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Cofactor Fingerprinting with STD NMR to Characterize Proteins of Unknown Function: Identification of a Rare cCMP Cofactor Preference

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Abstract

Proteomics efforts have created a need for better strategies to functionally categorize newly discovered proteins. To this end, we have employed saturation transfer difference NMR with pools of closely related cofactors, to determine cofactor preferences. This approach works well for dehydrogenases and has also been applied to cyclic nucleotide-binding proteins. In the latter application, a protein (radial spoke protein-2, RSP2) that plays a central role in forming the radial spoke of *Chlamydomonas*

reinhardtii flagella was shown to bind cCMP. cCMP-binding proteins are rare, although previous reports of their presence in sperm and flagella suggest that cCMP may have a more general role in flagellar function. ^{31}P NMR was used to monitor the preferential hydrolysis of ATP versus GTP, suggesting that RSP2 is a kinase.

1 Introduction

With the sequencing of the human and other genomes now completed, emphasis has switched to determining the structure and function of the protein complement of these genomes. To this end, chemical proteomic methods are being developed which use ligands as probes to define protein function. Although, these probes are often activity-based affinity labels [1, 2], strategies have also been developed to profile proteins based on the ligands they bind non-covalently, thereby defining an affinity "fingerprint" [3, 4]. NMR screening has recently been proposed as a means to create such a fingerprint, using panels of ligands [5]. Herein, we apply an efficient NMR strategy to assign proteins to functional classes, based on cofactor specificity that is probed by screening combinations of related cofactors, using competitive saturation transfer difference (STD) NMR [6-8]. Such data could provide annotation to functional genomic databases, since cofactor binding preferences provide useful insights into the biochemical function for an enzyme of unknown function [9]. For example, dehydrogenases would be expected to bind NAD(P)H, kinases should bind MgATP, and so on. We have validated this strategy on known dehydrogenases as well as a cyclic nucleotide dependent protein kinase. We then used it in a functional proteomic project to identify an unexpected cCMP binding preference for a protein of unknown function, but known to contain a GAF domain for cyclic nucleotides [9-12].

Cofactor fingerprinting with STD NMR is best done using mixtures of cofactors that represent the most commonly used cofactors in biochemistry [9]. Since this strategy relies on the STD **7** NMR binding assay, there is no need to know an enzymatic activity or function a priori. Also, screening can be done with pools of cofactors that are suspected ligands for the protein in question, since STD selectively identifies which ligands bind. Of special relevance for our application is that the tighter binding ligand(s) will dominate the STD NMR spectrum, as long as binding is in "fast exchange" on the NMR chemical shift timescale – meaning exchange rate is greater than the differences in chemical shifts (in units of s^{-1}) for resonances from free and bound ligand. Since cofactors usually bind weakly to proteins (K_d> 1 μM) [9], and since binding (k_{on}) is often close to diffusion-controlled, cofactor binding/release will usually be in fast exchange and therefore give a strong STD signal. But, occasional cases of slow exchange will be easily diagnosed upon deconvolution of STD data (repeating with individual cofactors), since the STD experiment is being run in competition mode. A cofactor that binds so tight that it is in slow exchange (generally with K_d < 1 μM) will decrease the STD signal of a related but weaker binding cofactor.

2 Materials and methods

2.1 Protein and reagents

RSP2 was a generous gift from Dr. Pinfen Yang (Marquette University). The full-length RSP2 protein had been cloned from *Chlamydomonas reinhardtii* into a pET vector and overexpressed in *E. coli* (strain BL21(DE3)), then purified as described [12]. Purified RSP2 was exchanged into NMR buffer using a gel filtration column, then concentrated using Amicon YM-30 Centricon filters. ATP, GTP, cAMP, cGMP, and cCMP are from Sigma–Aldrich and D_2O (99.9 at.% D) is from Cambridge Isotope Laboratories, Inc.

Glucose-6-phosphate dehydrogenase (G6PDH) from *Leuconostoc mesenteroides*, lactate dehydrogenase (LDH) from rabbit muscle and protein kinase A (PKA) from bovine heart are all from Sigma–Aldrich.

2.2 STD NMR studies

STD NMR studies (Figs. 1–4) were performed at the NMR Facility at Madison on Varian 600 or 800 MHz spectrometers. Unless specified otherwise, all experiments were performed at 298 K in an NMR buffer of 20 mM sodium phosphate, 200 mM NaCl and 100% D_2O at pH 7.4. STD NMR experiments were performed using the Varian cyclenoe pulse sequence, with alternating on-resonance irradiation of the protein methyl region (around 1 ppm) and off-resonance control irradiation at −2.0 ppm. None of the proteins analyzed had resonances in this chemical shift range and control irradiation here gave the same result as irradiation in the low-field range above 10 ppm (not shown). Total irradiation time was for 4–6 s, using a train of 100 ms pulses. All proton spectra were referenced to the residual water signal at 4.76 ppm (at 298 K).

