The Negative Transcriptional Regulator NmrA Discriminates between Oxidized and Reduced Dinucleotides*

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NmrA, a transcription repressor involved in the regulation of nitrogen metabolism in Aspergillus nidulans, is a member of the short-chain dehydrogenase reductase superfamily. Isothermal titration calorimetry and differential scanning calorimetry have been used to show NmrA binds NAD+ and NADP+ with similar affinity (average $K_\text{D}$ 65 $\mu$M) but has a greatly reduced affinity for NADH and NADPH (average $K_\text{D}$ 6.0 mM). The structure of NmrA in a complex with NADP+ reveals how repositioning a His-37 side chain allows the different conformations of NAD+ and NADP+ to be accommodated. Modeling NADP+/H into NmrA indicated that steric clashes, attenuation of electrostatic interactions, and loss of aromatic ring stacking can explain the differing affinities of NAD(P)+/NAD(P)H. The ability of NmrA to discriminate between the oxidized and reduced forms of the dinucleotides may be linked to a possible role in redox sensing. Isothermal titration calorimetry demonstrated that NmrA and a C-terminal fragment of the GATA transcription factor AreA interacted with a 1:1 stoichiometry and an apparent $K_\text{D}$ of 0.26 $\mu$M. NmrA was unable to bind the nitrogen metabolite repression signaling molecules ammonium or glutamine.

Two major classes of mutant affecting nitrogen metabolite repression have been isolated. The first class exemplified by the nmr-1 and nmrA genes of N. crassa and A. nidulans, respectively, has a partially de-repressed phenotype (2, 3) implying they act as negative transcription regulators.

The structures of the native form of the nmrA-encoded NmrA protein as well as a complex with NAD+ have been reported (4, 5). Structural comparisons reveal that NmrA shows an unexpected similarity to the short-chain dehydrogenase reductase (SDR) superfamily (5), with the closest relationship to UDP-galactose 4-epimerase (root mean square deviation for 251 equivalent C-atoms of 2.7 Å). Whereas UDP-galactose 4-epimerase has the conserved Tyr-X-X-Lys SDR catalytic motif (6, 7), in NmrA this is changed to Met-X-X-Lys. The loss of a key catalytic tyrosine implies NmrA is unlikely to be an active dehydrogenase. The N-terminal domain of NmrA consists of a Rossmann fold, which, despite lacking the characteristic nucleotide-binding motif Gly-X-Gly-X-Gly, is able to bind NAD+ (5). Prior to the structural work on NmrA, NAD+ binding was an unreported property of the protein that could not be predicted from bioinformatics or genetic studies. However, in order to assess the possible physiological significance of such binding, it is necessary to characterize the relative strength of NAD+ as well as NADP+ binding to NmrA in both their oxidized and reduced forms.

The nit-2 and areA genes that encode the GATA-binding NIT2 and AreA proteins of N. crassa and A. nidulans, respectively, exemplify the second class of mutants. These proteins contain single zinc fingers and are required to stimulate transcription of genes controlled by nitrogen metabolite repression (8–12). Loss of function mutants are unable to use non-preferred nitrogen sources and are said to have a repressed phenotype in contrast to the wild-type repressible phenotype.

The molecular mechanism of the signal transduction pathway responsible for nitrogen metabolite repression is complex and in A. nidulans includes control of mRNA stability mediated through the 3'-untranslated region of the areA mRNA and AreA-dependent re-modeling of chromatin domains (13, 14). In vivo post-translational modulation of NIT2 activity has been implicated, as in vitro the nmr-1-encoded N-terminally deleted forms of NMR1 protein bind directly to the zinc finger region and the extreme C-terminal 30 amino acids of NIT2 (15–17). The interactions were demonstrated in a qualitative manner by using the yeast two-hybrid system, as well as steady state techniques that included electrophoretic mobility shift assay and column binding using His$_6$- and glutathione S-transferase-tagged fragments of the NIT2 protein as a bait. No kinetic data nor the strength of the interaction with the wild-type NMR1 have been reported. Here we investigate the ligand binding...
properties of NmrA and show that it binds to a C-terminal fragment of AreA, the dinucleotides NAD$^+$ and NADP$^+$, but not their reduced forms, and not to the nitrogen metabolite signal molecules ammonium and glutamine. These properties of NmrA suggest a possible link to a redox-sensitive signal transduction pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—Chemicals and solvents were purchased from local suppliers and were of AnalaR or greater purity. Enzyme substrates were purchased from Sigma, and molecular biology reagents (which were used in accordance with the manufacturers recommendations) were purchased from Invitrogen, Amersham Biosciences, BCL, or the University of Newcastle upon Tyne Facility for Molecular Biology.

