Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Expression, purification and crystallization of *Aspergillus nidulans* NmrA, a negative regulatory protein involved in nitrogen-metabolite repression

C. E. Nichols,[a,b] S. Cocklin,[c] A. Dodds,[c] J. Ren,[a] H. Lamb,[c] A. R. Hawkins[c] and D. K. Stammers[a,b]*

[a]Structural Biology Division, The Wellcome Trust Centre for Human Genetics, University of Oxford, Roosevelt Drive, Oxford OX3 7BN, England, [b]Oxford Centre for Molecular Sciences, New Chemistry Building, South Parks Road, Oxford OX1 3QT, England, and [c]School of Biochemistry and Genetics, Medical School, Catherine Cookson Building, Framlington Place, University of Newcastle-upon-Tyne, Newcastle-upon-Tyne NE2 4HH, England

Correspondence e-mail: daves@strubi.ox.ac.uk

Received 13 June 2001
Accepted 28 August 2001

The NmrA repressor protein of *Aspergillus nidulans* was overproduced in *Escherichia coli* and purified to homogeneity. Gel-exclusion chromatography showed that NmrA was monomeric in solution under the buffer conditions used. The protein was crystallized in three forms, belonging to trigonal, monoclinic and hexagonal space groups. Two of these crystal forms (*A* and *B*) diffract to high resolution and thus appear suitable for structure determination. Crystal form *A* belongs to space group $P3_{(1)}21$, with unit-cell parameters $a = b = 76.8$, $c = 104.9$ Å. Crystal form *B* belongs to space group *C*2, with unit-cell parameters $a = 148.8$, $b = 64.3$, $c = 110.2$ Å, $\beta = 121.8^\circ$.

1. Introduction

Neurospora crassa, *A. nidulans* and other ascomycetous fungi are able to utilize a wide array of nitrogen sources and many of the pathways involved are regulated at the level of transcription by pathway-specific control proteins. When the preferred nitrogen sources ammonium or glutamine are present in the growth medium with an alternative nitrogen source, the pathway for the non-preferred source remains inactive. This situation is known as nitrogen-metabolite repression and the alternate nitrogen-utilization pathway is said to be repressed (Grove & Marzluf, 1981; Wilson & Arst, 1998). This nitrogen-metabolite repression is the result of various factors including the interaction of specific proteins that provide positive and negative control elements for modulating transcription.

N. crassa and *A. nidulans* encode DNA-binding proteins that recognize GATA-containing sequences (NIT2 and AreA, respectively). These proteins contain single zinc fingers that control the transcription of genes responsive to nitrogen-metabolite repression. The isolation of mutants that had a partially de-repressed phenotype in the presence of the nitrogen-metabolite signalling molecule glutamine (de Busk & Ogilvie, 1984; Dunn-Coleman *et al.*, 1981) identified the *nmr-1* gene in *N. crassa*. A homologous gene in *A. nidulans* was identified from database searching with *nmr-1* and is called *nmrA* (Andrianopoulos *et al.*, 1998). The gene product of *nmr-1*, called NMR1, has been shown to bind (as a chimeric NMR1-GST fusion protein) directly to the zinc-finger region and the last 30 C-terminal amino acids of NIT2 (de Busk & Ogilvie, 1984; Xiao *et al.*, 1995; Young & Marzluf, 1991). It is thought that the homologue NmrA functions in a similar manner. The three-dimensional structure of the AreA zinc-finger region has been determined by NMR methods (Starich *et al.*, 1998); as yet, however, there is no three-dimensional structure available for NMR1 or NmrA. Crystal structures of NmrA or homologues could provide information on the structural basis of transcriptional regulation in this system.

2. Materials and methods

2.1. Molecular biology and biochemistry

Growth of *A. nidulans* and routine molecular-biology protocols followed individual manufacturer's recommendations or were as previously described (Lamb *et al.*, 1996; Maniatis *et al.*, 1982). Purification of *A. nidulans* total RNA was as previously described (Cathala *et al.*, 1983) and polyA$^+$ mRNA was prepared from total RNA using Dynabeads Oligo (dT) (Dynal UK) following the manufacturer's protocol. cDNA was produced from polyA$^+$ mRNA using Superscript reverse transcriptase (Gibco BRL) using the manufacturer's recommended conditions. DNA sequencing was carried out on double-stranded plasmid DNA using an ABI PRISM 377 DNA sequencer and amino-acid sequencing used a Beckman LF 3000 sequencer, both in the University of Newcastle-upon-Tyne Facility for Molecular Biology.