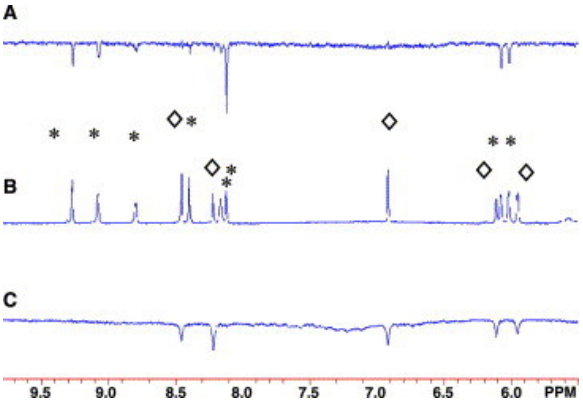

Figure 1. Cofactor fingerprinting with STD NMR (298 K, 800 MHz), performed on G6PDH and LDH. (A) STD NMR spectrum of the mixture of G6PDH and NADH/$NADP^+$. (B) 1D ^1H NMR spectrum of the NADH/$NADP^+$ cofactor mixture. The resonances marked with ◊ are from NADH, while those marked with * are from $NADP^+$. (C) STD NMR spectrum of the mixture of LDH and NADH/$NADP^+$. On-resonance irradiation was applied at 0.92 ppm and off-resonance irradiation was applied at −2.0 ppm, each for 6 s using a train of 100 ms rectangular pulses. Spectra represent the average of 16 acquisitions, using a 1 s relaxation delay.

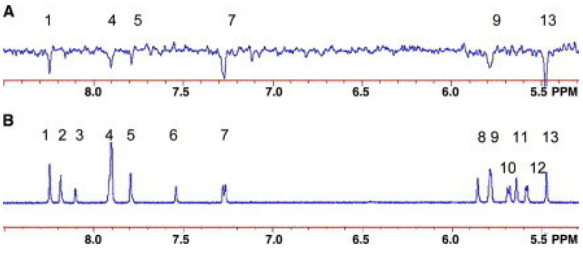

Figure 2. Cofactor fingerprinting with STD NMR (at 298 K and 600 MHz) performed on a mixture of RSP2 (~10 µM) and six cofactors (ATP, GTP, cAMP, cGMP, cCMP and 5'AMP, each 1 mM). (A) 1D STD ^1H NMR spectrum of the mixture of RSP2 and cofactors (with 4 mM Mg^{2+} present). (B) 1D ^1H NMR spectrum of the same mixture. The assignments for the proton resonances are as follows: 1, 5' AMP; 2, ATP; 3, impurity in cAMP; 4, cAMP, ATP and 5'AMP; 5, GTP; 6, cGMP; 7, cCMP; 8, cAMP; 9, 5'AMP and ATP; 10, cCMP; 11, cGMP; 12, GTP; 13, cCMP. On-resonance irradiation was applied at 1.2 ppm and off-resonance irradiation was applied at −2.0 ppm, each for 4 s using a train of 100 ms rectangular pulses. Spectra represent the average of 128 acquisitions, using a 1 s relaxation delay.

