA and RNA oligonucleotides. These methods include attaching spin labels to the nucleotide base [9], the sugar moiety [4, 5], and the terminal (5') phosphate [4, 6]. For the case of individual nucleotides, labels have been developed to target the C2' and C3' positions of the ribose component [7, 8]. In the case of oligonucleotides, the 5' phosphate has been labeled with MTSSL after conversion to a reactive phosphorothioate [11]. Thus, synthesis of this spin-labeled oligonucleotide, which has found utility in ESR experiments, proceeds analogously to cysteine labeling by MTSSL [11]. Using a related approach, we herein present a spin labeling method for ATP-binding proteins that adds a label directly to the substrate nucleotide (ADP) at the β -phosphate position. Thus, this method allows for direct labeling of the nucleotide-binding region of ATP-binding proteins without modification of the protein itself. Adding the spin-label to the β -phosphate, as opposed to the sugar or base moieties, is advantageous because such a modification is less likely to interfere with binding determinants provided by these constituents. Furthermore, in many ATPases and kinases the position of the γ -phosphate is accessible for hydrolysis or phosphorylation and thus replacement by nitroxide is likely to be accommodated. The feasibility of this approach is demonstrated with the ATP-binding histidine kinase CheA from *Thermotoga maritima*, a large protein that contains several domains connected by long flexible linkers. CheA is a well-characterized component of the phospho-relay system of bacterial chemotaxis and a previous subject of spin-labeling studies [14]. CheA functions as a dimer and each subunit consists of five domains (P1–P5) with distinct function. Herein, we utilize CheA to demonstrate the effectiveness of this method.

2 Results and Discussion

2.1 Synthesis of ADP- β -S-SL

The ESR spin label MTSSL was coupled to the ADP analog ADP- β -S by reaction of ADP- β -S with MTSSL in aqueous solution for 10 hours (Fig. 1). The product, ADP- β -S-SL, was readily detected by thin-layer chromatography and then distinguished from the precursors and methanesulfonyl byproduct by liquid-chromatography mass-spectrometry (LC-MS) (Fig. 2a). Solid ADP- β -S-SL was then isolated by high-performance liquid chromatography (HPLC) followed by rotary evaporation to produce ~70% of the expected yield. Subsequent analysis of the isolated product by LC-MS demonstrates that ADP- β -S-SL is present in high purity and stable in solution after freezing, storage at -80°C , and 24 hours incubation at room temperature. LC-MS experiments identify adenosine monophosphate (AMP) as the primary degradation product of ADP- β -S-SL (Fig. 2b).

2.2 Binding of ADP- β -S-SL to CheA

To evaluate whether ADP- β -S-SL binds in the ATP-binding pocket of *T. maritima* (Tm) CheA, radioisotope assays of CheA autophosphorylation were conducted with ADP- β -S-SL

as a competitive inhibitor. ATP binds to Tm CheA dimer with a K_D of $\sim 1 \mu\text{M}$ [15]. Addition of ADP- β -S-SL reduces CheA autophosphorylation activity as expected (Fig. 3a). Fitting the data to a simple competitive inhibition model indicates an apparent $K_i = 29 \pm 6 \mu\text{M}$.

To confirm that the nucleotide analog indeed targets the ATP binding domain, the ATPase domain of CheA (P4) was co-crystallized with ADP- β -S-SL and the structure determined to 2.9 Å resolution by x-ray crystallography (Table 1). The structure of CheA[ADP- β -S-SL] reveals a P4 structure similar to that observed in the structure of CheA P4 with the fluorescent ATP analog TNP-ATP [16]. ADP- β -S-SL binds analogously to ADP with a key hydrogen bond formed between Asp449 and the adenine ring at the base of the P4 β -sheet. Typical of diphosphate nucleotide binding, the P4 ATP-lid region is open and not engaged with a divalent metal ion [16]. Although the phosphate moieties of ADP are well defined, electron density for the nitroxide moiety itself is weak and only evident at low contour levels (Fig 3c). Thus, given that ADP- β -SSL is stable under the conditions of crystallization and the nitroxide projects from the surface of the protein with little direct interaction to any protein residues, it is likely that the nitroxide is conformationally disordered (Fig 3c).