2.2. Construction of recombinant plasmids for expression in *E. coli*

Routine molecular-biology protocols followed individual manufacturer's recommendations or were as previously described

(Maniatis *et al.*, 1982). The coding sequence of the *nmrA* gene was amplified by the PCR from chromosomal and cDNA using the 5′ sense oligonucleotide ATTCAGCCGTCCCCATGGCCCAGCAAAAG and the 3′ antisense oligonucleotide AAAATTTTCTTAGGATCCGTCTGTCACAGC according to the following protocol: cycle 1, 367 K 2 min, 323 K 2 min, 345 K 4 min; cycles 2–30, 367 K 1 min, 323 K 2 min, 345 K 4 min using 'Expand' high-fidelity Taq polymerase (Boehringer). Following digestion with *Nco*I and *Bam*H1 the PCR fragments were subcloned into the *E. coli* expression plasmid pTr*c*99*a* (Pharmacia) to produce pTR121 (cDNA copy) and pTR134 (chromosomal copy).

2.3. Purification of the NmrA protein

E. coli strain BL21 DE3 containing plasmid pTR121 was grown to an attenuance (D_{500}) of 0.2 in rich medium containing 0.1 mg ml^{-1} ampicillin and the pTrc promoter induced to full strength by incubation in the presence of 0.2 mg ml^{-1} IPTG for a further 5 h. After recovery by centrifugation, the cell paste (approximately 23 g) was disrupted by sonication in a buffer containing 50 m*M* potassium phosphate buffer pH 7.2 and 1 m*M* DTT; 1 m*M* benzamidine was also added as a protease inhibitor (buffer 1). Following centrifugation at 2500*g* for 30 min at 277 K, the clarified supernatant was applied to a DEAE Sephacel column of dimensions 13 × 4.5 cm equilibrated with buffer 1. The column was washed with 500 ml of buffer 1 and then eluted with a linear 1 l 0.0–1.0 *M* NaCl gradient in buffer 1. Individual fractions were analysed by SDS–PAGE and NmrA-containing fractions were pooled and brought to 25%(*w*/*v*) with ammonium sulfate. Following centrifugation at 2500*g* for 30 min at 277 K, the clarified supernatant was brought to 50%(*w*/*v*) ammonium sulfate and the centrifugation was repeated. The pellet was redissolved in buffer 1 containing 1.0 *M* ammonium sulfate (buffer 2) and applied to a phenyl Sepharose column of dimensions 13 × 4.5 cm that had been equilibrated with buffer 2. The column was eluted with a 1 l reverse linear 1.0–0.0 *M* ammonium sulfate gradient using buffers 1 and 2. Individual fractions from the elution profile were analysed by SDS–PAGE and NmrA-containing fractions were pooled and dialysed against 2 × 5 l changes of buffer 1. The dialysed protein was applied to a hydroxyapatite column of dimensions 6.5 × 4.5 cm and eluted with the column flow-through during a 400 ml wash with buffer 2. The pooled NmrA protein was then pressure concentrated using argon and a ChemLab concentrator in conjunction with a YM10 DIAFLO ultrafiltration membrane. The concentrated NmrA protein was then applied in batches of approximately 20 mg to a 4 ml AQ[4] weak anion FPLC column equilibrated in buffer 1. Following a 16 ml wash with buffer 1, the column was eluted with an 80 ml 0.0–0.5 *M* NaCl gradient in buffer 1. Individual fractions were screened by SDS–PAGE and those containing pure NmrA protein were pooled. Using this protocol, approximately 80 mg of NmrA was isolated at a purity of greater than 99%. 100 pmol of NmrA was subject to N-terminal amino-acid sequencing and the following sequence (which matches that predicted) was obtained: Met-Ala-Gln-Gln-Lys-Lys-Thr.

2.4. Molecular-exclusion chromatography

Approximately 1 mg of NmrA was chromatographed on a 122 × 1.5 cm Sephacryl S300 column in a buffer consisting of 50 m*M* potassium phosphate pH 7.2, 1 m*M* DTT, 100 m*M* NaCl, collecting 40-drop (approximately 4.6 ml) fractions. The void volume of the column was estimated using blue dextran and calibrated using the markers bovine serum albumin (M_r = 66 000), carbonic anhydrase (M_r = 29 000) and cytochrome *c* (M_r = 12 400).