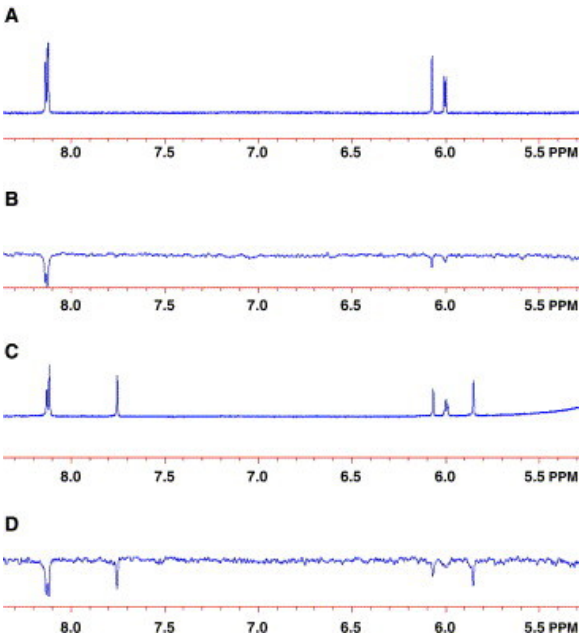

Figure 3. Cofactor fingerprinting with STD NMR (at 298 K and 600 MHz) performed on the mixture of RSP2 (~25 µM), cAMP and cGMP. (A) 1D ^{1}H NMR spectrum of RSP2 with 1 mM cAMP. (B) STD NMR spectrum of the sample in panel A. (C) 1D ^{1}H NMR spectrum of RSP2 (~25 µM) with 1mM cAMP and 1 mM cGMP. (D) STD NMR spectrum of the sample in panel C. NMR experimental parameters were as in Fig. 2.

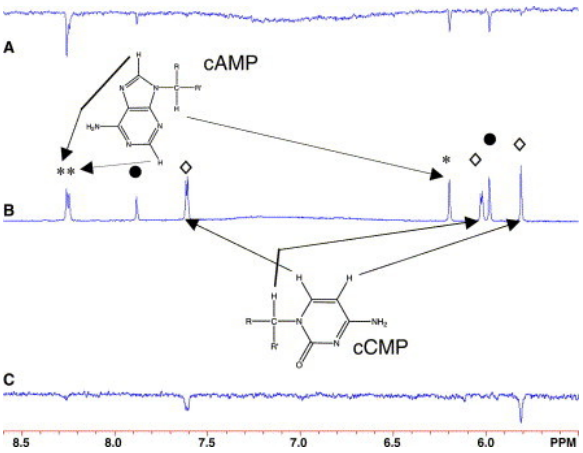

Figure 4. Cofactor fingerprinting with STD NMR applied to PKA and RSP2. (A) STD NMR spectrum of the mixture of PKA and cAMP/cGMP/cCMP. (B) 1D ^{1}H NMR spectrum of the mixture of cAMP (*), cGMP (•) and cCMP (◊). (C) STD NMR spectrum of the mixture of RSP2 and the three cyclic nucleotides. NMR experimental parameters were as in Fig. 2.

2.3 ^{31}P NMR study of ATP hydrolysis by RSP2

^{31}P NMR studies (**Fig. 5** A) were performed on a Varian 300 MHz spectrometer operating at 121.5 MHz and at 290 or 298 K. Spectra represent an average of 1024 transients collected over 38 min, using a broad spectral width of 18 248 Hz, to provide over-sampling for a flat baseline. Internal referencing was to orthophosphate. Processing was with MestRe-C (University of Santiago, Spain), using exponential multiplication with a line-broadening of 5 Hz. Sample was maintained at 4 °C between NMR

experiments, to minimize protein degradation. Integrated ^{31}P NMR signals were fitted as a function of time to the exponential function described below (**Fig. 5**B), using SigmaPlot 8.0.

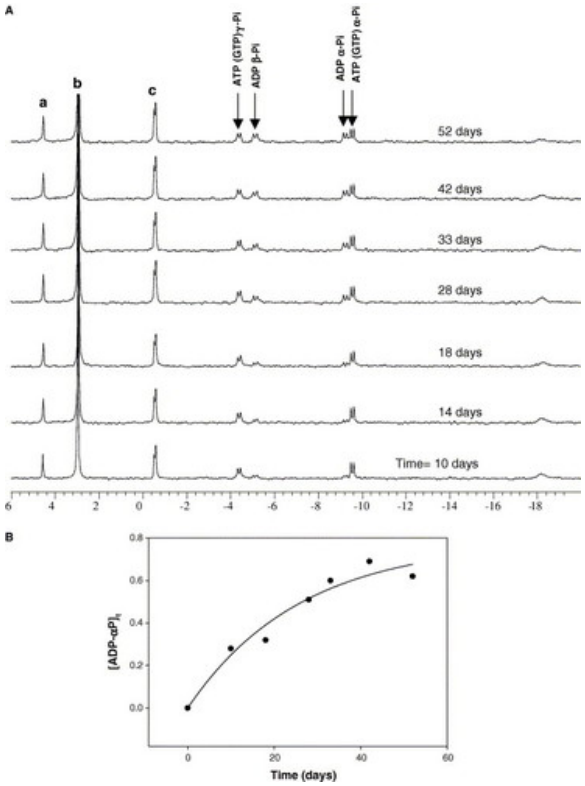

Figure 5. (A) 1D ^{31}P NMR spectra (at 290 K and 300 MHz) for RSP2 (~10 µM) and the ligand mixture used in Fig. 2, supplemented with 3 mM Mg^{2+}. The ^{31}P NMR spectrum at the bottom (time = 10 days) was the first spectrum in which the ADP phosphate resonances were observed. The α and γ phosphate signals of ATP and the α and β phosphate signals of ADP are marked. Additionally, **a** is the 5'AMP signal and **b** is the inorganic phosphate signal, while **c** is assigned to cAMP, cGMP and cCMP. (B) Corresponding fit to an exponential function to give the pseudo-first order rate constant for RSP2 catalyzed ATPase (R^2 = 0.96). Control sample with no RSP2 showed no ADP-phosphate signal after 28 days.