2.3 ESR measurements of ADP- β -S-SL bound to CheA

A conformationally disordered nitroxide moiety is consistent with results from cw-ESR at 25 °C carried out with full-length CheA bound to stoichiometric amounts of ADP- β -S-SL. The resulting spectra are indicative of highly mobile spin-label with some indication of broadening due to the binding interaction (Fig 3b). To ensure that the ADP- β -S-SL can bind to other ADP-binding proteins, similar cw-ESR experiments were carried out with the eukaryotic Caesin Kinase 1 (CK1) [17] and FRQ-interacting RNA helicase (FRH) [18], both from *Neurospora crassa* (Fig 3b). In these two cases of ATP-binding proteins broadening of the spectra is clearly evident. Pulsed ESR measurements on ADP- β -S-SL- bound CheA indicated that the isolated ADP- β -SSL has a strong primary echo signal that decays in a manner similar to that of MTSSL. DEER spectroscopy was then carried out with full-length CheA containing an nitroxide-labeled cysteine at residue 387 (E387C-SL) both with and without purified ADP- β -S-SL. CheA is a dimeric kinase with each subunit (MW of 79 kDa), containing five domains connected by flexible linkers. Without ADP- β -S-SL present, E387C-SL CheA produces a distance distribution ($P(r)$) with a peak centered at 45 Å, which represents the distance between the spin-labeled E387C positions of the two P4 domains in the CheA dimer (Fig 4a) [14]. However, when ADP- β -S-SL is added to E387C-SL an additional shorter distance of about 35 Å becomes apparent (Fig. 4b). *T. maritima* CheA binds ATP with a high degree of negative cooperativity [15]; under these conditions we expect primarily one nucleotide to be bound per CheA dimer. In addition, based on the structure of the CheA dimer, the nucleotide binding sites are likely $> 70 \text{ Å}$ apart; thus, we do not expect to see an intersubunit signal from the nucleotide. The 35 Å distance agrees with the expected intrasubunit separation between the E387C-SL and ADP- β -S-SL bound to P4 in the crystal structure (Fig. 2d). Upon addition of excess ATP (5 mM) the short distance component no longer contributes to the distribution (Fig 4c).

2.4 Discussion

In principle, any ATP binding protein would be a suitable subject for spin-labeling by this strategy. Importantly, proteins that catalyze ATP hydrolysis commonly bind to the adenine moiety so that the γ -phosphate is exposed near the surface of the protein. Therefore, nitroxide labeling at the β -phosphate is in many cases not likely to interfere with nucleotide binding by these proteins. Whereas the method presented here utilized an ADP analog, non-hydrolyzable forms of ATP could in principle also be spin-labeled on the γ -phosphate in a similar approach. Here, we elected to add a spin-label to an ADP β -phosphate to produce a more analogous steric analog of ATP than one that extends the nucleotide by a full nitroxide moiety.

Furthermore, current commercial ATP analogs suitable for labeling may be subject to hydrolysis by kinases. Overall, the technique is straightforward and can provide immediate structural constraints and information on nucleotide environment for complex molecules without further genetic engineering. Many ATP-binding proteins are oligomers and hence pulsed-ESR spectroscopy in concert with ADP- β -S-SL binding can report on their assembly state as well as active site dispositions. Combined with Cys-directed spin-labeling, both internal distance constraints and intermolecular constraints to interacting partner proteins are readily accessible. Although this method is presented specifically for ATP/ADP-binding proteins, it should also be applicable to any nucleotide binding protein, including GTP-binding proteins.