2.5. Crystallization of NmrA

NmrA was concentrated and buffer exchanged into 10 m*M* Tris pH 7.4, 25 m*M* KCl using Centricon-10 centrifugal concentrators (Millipore). Initial surveys of crystallization conditions used standard screening kits purchased from Hampton Research Corporation, *i.e.* Crystal Screen I, Crystal Screen II, ammonium sulfate and PEG 6000 GRID Screens. NmrA at 18 mg ml^{-1} was used to set up droplets of 3 µl of protein mixed with 3 µl of reservoir solution which were placed on microbridges (Harlos, 1992) for sitting-drop vapour-diffusion experiments. All crystallization trials were incubated at 277 K. Crystallization conditions identified from screens were optimized by employing finer intervals of pH and precipitant concentration.

2.6. X-ray diffraction studies

Crystals were initially characterized using in-house Rigaku X-ray generators equipped with Cu anodes and either a graphite monochromator or mirrors to give *Kα* radiation (λ = 1.5418 Å). MAR image plates were used as detectors. Crystals were either examined at room temperature in sealed quartz tubes or at 100 K with an Oxford Cryosystems Cryostream. Prior to freezing, crystals were transferred for a few seconds into a reservoir solution to which 20%(*v*/*v*) glycerol had been added as cryoprotectant.

Indexing and integration of data images were carried out with *DENZO* and the data were merged using *SCALEPACK* (Otwinowski & Minor, 1996). The number of NmrA molecules in the asymmetric unit was estimated from considering the normal

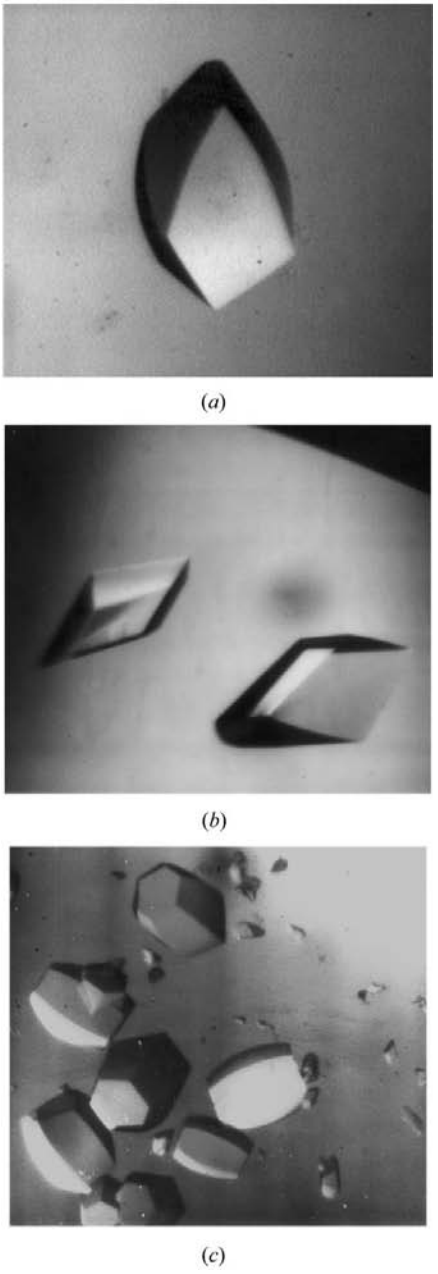

Figure 1
Photomicrographs of NmrA crystals: (*a*) form *A*, (*b*) form *B*, (*c*) form *C*. Magnification ×65, ×57 and ×43, respectively.

Table 1
Growth conditions, space group and unit-cell parameters for NmrA crystals.

Crystal form	*A*	*B*	*C*
Crystallization conditions			
Precipitant	3.6 *M* KCl	3.6 *M* NaCl	2.6 *M* Na formate
Buffer	0.1 *M* MES pH 6.5	0.1 *M* Tris pH 8.0	0.1 *M* Tris pH 8.3
Salt	0.1 *M* NaH_2PO_4, 0.1 *M* KH_2PO_4	—	—
Space group	$P3_{(1)}21$	*C*2	$P6_{(1)}22$
V_M† (Å^3 Da^{-1})	2.30	2.89	2.29
Asymmetric unit‡	1	2	2
Solvent content (%)	44	56	44
Unit-cell parameters§			
a (Å)	76.8	148.8	82.3
b (Å)	76.8	64.3	82.3
c (Å)	104.9	110.2	362.9
β (°)	90.0	121.8	90.0

† Matthews coefficient (Matthews, 1968). ‡ Estimated number of molecules. § Measured at 100 K for forms *A* and *B* and at 292 K for form *C*.

