Biomol NMR Assign (2007) 1:81–83
DOI 10.1007/s12104-007-9022-3

ARTICLE

NMR assignment of the apo-form of a *Desulfovibrio gigas* protein containing a novel Mo–Cu cluster

Sofia R. Pauleta · Américo G. Duarte · Marta S. Carepo · Alice S. Pereira · Pedro Tavares · Isabel Moura · José J. G. Moura

Received: 5 April 2007 / Accepted: 6 May 2007 / Published online: 7 July 2007

Abstract We report the 98% assignment of the apo-form of an orange protein, containing a novel Mo–Cu cluster isolated from *Desulfovibrio gigas*. This protein presents a region where backbone amide protons exchange fast with bulk solvent becoming undetectable. These residues were assigned using ^{13}C-detection experiments.

Keywords NMR assignment · Carbon detection · Novel Mo–Cu cluster · *Desulfovibrio*

Biological context

An orange-coloured protein (ORP), 117 residues long, was isolated from the sulphate reducing bacterium *Desulfovibrio gigas* grown in a lactate-sulphate medium under anaerobic conditions (Bursakov et al. 2004). Sequence homology search through sequenced or partially sequenced genomes shows that this gene is present in organisms that are synthrophic with methanogenic archaea, such as other species of *Desulfovibrio* (*D.*), as *D. vulgaris* Hildenborough and *D. desulfuricans* G20. However, the sequence identity between *D. gigas* ORP and the corresponding ORF in these organisms is 48% and 42%, respectively and no structural information is available for any of them. Moreover, a recent study using *D. vulgaris* Hildenborough and *Methanosarcina barkeri* has showed that the gene that codes for this protein is one of three putative encoding-genes whose expression is increased when the growth conditions are switched from syntrophic to sulphate reducing (Scholten et al. 2007), making the study of this protein more imperative in order to understand its physiological role.

To our knowledge, the *D. gigas* ORP was the first of this family of proteins to be isolated in the holo-form. The metal cluster is composed of two molybdenum and one copper ions arranged in a novel mixed-metal sulphide cluster of the type $[S_2MoS_2CuS_2MoS_2]^{3-}$, which is non-covalently bound to the polypeptide chain (Bursakov et al. 2004, George et al. 2000). However, the ORP expressed heterologously in *Escherichia. coli* does not contain the metal cluster, but attempts are currently being made in order to reconstitute the protein with a metal cluster.

Solution structural studies have been performed in two other proteins of this family, the homologue from *Thermotoga maritima* (TM1290) and the one from *Methanobacterium thermoautotrophicum* (MTH1175) (Cort et al. 2000, Etezady-Esfarjani et al. 2004). Both proteins were obtained through the structural genomic approach: the ORFs with no known function were chosen from the complete genome of these bacteria, heterologously expressed and their structure determined in order to obtain functional insights. However, besides presenting low sequence identity with *D. gigas* ORP, 35% (TM1290) and 32% (MTH1175), no function has been identified so far for these proteins. Attending to the fact that these homology values are in the limit to apply homology modelling, we started our studies by the resonance assignment in order to obtain preliminary structural information about the apo-ORP from *D. gigas*. This is the starting point to further pursue the studies using the reconstituted holo-protein, in order to determine the binding site of the metal cluster and to obtain further structure-function insights on this new

S. R. Pauleta (✉) · A. G. Duarte · M. S. Carepo · A. S. Pereira · P. Tavares · I. Moura · J. J. G. Moura
REQUIMTE/CQFB, Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Caparica 2829-516, Portugal
e-mail: srp@dq.fct.unl.pt

family of metalloproteins. Therefore, we report here an extensive NMR assignment of the ORP apo-form.

Methods and experiments

The PCR isolated ORP gene was cloned into the *Nhe* I/*Hind* III restricted pET 21-d expression vector (Novagen) and the apo-ORP was heterologously expressed in the *E. coli* strain, BL21(DE3) (Invitrogen). Expression of uniformly ^{15}N or $^{13}C/^{15}N$-labelled apo-ORP was carried out by growing the cells in M9 minimum medium containing either 1 g/l $^{15}NH_4Cl$ or 1 g/L $^{15}NH_4Cl$ and 4 g/l [$^{13}C_6$]-glucose, respectively, as the sole nitrogen and carbon sources. The cells were grown at 37°C, at 250 rpm, to an OD_{600} of 0.4–0.6, the protein expression was induced with 0.5 mM IPTG for 18 h–20 h. The cells were harvested by centrifugation and the pellet was resuspended in 10 mM Tris-HCl at pH 7.6, containing protease inhibitors (Complete, Roche). The cell-free soluble extract was obtained by breaking the cells with a French-Press, the cell debris and membrane fraction were removed by low-speed centrifugation followed by an ultracentrifugation at 138000 g.