**Molecular Biology and Biochemistry**—Routine molecular biology protocols followed individual manufacturer’s recommendations or were as described previously (18, 19). All DNA sequencing was carried out on double-stranded plasmid DNA using an ABI PRISM 377 DNA sequencer in the University of Newcastle upon Tyne Facility for Molecular Biology.

**Construction of a Reconstituted Plasmid for Expression in Escherichia coli**—The areA deletion mutant areA$^{<}$ was amplified by the PCR using 5′ sense oligonucleotides (containing an Ncol recognition site) of sequence CCAGTCCAGCCGGCCTAGGAAACGGAGAGC and a 3′ antisense oligonucleotide (containing a BamHI site) of sequence TTTTAGAATCTTCCAACTCA. The PCR used the following conditions: cycle 1–94°C for 2 min, 50°C for 2 min, 72°C for 4 min; cycles 2–30 at 94°C for 1 min, 50°C for 2 min, and 72°C for 4 min using “ Expand” high fidelity Taq polymerase (Roche Applied Science). After suitable digestion, the DNA sequence was subcloned into the E. coli expression vector pRSETB (Invitrogen) placing it under the control of the isopropyl-1-thio-$\beta$-galactopyranoside-inducible T7 promoter. This plasmid was designated pRF48.

**Overproduction and Purification of the AreA$^{<}$ Protein**—500-ml cultures of E. coli strain BL21DE3 pLysS containing plasmids pRF48 were grown to an attenuation (OD$$_{600}$) of 0.2 in rich medium at 37°C, grown to an OD$$_{600}$ of 1 and 35°C with 1 mM isopropyl-$\beta$-D-galactopyranoside added, and growth was continued for a further 4 h. After harvesting by centrifugation, the cell pellets were resuspended in 40 ml of 50 mM potassium phosphate, pH 7.2, 0.5 mM NaCl, 1 mM DTT, 1 mM benzamidine (buffer 1), and following sonication a cell-free extract was prepared by centrifugation at 2,500 × g for 30 min at 4°C. Proteins in the sonicate capable of zinc binding were adsorbed onto a 27.15-ml chelating Sepharose (Sigma) of 0.2 in rich medium at 37°C and were used at concentrations between 0.57 and 0.78 mg ml$^{-1}$.

**Isothermal Titration Calorimetry—**Isothermal titration calorimetry experiments at 25°C were performed using a high precision VP-ITC system (Microcal Inc.). For potential small effector molecule binding studies, NmrA in the calorimetric cell (1.4 ml) in the range 80–163 μM was titrated with either ammonium chloride, L-glutamine, NAD$^+$, NADH, NADP$^+$, or NADPH dissolved in the same buffer (50 mM potassium phosphate, 1 mM DTT, pH 7.2) at 1.5–4 mM initial concentrations in the injection syringe. Experiments designed to detect any heat of ionization associated with the buffer and NAD$^+$, NADH, NADP$^+$, or NADPH used 50 mM PIPES, HEPES, TES, or Tris buffers, 1.0 mM DTT, and were carried out at 25°C. To study the properties of protein interactions, AreA$^{<}$ in the calorimetric cell (1.4 ml) at concentrations in the range 37.5–50 μM was titrated with NmrA at a concentration of 454 μM in the injection syringe. The heat evolved following each 10-μl injection was obtained from the integral of the calorimetric signal. The heat due to the binding reaction was obtained as the difference between the heat of reaction and the corresponding heat of dilution. Analysis of data was performed using Microcal Origin Software. AreA$^{<}$ was encoded by a fragment of the areA gene subcloned into the E. coli expression vector pRSETB (Invitrogen) and has a heterologous N-terminal additional sequence that contains 6 histidine residues facilitating purification by immobilized metal affinity chromatography (see above). As a control we purified the protein encoded by the cloned prpF gene by using the same method as AreA$^{<}$, because it has the same N-terminal heterologous extension. When this protein was used in the ITC cell at a concentration of 35 μM, no heat exchange above baseline values was observed when NmrA was titrated into the protein solution. This control demonstrates that the heat exchange seen when NmrA is titrated into AreA$^{<}$ in the ITC cell was due to a specific interaction between NmrA and AreA$^{<}$.