Table 2
Data-collection statistics for form *A* and *B* NmrA crystals.

Values in parentheses are for outer-shell data.

NmrA crystal	Form *A*	Form *B*
Detector	MAR 345	MAR 300
Resolution range (Å)	40.0–2.3 (2.38–2.30)	40.0–2.6 (2.69–2.60)
No. of unique reflections	16329 (1600)	28362 (2817)
Fold redundancy	16.6 (15.3)	4.4 (4.1)
Data completeness (%)	99.5 (98.9)	99.8 (99.7)
Average $I/\sigma(I)$	47.9 (19.4)	18.3 (6.4)
Data with $I > 3\sigma$ (%)	96.5 (92.6)	89.2 (66.5)
R_{merge}†	0.063 (0.14)	0.064 (0.15)

† $R_{merge} = \sum |I - \langle I \rangle| / \sum \langle I \rangle$.

range of packing densities for protein crystals (Matthews, 1968).

3. Results and discussion

3.1. Overproduction, purification and physical characterization of the NmrA protein

We isolated cDNA (from strain R153) and chromosomal (from strain G191) copies of the *nmrA* gene by the PCR using oligonucleotide primers designed from the published sequence (Andrianopoulos *et al.*, 1998). Both copies of the *nmrA* gene were subcloned into the *E. coli* expression vector pTr*c*99a to produce plasmids pTR121 (cDNA copy) and pTR134 (chromosomal copy). The *nmrA* genes in each plasmid were sequenced and found to contain the same single nucleotide difference ($T^{1578} \rightarrow G$) from the published sequence (EMBL accession number AF041976). This sequence change alters Leu238 to Arg, which is the same amino acid in the corresponding position in the *N. crassa* NMR1 and *Giberella fujikuroi* (accession number Y15902) NMR proteins.

Plasmid pTR121 directed soluble overproduction of NmrA protein in *E. coli*. The NmrA protein was purified in bulk using a combination of ammonium sulfate precipitation, anion-exchange, hydrophobic, hydroxyapatite and anion-exchange FPLC chromatography according to the protocol described in §2. SDS–PAGE analysis demonstrates that the final product was in excess of 99% pure. Molecular-exclusion chromatography estimated the native molecular weight of NmrA as 39.7 (±6.6) kDa averaged over two separate purifications. As the predicted M_r of NmrA is 38 800, the data imply that NmrA is monomeric in solution under the conditions of the chromatography.

3.2. Crystallization of NmrA

NmrA crystallized readily using certain salts as precipitants. Initially, crystals were observed from 4 *M* sodium formate, although crystals could also be grown from NaCl or KCl. Three different crystal forms were obtained (Fig. 1). A particular set of crystallization conditions can produce all three crystal forms, although in some cases one form appeared favoured. We have generally used KCl as the precipitant to grow form *A* trigonal pyramidal crystals, whilst NaCl was used to grow form *B* crystals in the form of monoclinic plates. Hexagonal barrel-like form *C* crystals were grown from sodium formate. Crystals of forms *A* and *B* could grow up to 0.5 mm in their longest dimension, whilst form *C* were up to 0.6 mm in length. Space group and unit-cell parameters for the three different forms are shown in Table 1. Crystal forms *A* and *B* diffracted strongly (Figs. 2*a* and 2*b*). Statistics for data collected from an in-house source are shown in Table 2. From considerations of packing densities (Matthews, 1968), form *A*, *B* and *C* crystals most probably have one, two and two molecules in the asymmetric unit, respectively. Form *C* crystals diffracted to lower resolution than did forms *A* and *B* and this, together with the difficulties in resolving the *c* axis of >360 Å using an in-house data-collection system (Fig. 2*c*), meant this form was less suitable for high-resolution structural analysis and thus was not studied further.