3 Results and discussion

3.1 Application of cofactor fingerprinting with STD NMR to dehydrogenases

We initially applied the cofactor fingerprinting with STD NMR approach to well-characterized proteins (dehydrogenases), then to a protein of unknown function, as part of a larger functional proteomics project [12-15]. Dehydrogenases are an excellent gene family for applying functional proteomic methods, since they represent 3–6% of most proteomes, and are easily identified using bioinformatics tools [16, 17]. But, it is often difficult to predict based on sequence whether there will be preference for NADH or for its 2'-phosphorylated form (NADPH). Cofactor fingerprinting with STD NMR can be used to determine which cofactor is preferred, by screening enzyme in the presence of both, without any need to have the enzyme's substrate present. Since the protons on both NADH and NADPH have similar chemical shifts, it is necessary to use mixtures of either NADH/$NADP^+$ or NAD^+/NADPH. We applied cofactor fingerprinting with STD NMR to two common dehydrogenases, G6PDH and LDH, using a NADH/$NADP^+$ mixture (**Fig. 1**). The concentration of protein and cofactor was 2 mg/mL and 1 mM, respectively. Protein was irradiated

at the frequency (~1 ppm) of the methyl protons for amino acids like Val, Ile, Leu, Thr and Ala, being careful to not inadvertently irradiate cofactor resonances. The spectrum with on-resonance irradiation at ~1 ppm is subtracted from a spectrum with off-resonance irradiation. As long as cofactor binding is in fast exchange, the STD NMR spectrum gives signal only for the cofactor that binds with highest affinity to the protein, thereby allowing determination of cofactor preference in a short experiment (5–10 min) with no more than 1 mg of protein.

The STD NMR spectra indicate that $NADP^+$ is the preferred cofactor for G6PDH (**Fig. 1**A) and NADH is the preferred cofactor for LDH (**Fig. 1**A). Consistent with these results, G6PDH is known to be specific for NADP(H) in the redox interconversion of glucose 6-phosphate and 6-phosphoglucono-δ-lactone, while LDH is specific for NAD(H) in the redox interconversion of pyruvate and lactate [9]. This study therefore suggests the general utility of cofactor fingerprinting with STD NMR for determining cofactor preferences of dehydrogenases.

3.2 Application of cofactor fingerprinting with STD NMR in functional proteomics: RSP2

We next applied the cofactor fingerprinting with STD NMR strategy in a functional proteomics project, devoted to determining functions for the radial spoke proteins in the flagella of *Chlamydomonas reinhardtii* [12-15]. One of these proteins is RSP2 (radial spoke protein-2). RSP2 has a sequence motif classified as a GAF domain, which is present in a new class of cGMP receptors [11, 12]. The initial hypothesis was therefore that RSP2 binds cGMP and/or cAMP, as is typical of GAF domains. To test this bioinformatic hypothesis, we applied the cofactor fingerprinting with STD NMR approach to RSP2 to explore preferences for cyclic nucleotides and other potential cofactors. The STD NMR spectrum for RSP2 in a pool of six cofactors (**Fig. 2**) indicates cCMP (peaks 7, 10, and 13) and 5'-AMP (peaks 1, 4, and 9) binding, and possibly also GTP and ATP binding. The absence of cAMP and cGMP binding was surprising, so these cofactors were assayed alone with RSP2, and it was found that both cAMP and cGMP bind to RSP2 (**Fig. 3**). The absence of cAMP and cGMP binding in the pool of six cofactors could be easily explained by the competitive displacement of cAMP and cGMP by a tighter binding cofactor in the pool. This was confirmed in an STD NMR experiment that included all three cyclic nucleotides (cCMP, cAMP, and cGMP), but showed only binding of cCMP (**Fig. 4**C). Thus, a competition between related cofactors can reveal which is preferred – in this case cCMP. To validate this strategy of determining cyclic nucleotide preferences, a well-characterized model protein was also analyzed. STD NMR of PKA (cyclic AMP-dependent protein kinase from bovine heart) in the presence of related cyclic nucleotides indicates binding of both cAMP and cGMP, suggesting a modest level of non-specificity. cAMP showed the stronger STD effect (**Fig. 4**A), especially for the H8 proton of the adenine group, but the ribose glycosidic protons on cGMP and cAMP show similar STD effects. This suggests that while both cAMP and cGMP bind, the adenine ring (H8) is more sequestered in the protein binding site than the guanine ring. As demonstrated here and elsewhere, STD approaches [7, 18, 19] can also provide structural information on binding mode. The second messenger cAMP is well known to activate PKA by binding to regulatory subunits. Although, little is known regarding cGMP binding to bovine PKA, PKA is activated by both cAMP and cGMP in *Orconectus limosis*, *Amblyomma americanum*, *Saccharomyces cerevisiae*, and *Pichia pastoris* [20-23]. We find that both cAMP and cGMP bind to bovine PKA, with a preference for cAMP. Perhaps more importantly, cAMP binds with its purine ring more sequestered in the protein than that on cGMP – which may be crucial for productive binding that leads to measurable activation. As expected, we see no cCMP binding.