**Differential Scanning Calorimetry—**Differential scanning calorimetry measurements on NmrA were made using Microcal VP-DSC or MCS instruments at a scan rate of 1°C per min and a protein concentration of 17.3 (VP-DSC) or 168 μM (MCS). Protein was dialyzed in 50 mM KPO$_4$, pH 6.6, 1 mM DTT, and the dialysis buffer was retained to displace ligands, salt, and enzyme substrates, and for base-line controls. DSC experiments were repeated with addition of 3 mM putative effector (NAD$^+$, NADH, NADP$^+$, and NADPH) under otherwise identical conditions. Deconvolution analysis was performed using the non-two-state model using the Microcal Origin software.

**Protein Crystalization and Structural Determination—**The NmrA-NADP$^+$ complex was crystallized in form A (trigonal, space group $P_3_1_2_1$, one molecule per asymmetric unit) using the conditions described previously (4, 5). X-ray data were collected at European Synchrotron Radiation Facility, Grenoble, France, on beamline ID29 using a wavelength of 0.99817 Å. The crystals were flash-frozen using 20% (v/v) glycerol as a cryoprotectant prior to data collection and maintained at 100 K. Data indexing/integration and merging were carried out using MOSFLM (39) and the data were processed using CCP4 (40) and SCALCEPK, respectively (21). Structure refinement used CNS (22), whereas model building was carried out using O (23) on an SGI Octane2 work station. The native NmrA coordinates (Protein Data Bank code 1KG1) were used for rigid body refinement, followed by simulated annealing and positional and individual B-factor refinement. Data collection and refinement statistics are shown in Table I. Figures of molecular models were created using VMD (24).

**Energy Minimization of NmrA Dinucleotide Complexes—**Minimization was carried out using the program NAMD2 (25) utilizing the CHARMM22 force field. NADH was initially placed in the same position as NAD$^+$ observed from the crystal structure with NmrA (5). The system was then subjected to conjugate gradient minimization in the presence of water (coordinates obtained from the crystal structure). The very similar structures of NmrA seen in different crystal forms indicated a relatively rigid conformation for the protein, and hence the NAD$^+$/NADH-binding site was fixed except for the following key residues: His$^57$, Lys$^{131}$, Gly$^{151}$, Tyr$^{153}$, Asn$^{156}$, and Tyr$^{276}$. As a control, NADH was also subject to the same protocol.

**RESULTS**

**NmrA Discriminates between Oxidized Dinucleotides and Their Reduced Forms—**Purified NmrA was used in microcalorimetry experiments to quantify any interaction with NAD$^+$, NADP$^+$, NADH, and NADPH in HEPES, phosphate, PIPES, TES, and Tris buffers. The results of an ITC analysis (see Tables I–III) showed that NmrA was able to bind exothermically with 1:1 stoichiometry and moderate affinity to oxidized

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1 The abbreviations used are: DTT, dithiothreitol; DSC, differential scanning calorimetry; ITC, isothermal titration calorimetry; SDR, short-chain dehydrogenase reductase; PIPES, 1,4-piperazinediethanesulfonic acid; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]aminoethanesulfonic acid.
Ligand Binding Properties of NmrA