Searching databases for amino-acid sequences related to NmrA revealed no similarities with proteins of known three-dimensional structure and therefore we are

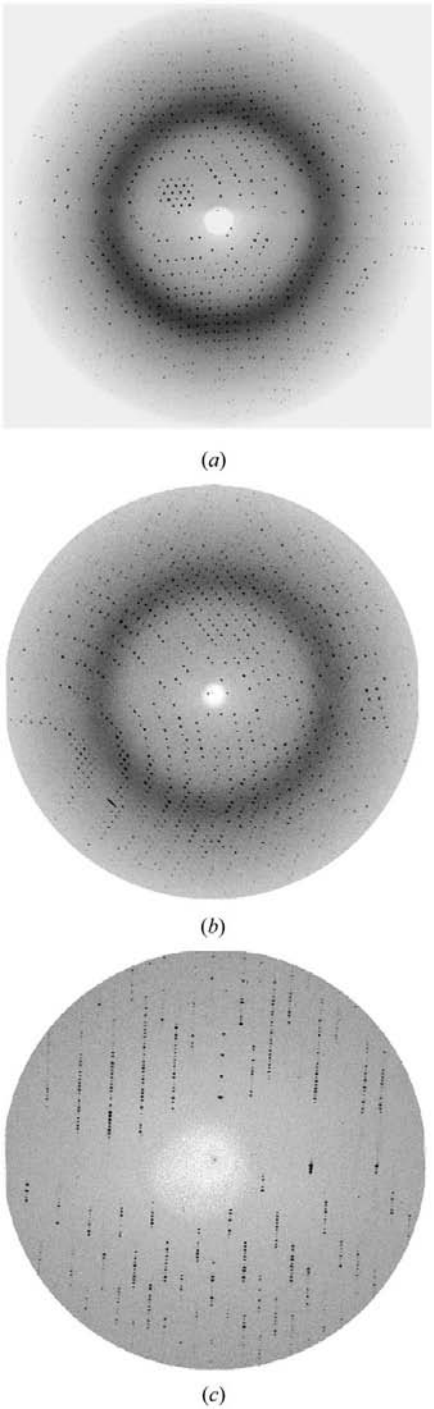

Figure 2
X-ray diffraction patterns of NmrA crystals. All images were recorded from unfrozen NmrA crystals using in-house X-ray sources and MAR image plates. The resolution at the edge of the plate is shown in brackets: (*a*) form *A* (2.3 Å), (*b*) form *B* (2.55 Å), (*c*) form *C* (6.2 Å). For (*c*) the systematic absences along the sixfold screw axis can be seen in the top half of the diffraction image.

using isomorphous replacement methods to solve the three-dimensional structure of this protein. A high-resolution crystal structure of NmrA should provide new insights into the molecular mechanism of transcriptional regulation for this system.

This research was funded by grants from the BBSRC and the Wellcome Trust. DKS is supported by the MRC.

References

Andrianopoulos, A., Kourambas, S., Sharp, J. A., Davis, M. A. & Hynes, M. J. (1998). *J. Bacteriol.* **180**, 1973–1977.

Busk, R. M. de & Ogilvie, S. (1984). *J. Bacteriol.* **160**, 656–661.

Cathala, G., Savouret, J. F., Mendez, B., West, B. L., Karin, B., Martial, J. A. & Baxter, J. D. (1983). *DNA*, **2**, 329–335.

Dunn-Coleman, N. S., Tomsett, A. B. & Garrett, R. H. (1981). *Mol. Gen. Genet.* **182**, 234–239.

Grove, G. & Marzluf, G. A. (1981). *J. Biol. Chem.* **256**, 463–470.

Harlos, K. (1992). *J. Appl. Cryst.* **25**, 536–538.

Lamb, H. K., Newton, G. H., Levett, L. J., Cairns, E., Roberts, C. F. & Hawkins, A. R. (1996). *Microbiology*, **142**, 1477–1490.

Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

Otwinowski, Z. & Minor, W. (1996). *Methods Enzymol.* **276**, 307–326.

Starich, M. R., Wikstrom, M., Arst, H. N., Clore, M. & Gronenborn, A. M. (1998). *J. Mol. Biol.* **277**, 605–620.

Wilson, R. A. & Arst, H. N. (1998). *Microbiol Mol. Biol. Rev.* **62**, 586–596.

Xiao, X., Fu, Y. H. & Marzluf, G. A. (1995). *Biochemistry*, **34**, 8861–8868.

Young, J. L. & Marzluf, G. A. (1991). *Biochem. Genet.* **29**, 447–459.