3 Methods and Materials

3.1 Synthesis and isolation of ADP- β -S-SL

ADP- β -S (Adenosine-5'-O-(2-thiodiphosphate) trilithium salt, Krackeler Scientific) and MTSSL (Chem Cruz) were mixed in a 1:2 molar ratio (7.5 mM, 15 mM) in 300 μ l of a solution containing 50 mM potassium phosphate buffer pH 7.5 and 20% acetonitrile. The reaction was allowed to proceed at room temperature for 10 hours in a rocked tube covered with aluminum foil. Formation of a new nucleotide species in the reaction mixture was monitored by thin-layer chromatography [19]. The reaction mixture was then injected on an Agilent 1100 series HPLC system with a reverse-phase column (Agilent Eclipse, XDB-C18, 5 μ M 9.4 $\times$ 250 mm) preequilibrated with water and eluted at 3.2 ml/min for 40 minutes with an increasing gradient of acetonitrile (0–20%). The fractions containing product, assumed to be ADP- β -S-SL, were dried by rotary evaporation. The isolated ADP- β -S-SL was then resuspended in 50 mM potassium phosphate buffer pH 7.5 and 20% acetonitrile, separated into aliquots, flash frozen, and stored at -80°C .

LC-MS analyses of the reaction mixture and isolated ADP- β -S-SL was carried out with a chromatography protocol similar to that above using an analytical HPLC column (Agilent Eclipse, XDB-C18, 5 μ M 4.6 $\times$ 150 mm) connected to a Quattro Ultima mass spectrometer (Micromass/Waters) operated in electrospray negative and positive ionization (ESI $^{-}$; ESI $^{+}$) modes.

3.2 Purification of Proteins

CheA from *T. maritime* was cloned from genomic DNA into a pet28a vector that contains an N-terminal poly-His tag and a kanamycin resistance marker. *N. crassa* FRH was prepared as previously described (ref). *N. crassa* CK1a (clone generously provided by J.C. Dunlap Dartmouth Medical College) was also cloned into pet28a and expressed and purified as described for CheA. pet28a expressing the desired protein was transformed into *E. coli* BL21 competent cells. The cells were grown in 100 ml of LB at 37 °C overnight and then transferred into 8 L of LB. The cells were grown at 37 °C to an O.D. of 0.8 and then induced by the addition 100 µM IPTG at 25 °C and 17°C for CK1 and FRH. After 16 hours of protein growth the cells were collected by centrifugation and lysed by sonication. The lysate was subjected to centrifugation to pellet insoluble cellular components. The soluble portion of the lysate was run over a column containing Ni-NTA. The resin was washed with buffer to remove all proteins except for the His-tagged CheA. Protein was subjected to size-exclusion chromatography and concentrated via high-speed centrifugation. CheA P4 (residues 350–540), was also expressed from pet28a and purified using a similar procedure as above. For both proteins, the poly-His tag was removed via protein cleavage with thrombin.

3.3 Radioisotope assays with CheA

Radioisotope assays that monitor CheA autophosphorylation over time were used to evaluate the ability of ADP-β-S-SL to compete with ATP for binding CheA. Purified CheA (2 µM) was incubated in 23 µl buffer (50mM Tris pH 7.5, 150 mM NaCl, 50 mM KCl, 10 mM MgCl₂) with varying concentrations of ADP-β-S-SL (0–2 mM) for 1 hour at 25°C. To initiate autophosphorylation, 2 µl of a solution containing cold ATP (312.5 µM) and 1 µl [γ -32 P] ATP (3000 Ci/mmol, 10 µCi/µL, Perkin Elmer) was added to the mixture. Reactions were then quenched with 25 µL of 3×SDS buffer containing 50 mM EDTA pH 8.0 and 30 µL of the quenched samples were subjected to gel electrophoresis on a gradient 4–20% Tris-glycine SDS-PAGE gel. The gel was dried with a GelAir gel dryer and then placed in a cassette for 24 hours. The gels were imaged with a Storm phosphor-imager (GE Healthcare), and the relative band intensities were analyzed with ImageJ. The relative initial velocity for autophosphorylation was calculated in the presence of ATP (25 µM) and varying concentrations of ADP-β-S-SL (0–2 mM). These values were then used to calculate the K_i of ADP-β-S-SL binding to CheA by using the equations $V=(V_{max}+[S])/(K_{m app}+[S])$ and $K_{m app}=1+([I]/K_i)$. The apparent K_i was calculated using four concentrations of ADP-β-S-SL.