TABLE I
Thermodynamic parameters for the binding of NmrA to AreA<sub>mac</sub> as determined by ITC at 25 °C

| [NmrA]<sup>a</sup> | [AreA]<sup>a</sup> | n | K<sub>D</sub> | ΔH<sub>obs</sub> | ΔS<sup>0</sup> | c<sup>c</sup> |
|-------------------|------------------|---|-----------|-------------|-------------|---------|
| μM               | μM               |   | μM        | kcal mol<sup>-1</sup> | cal K<sup>-1</sup> mol<sup>-1</sup> | |
| 454              | 50               | 0.97 (±0.003) | 0.28 (±0.02) | 16.2 (±0.08) | 84.5 | 173 |
| 454              | 50               | 0.99 (±0.002) | 0.25 (±0.02) | 15.5 (±0.07) | 82.3 | 198 |
| 454              | 37.5             | 0.98 (±0.001) | 0.26 (±0.01) | 15.9 (±0.03) | 83.4 | 141 |

<sup>a</sup> Protein concentration in injection syringe.

<sup>b</sup> Protein concentration in ITC cell.

<sup>c</sup> [NmrA] × K<sub>x</sub> × n (the stoichiometry parameter).

dinucleotides with an average K<sub>n</sub> in the region of 47 and 82 μM for NAD<sup>+</sup> and NADP<sup>+</sup>, respectively. No binding of the reduced forms could be detected.

The ITC results were confirmed by differential scanning calorimetry (DSC) experiments (using a phosphate buffer) showing the effects of oxidized and reduced dinucleotides on the thermal stability of NmrA in solution under similar conditions (see Fig. 1). In the absence of added ligand, NmrA undergoes an endothermic thermal unfolding transition with T<sub>m</sub> ≈ 48.5 (±0.5) °C and mean enthalpy, ΔH = 670 (±50) kJ mol<sup>-1</sup>. Although the unfolding transition is irreversible under these conditions, the ratio of calorimetric to van’t Hoff enthalpies, ΔH<sub>cal</sub>/ΔH<sub>th</sub> = 1.05 (±0.12) is consistent with cooperative unfolding of a monomeric protein unit.

Addition of 3 mM NAD<sup>+</sup> or NADP<sup>+</sup> raises the T<sub>m</sub> by about 4 °C, whereas the same concentration of reduced dinucleotides has only a marginal effect (ΔT<sub>m</sub> = 0.5–1 °C). For a ligand that binds to the native (folded) state of a protein, the increase in T<sub>m</sub> can be related to the dissociation constant (K<sub>n</sub>) and concentration of ligand, [L], as shown in Equation 1,

\[
\Delta T_m = \frac{RT_m\ln(1 + [L]/K_n)}{\Delta H_{unf}}
\]

in which ΔT<sub>m</sub> = T<sub>m</sub> − T<sub>m,0</sub> is the change in unfolding transition temperature; R is the gas constant, and ΔH<sub>unf</sub> is the unfolding enthalpy of the protein in the absence of ligand (26, 27). By using the data from DSC in the presence and absence of ligand with [L] = 3 mM, this expression gives estimates for K<sub>n</sub> = 15 (±0.08) and 0.37 (±0.07) mM for NAD<sup>+</sup> and NADP<sup>+</sup>, respectively, compared with much weaker values of 4.8 (±2.7) and 7.2 (±1.1) mM for NADH and NADPH under the same conditions.

This is consistent with the more direct calorimetric titration data and shows clear discrimination in binding affinities for the oxidized and reduced forms of these ligands. It must be borne in mind that the DSC data relate to ligand binding at the oxidized and reduced forms of these ligands. It must be borne in mind that the DSC data relate to ligand binding at the oxidized and reduced forms of these ligands.
Table II

Thermodynamic parameters for the binding of NmrA to NAD+ as determined by ITC at 25 °C

The binding of NmrA to NAD+ was measured in the buffers shown by ITC at the concentrations indicated. The ±S.D. error estimates are as determined by the fitting software for individual experiments. Shown are the values for [NmrA], the stoichiometry of binding; KD, the equilibrium dissociation constant; ΔH°obs, the observed enthalpy; and ΔS°, the standard entropy change for single site binding. The c values fall within the range 1–1000 that allows the isotherms to be accurately deconvoluted to derive K values (41).