cCMP binding to RSP2 is specific, since a control protein preparation from *E. coli* lacking the RSP2 expression construct showed no STD effect (not shown). Binding cCMP in preference to cAMP and cGMP is unexpected, since cCMP is not a widely used cofactor in biochemistry [9]. Furthermore, it had previously been shown that cAMP and cGMP were the cyclic nucleotides that affect ciliary and flagellar motility, presumably through kinases [24]. Interestingly, there has been a report of a regulatory role for cCMP in motility of sperm flagella [25], and several other studies suggest that cCMP may play a role in cell development [26-28].

To broaden the scope of the cofactor fingerprinting approach used herein, we explored the use of a basis set of heterocyclic rings that comprise the most commonly used cofactors in biochemistry (determined using the fragmentation algorithm in Pipeline Pilot [29], version 3.0.6). But, we found that the fragments (ex. adenine) do not bind well enough to give strong STD signals, so the preferred implementation of cofactor fingerprinting with STD NMR is with small pools of related and fully intact cofactors, as in **Figs. 1–4**.

3.3 Further NMR-based functional characterization of RSP2: slow ATPase activity

Since earlier studies had suggested that RSP2 has kinase activity [12], ^{31}P NMR studies were undertaken to determine if RSP2 is capable of catalyzing the slow hydrolysis of ATP. Such slow ATPase activity is typical of kinases, in the absence of the substrate that normally receives the phosphate. In cases where the phosphate acceptor for a suspected kinase is unknown, screening for ATPase activity is the best way to experimentally verify kinase activity for a purified protein. Conversion of ATP to ADP or GTP to GDP was monitored with ^{31}P NMR. Simultaneous monitoring of GTPase and ATPase activity indicated a clear preference for ATPase activity, with a decrease in signal for the ATPγ-phosphate occurring simultaneously with an increase in signal for the β-phosphate from ADP. The 1:1 ratio of resonances at −4.4 and −5.2 ppm at the last time-point reflects the complete conversion of ATP to ADP, with GTP still intact and serving as an internal control. That it was ATP and not GTP being hydrolyzed was confirmed with a similar study done in the absence of GTP (not shown). Similar changes are observed for the α-phosphates. The increase in α-phosphate signal was fitted to an exponential function:

$$[\mathrm{ADP}]_t = [\mathrm{ADP}]_{1-\infty}(1 - e^{-k't})$$

giving a pseudo-first order rate constant (*k*') for ATPase activity of 4.5×10^{-7} s^{-1}. This value is certainly a lower limit, since it is not clear if specific activity of RSP2 decreased during purification or storage, since there is currently no activity assay for it. The fact that hydrolysis is observed for ATP and not GTP, and also that no ATP hydrolysis is observed in the absence of RSP2 is evidence that RSP2, possesses ATPase activity, and is therefore a kinase.

In summary, cofactor fingerprinting with STD NMR has been shown to be an efficient way to establish cofactor preferences for proteins. It has been validated with both dehydrogenases and with cyclic nucleotide-binding proteins, and then applied in a functional proteomics project. With regard to the latter, it has been used to establish that *Chlamydomonas reinhardtii* RSP2 binds cCMP in preference to cAMP and cGMP, which is an unexpected cofactor preference for a GAF domain. The slow ATPase activity of RSP2 also suggests that it is a kinase.

Acknowledgments

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