3.4 MTSSL labeling of CheA

To label CheA E387C with MTSSL, 5 mg of MTSSL (Chem Cruz) was dissolved in 100 µl of acetonitrile and added to 5 ml of buffer. The mixture was added to Ni-NTA resin containing bound poly-His CheA and incubated for 16 hours at 4 °C. The unreacted MTSSL was washed from the resin with buffer and the protein was eluted from the Ni-NTA with imidazole. The eluted protein was further purified using an S200 GE-Sephadex column and concentrated via highspeed centrifugation to ~200 µM.

3.5 Crystallization of CheA P4 with ADP- β -S-SL

The P4 (ATP-binding) domain of CheA was co-crystallized with ADP- β -S-SL via hanging drop vapor diffusion by mixing 2 μ l of 1 mM P4 solution (50 mM Tris pH 7.5, 150 mM NaCl) containing 3 mM ADP- β -S-SL with 2 μ l of crystallization buffer (50 mM citrate pH 5.5, 2 M ammonium sulfate). The rectangular crystals formed within 24 hours and diffracted to about 2.9 Å resolution on the 24-ID-E beam line of Advanced Photon Source (APS) at Argonne National Laboratory. Diffraction data was collected on an Eiger-16M Pixel Array Detector, and processed with HKL2000. The structure was determined by molecular replacement in PHENIX using a probe of CheA P4 bound to the nucleotide analog TNP-ATP (PDB code: 1I5D). The protein crystallized as a homo-dimer in a P 2₁ space group. The structure was refined in PHENIX [20] and the model built in COOT [21]. Parameters for ADP- β -S-SL were derived from those for ADP and the nitroxide spin label bound to cysteine (MTN) as implemented in PHENIX.

3.6 Electron-spin resonance spectroscopy

For Pulsed Dipolar Spectroscopy (PDS) measurements, four pulse double electron-electron resonance (DEER) experiments were conducted at 60 K on a 17.3 GHz FT EPR spectrometer, which is modified to perform PDS experiments [22]. Data were collected on 100 μ M protein over 16 hours for each sample. The signal background was approximated by a polynomial function in the semi-log scale and subtracted out [23]. Noise from the time domain data was then removed by the WavPDS method [24] (a wavelet denoising procedure for PDS) and distance distributions of spin separations calculated by the new singular-value decomposition (SVD) method [25] developed to solve ill-posed problems such as for the PDS signal.

The cw-ESR spectra were obtained on a Bruker E500 ESR spectrometer operating at X-band (~9.4 GHz). To prepare the samples, 100 μ l of buffer containing 100 μ M of ADP- β -S-SL and 100 μ M CheA monomer were incubated for 30 min and then washed via 5 $\times$ dilution followed by re-concentration to remove any unbound ADP- β -S-SL. Samples using CK1 and FRH with ADP- β -S-SL were prepared using an identical method. 30 μ l of each sample were used for data collection. The ESR measurements were carried out at room temperature with a modulation amplitude of 0.5 G and modulation frequency of 100 kHz. All spectra were digitized to 1024 points with a sweep width of ~100 G and an average of 4–16 scans.

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Fig. 1.

The reaction scheme for the synthesis of ADP-β-S-SL. ADP-β-S reacts with MTSSL in a 1:1 stoichiometric ratio to form an ADP analog with a nitroxide spin-label linked to the β-phosphate via a disulfide bond

Fig. 2.