| Buffer     | Phosphate | PIPES | HEPES | TES | Tris |
|------------|-----------|-------|-------|-----|------|
| KD (μM)    | 71 ± 3    | 68.9 ± 3 | 35 ± 1 | 31 ± 0.1 | 35 ± 0.8 | 44 ± 2.6 | 48 ± 3.28 | 54 ± 4.8 | 58 ± 5.3 |
| ΔH°obs (kcal mol⁻¹) | -7.01 ± 0.2 | -7.01 ± 0.14 | -14.3 ± 0.02 | -13.7 ± 0.09 | -13.9 ± 0.59 | -15.0 ± 0.12 | -18.0 ± 0.3 | -19.0 ± 0.55 | -19.5 ± 1 | -20.0 ± 1 |
| ΔS° (K⁻¹ mol⁻¹) | -4.7 | -4.6 | -27.4 | -27.3 | -29.27 | -30.67 | -41.03 | -44.09 | -45.75 | -48.78 |
| c          | 1.1       | 1.1    | 3.1   | 3.6   | 3.0   | 3.0   | 3.5   | 2.8   | 1.5   | 1.4   |

a NmrA concentration in the ITC cell.
b Nucleotide concentration in the injection syringe.
c c = [NmrA] × Ka × n (the stoichiometry parameter).

Table III

Thermodynamic parameters for the binding of NmrA to NADP+ as determined by ITC at 25 °C

The binding of NmrA NADP+ was measured in the buffers shown by ITC at the concentrations indicated. The ±S.D. estimates are as determined by the fitting software for individual experiments. Shown are the values for [NmrA], the stoichiometry of binding; KD, the equilibrium dissociation constant; ΔH°obs, the observed enthalpy; and ΔS°, the standard entropy change for single site binding. The c values fall within the range 1–1000 that allows the isotherms to be accurately deconvoluted to derive K values (41).

| Buffer     | Phosphate | PIPES | HEPES | TES | Tris |
|------------|-----------|-------|-------|-----|------|
| KD (μM)    | 100 ± 2   | 119 ± 1.5 | 60 ± 1.7 | 58 ± 2.2 | 103 ± 1.2 | 105 ± 1.3 | 107 ± 1.7 | 109 ± 0.3 | 34 ± 1.6 | 32 ± 2.1 |
| ΔH°obs (kcal mol⁻¹) | -12.0 ± 0.1 | -11.3 ± 0.1 | -28.7 ± 0.05 | -28.1 ± 0.05 | -24.3 ± 0.19 | -24.0 ± 0.12 | -22.6 ± 0.36 | -22.7 ± 0.49 | -12.0 ± 0.33 | -12.0 ± 0.34 |
| ΔS° (K⁻¹ mol⁻¹) | -22 | -20 | -76.97 | -74.79 | -63.23 | -62.51 | -57.63 | -58.3 | -19.86 | -19.67 |
| c          | 1.8       | 1.9    | 1.8   | 1.9   | 1.7   | 1.7   | 1.5   | 1.2   | 2.5   | 2.6   |

a NmrA concentration in the ITC cell.
b Nucleotide concentration in the injection syringe.
c c = [NmrA] × Ka × n (the stoichiometry parameter).
the additional hydrogen atoms present at the C4-position and the backbone oxygen atom in residue Gly-151 as well as the Tyr-276 side-chain hydroxyl group. The loss of aromaticity of the nicotinamide ring in NAD(P)H is also likely to weaken binding as ring stacking interactions with the side chain of Tyr-153 will be attenuated (28). There is also a distortion of the hydrogen bonding interactions of the amide group in the puckered conformation (Fig. 3D). The polar nature of these hydrogens may allow weak electrostatic interactions between NAD(P)+ and the backbone oxygen atoms of residues Gly-151 and Ala-150 in NmrA. Similar interactions of this nature are observed with other protein ligand complexes (30). However, in NADH the C4 hydrogen atoms can no longer partake in such an interaction, which results in a steric clash with the backbone oxygen of Gly-151, when in the planar conformation (Fig. 3D). By using the ligand docking program G.O.L.D (31), the experimentally determined binding position of the nicotinamide mononucleotide portion of NAD+ and NADP+ in NmrA could be accurately reproduced. G.O.L.D. also gave consistently lower fitness scores with NADH compared with NAD+ and docked NADH in a displaced position from NAD+.

In the case of nicotinamide adenine dinucleotide-binding enzymes in general, there is normally high selectivity for NAD+ relative to NADP+ and vice versa. Whereas this discrimination cannot be put down to a particular common set of residues, nevertheless “fingerprint” interactions for NAD+ (a side-chain carboxylate interacting with the adenosine ribose hydroxyls) and for NADP+ (an arginine facing the adenine plane and interacting with the pyrophosphate) have been identified (32, 33). Unusually dihydrodipicolinate reductase shows a relatively low discrimination between NADH and NADPH of 4-fold, and in this case the carbohydrate of Glu-38 interacts with the adenosine ribose hydroxyls of NADH but does not make this interaction in the NADPH complex (34). The dihydridopicolinate reductase NADPH complex has an interaction that is not present in the NADH complex involving Arg-39. In this case the arginine hydrogen bonds to the 2'-hydroxyl rather than the pyrophosphate group, which is characteristic of the normal fingerprint interaction for NADP+. NADP+-specific SDRs have a pair of conserved basic residues (Lys-17 and Arg-39 in carbonyl reductase) that have been suggested as
providing the recognition for this dinucleotide by interacting with the 2'-phosphate (35). Interestingly in NmrA, which does not discriminate between NAD⁺ and NADP⁺, there are no homologous residues to these positions in carbonyl reductase. Also, significantly, for NmrA both fingerprint interactions for NAD⁺ and NADP⁺ are absent. The observed rearrangement of His-37 allows NmrA to bind either NAD⁺ or NADP⁺ by accommodating the differing conformations of the adenine ring moieties of these two dinucleotides.

*NmrA Binds to a C-terminal Fragment of AreA—Previous
work with NMR1 showed that in vitro it interacted with the C-terminal region of NIT2 (17). In order to characterize the kinetics of any interaction between NmrA and the C-terminal region of AreA, we deleted the N-terminal 662 amino acids of AreA to produce a small C-terminal fragment (designated AreA662) for this analysis (see “Experimental Procedures”). This C-terminal fragment of AreA contains the zinc finger region and was able to bind to the niaD promoter (36) in vitro in electrophoretic mobility shift assay experiments (data not shown).

NmrA and AreA662 were purified in bulk and used in ITC experiments to determine whether they interacted with one another. We carried out experiments in which NmrA in the injector syringe was titrated into AreA662 in the reaction cell. The results of these experiments are shown in Tables I–III, where it can be seen that NmrA and AreA662 interacted endothermically with a 1:1 stoichiometry and an average $K_D$ of 0.26 μM. Fig. 4 shows typical results of the titration of NmrA into AreA662.

In order to determine whether nucleotide binding was affected by the interaction of AreA with NmrA, we carried out experiments in which NAD$^+$, NADH, or NADP$^+$ in the injector syringe was titrated into an approximately equimolar mixture of NmrA and AreA662. The resulting thermograms were not significantly different from those observed in the absence of AreA, indicating that formation of the NmrA-AreA complex does not inhibit (oxidized) nucleotide binding nor does it affect the discrimination between oxidized and reduced forms. This is consistent with the structural studies that show that the nucleotide-binding site in NmrA is distant from the putative AreA interaction region.

NmrA Is Unable to Bind the Nitrogen Metabolite Repression Signal Molecules Ammonium or Glutamine—The fact that NmrA can bind NAD$^+$, NADP$^+$, and AreA662 implies that the protein is in a native state able to bind physiological ligands after our purification protocol. We used ITC to probe the ability of NmrA to bind the nitrogen metabolite repression signal molecule glutamine in a binary complex and in a ternary complex with AreA662. We were unable to detect any significant heat of binding when glutamine was added to a solution of AreA662 (40 μM) or NmrA. Also when the ITC cell contained NmrA (44.6 μM) previously titrated with AreA662 (312 μM), no heat of binding was observed when glutamine was titrated into the NmrA-AreA complex. We also used circular dichroism and fluorescence emission spectroscopy as independent techniques to probe the ability of NmrA to bind ammonium or glutamine in a binary complex; however, no conformational changes and no binding could be detected (data not shown).