Isolation and decomposition of ADP-β-S-SL analyzed by LC-MS. **a** A reaction mixture consisting of ADP-β-S-SL and MTSSL reveals the presence of the desired product, ADP-β-S-SL. The precursors and product are well separated using a reverse-phase HPLC system. **b** Isolated ADP-β-S-SL was analyzed by LC-MS after a freeze-thaw cycle and incubation at 25°C for 24 hours. (Top) Three degradation products are identified; a reduced form of ADP-β-S-SL is the most abundant. (Bottom) Extracted ion chromatograms of each molecule present in the degraded sample. For LCMS experiments, reaction species were identified by either positive or negative ion detection modes

Fig. 3.

ADP-β-S-SL binds to the histidine kinase CheA from *T. maritima*. **a** Competitive inhibition assays with ATP (25 μM) and ADP-β-S-SL demonstrate that ADP-β-S-SL binds to the nucleotide pocket of CheA with a K_i of 29 ± 6 μM. **b** cw-ESR of ADP-β-S-SL (100 μM) in the absence and presence of CheA (100 μM) after washing suggest that the nitroxide label is relatively mobile when bound to CheA. cw-ESR of ADP-β-S-SL bound to the *N. crassa* kinase CK1 and RNA helicase FRH, two other ATP binding proteins, reveal a more rigid nitroxides, in these cases. **c** The crystal structure of the CheA nucleotide-binding domain (P4) reconstituted with ADP-β-S-SL. The Fo-Fc omit map shown at $\sigma = 1.3$ (green contours) reveals density for the nucleotide and nitroxide moieties, although the nitroxide appears conformationally disordered to some degree. **d** The CheA[ADP-β-S-SL] structure predicts a ~35 Å distance between the nucleotide and residue 387.

Fig. 4. DEER measurements with nitroxide-labeled CheA and ADP- β -S-SL. a Dimeric CheA labeled with MTSSL on each P4 subunit at position E387C produces a distance distribution with a broad peak centered at 45 $\text{\AA}$. b When ADP- β -S-SL is reconstituted into the E387C-SL protein, the distribution become bimodal with a second peak centered at 35 $\text{\AA}$. c Upon addition of excess ATP, ADP- β -S-SL is competed from the nucleotide pocket and the 35 $\text{\AA}$ peak is lost. d Time domain data for the three samples shown above after baseline correction. Inset shows DEER data before baseline subtraction to 2 μs after the spectra were aligned for comparison purposes. Note that sample c differs from a in that the spin-

label is free from the protein for c. Uncertainty in the SVD of the distance distributions are shown in red

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Table 1.
Data collection and refinement statistics

Final refinement statistics for the structure of P4 [ADP- β -S-SL] with the statistics for the highest resolution shell shown in parentheses

Wavelength (Å)	0.97220
Synchrotron	APS
Beam-line	24-ID-E
Space group	P 1 2 ₁ 1
a, b, c	83.703, 75.145, 86.580
α , β , γ	90, 90.013, 90
Resolution (Å)	50–2.95(3.053–2.95)
R _{merge}	0.032 (0.875)
R _{p.i.m.}	0.040 (0.755)
R _{meas}	0.071 (1.341)
I/ σ (I)	5.7 (1.0)
Completeness (%)	93.41 (99.27)
Multiplicity	3.2 (2.0)
Mosaicity	0.475
Total reflections	25447
<u>Refinement</u>	
Resolution (Å)	10–2.95(3.053–2.95)
Reflections for refinement	21358
Reflections used for R _{free}	1692 (185)
R _{work}	0.2325 (0.5318)
R _{free}	0.2977 (0.6039)
No. non-hydrogen atoms	5687
Protein	5535 (737 residues)
Ligands	152
<u>B factors (Å²)</u>	
Average B factors	106.05
Protein	105.62
Ligands	121.70
TLS groups	25
<u>R.M.S Deviations</u>	
Bond lengths (Å)	0.015
Bond angles (°)	1.78
Ramachandran outliers (%)	0.56
Rotamer outliers (%)	2.52