**DISCUSSION**

We have quantified for the first time the ligand binding properties of NmrA, a protein that plays a major role in the signal transduction pathway controlling nitrogen metabolite repression in microbial eukaryotes. Calorimetry reveals that NmrA does not discriminate between NAD$^+$ and NADP$^+$ but can effectively discriminate between the oxidized and reduced forms. Structure determination of the complex of NmrA with NAD$^+$ and comparison with the previous NAD$^+$ complex reveals that the disposition of His-37 allows the significantly different conformations of the adenosine moiety of the two dinucleotides to be accommodated within the same binding site. Additionally, it may be noted that some structural features that have been ascribed to the NAD$^+$/NADP$^+$ discrimination in SDRs have been lost in NmrA (35). Because a range of mutations are needed to change NAD$^+/$/NADP$^+$ selectivity (32), it would seem that the ancestral ability of the SDR fold in NmrA to discriminate between NAD$^+$ and NADP$^+$ is unlikely to have been lost as the result of a single mutation that has become fixed through a stochastic event. The unusual ability of NmrA to bind NAD$^+$ and NADP$^+$ with equal affinity is more likely to be the result of evolutionary selection and implies that it may be of physiological importance. Similarly the ability of NmrA to discriminate between NAD(P)$^+$ and NADPH may have a biological function. The molecular basis for this discrimination between oxidized and reduced dinucleotides has been investigated by studies that show the reduced nicotinamide ring produces steric clashes and loss of aromatic ring stacking and electrostatic interactions with NmrA. Calorimetry also demonstrates that NmrA is able to bind to AreA662, a C-terminal fragment of AreA, a protein that plays an essential role in the positive control of transcription, with a 1:1 stoichiometry and an apparent $K_D$ of 0.26 μM.

The signal transduction pathway involving NmrA has been implicated in monitoring the intracellular levels of the nitrogen metabolite repression signal molecule glutamine (37). However, CD spectroscopy, fluorescence, and calorimetry provide no evidence that NmrA can recognize ammonium or glutamine in either a binary complex or a ternary complex with AreA662. The inability of NmrA to bind ammonium or glutamine in a binary complex or a ternary complex with AreA662 may mean that NmrA is not involved in the glutamine sensing signal transduction pathway that controls wild-type nitrogen metabolite repression. Glutamine-binding sites may exist on other...
regions of AreA or only in a higher order complex when additional components are present.

We note that two radically different models for the ability of NmrA to modulate the activity of AreA can be envisaged. NmrA may interact directly with AreA while it is complexed with DNA and disrupt its interaction with pathway-specific transcription-regulating proteins or the accessory transcription apparatus. On the other hand, NmrA may exert its effect by controlling the access of AreA to its target promoters by either a direct or indirect route. The direct route could involve occlusion of the zinc finger region (17), or it could act indirectly by controlling the rate of entry of AreA into the nucleus. The moderate affinity for the oxidized dinucleotides (average value 65 µM) means that in order to have a substantial proportion of the population in a binary complex with NAD+/NADP+. NmrA must be in an environment with oxidized nucleotides in the high micromolar range. The free intracellular and nuclear concentrations of oxidized and reduced nucleotides in *A. nidulans* are not known, and their determination is beyond the scope of this current study. However, overall intracellular concentrations of 1 mM for NAD+ and NADH have been reported in the microbial eukaryote *Saccharomyces cerevisiae* (38), and there is no reason to assume that *A. nidulans* will differ dramatically. If this situation is reflected in *A. nidulans*, then a dinucleotide signal recognition role for NmrA may be restricted to the cytosol where NmrA is most likely to encounter the highest concentrations of NAD+/NADP+. If NmrA functions to control the access of AreA to the nucleus, the binding of oxidized dinucleotides could modulate this ability.

The ligand binding properties of NmrA show some interesting parallels to those of the CtBP class of transcriptional co-repressor. The latter proteins are related to a metabolic apparatus. On the other hand, NmrA may exert its affect by controlling the rate of entry of AreA into the nucleus. The ligand binding properties of NmrA show some interest-

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