The purification of the apo-ORP was carried out in three-steps. The soluble cellular extract was first concentrated in a Diaflo apparatus and loaded into a Superdex 75 column (Amersham, ∅ 26 × 700 mm), equilibrated with 300 mM Tris-HCl, pH 7.6. The ORP containing fractions were pooled, concentrated and desalted using a dialysis membrane. The second purification step consisted of anionic exchange chromatography, with a Resource Q, equilibrated with 10 mM Tris-HCl, pH 7.6, to which the protein did not bind and therefore separating ORP from negatively charged proteins. As a final step, a high-resolution size-exclusion chromatography in a Superdex 75 10/30 GL (Amersham) was performed, using 20 mM sodium phosphate buffer, pH 7.2, 100 mM NaCl for the protein elution. The fractions were analysed by SDS-PAGE electrophoresis (12.5% acrylamide) and the ones containing the pure protein were combined, concentrated and stored at –80°C. Protein concentration was determined using the BCA Protein Assay (Sigma) using BSA (Bovine Serum Albumin) as the standard protein. The yield of the heterologous expression was 30 mg/l of minimum medium.

For the NMR experiments, three protein samples were prepared, the unlabelled, the uniformly ^{15}N-labelled, and the $^{13}C/^{15}N$-labelled apo-ORP following the procedure just described. Samples were 1 mM in protein concentration in 20 mM sodium phosphate, pH 7.2, 100 mM NaCl, 1 mM sodium azide, 5 μM benzamidine and 10% ^2H_2O.

NMR experiments were carried out at 298 K on Bruker Avance600, Bruker Avance700, Bruker Avance500 and Bruker Avance800 spectrometers using TXI-HCN-xyz gradient probes, which for the last two spectrometers are also cryoprobes. The carbon detection spectra were acquired on a Bruker Avance700 spectrometer, equipped with a triple-resonance cryoprobe optimised for ^{13}C direct-detection (TCI) experiments (Fig. 1A). The sequence assignments of the protein backbone resonances were obtained using 2D [^{15}N, ^1H]–HSQC, 3D HNHA, 3D HNCO, 3D HN(CA)CO, 3D CBCACONH, 3D CBCANH spectra (Ferentz et al. 2000). For the side chain assignment 2D [^{13}C, ^1H]–HSQC and 3D (H) CCH-TOCSY experiments

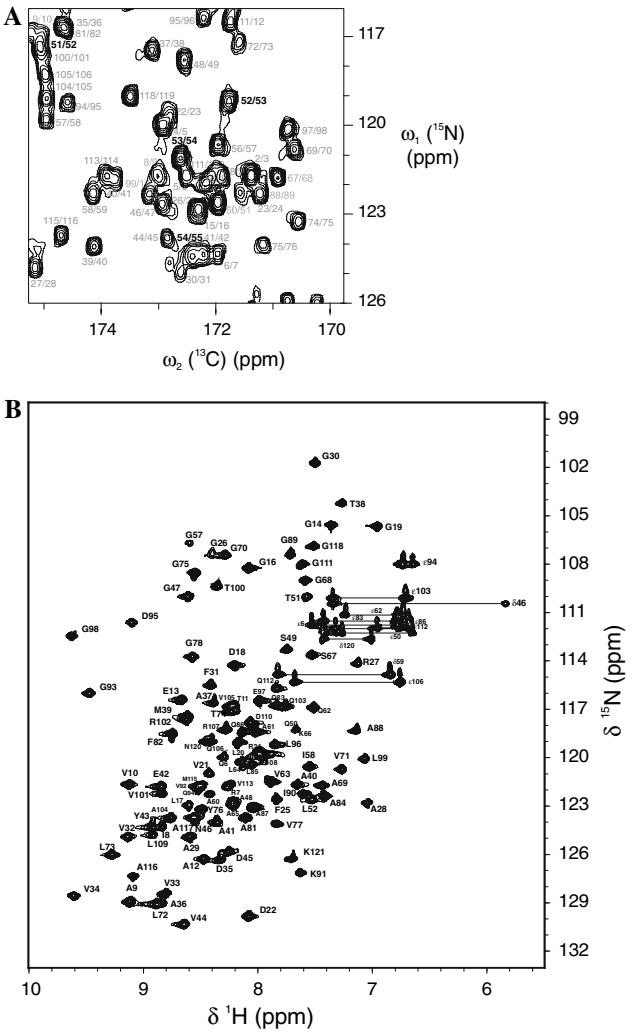

Fig. 1 (**A**) 2D CON spectrum of 1 mM apo-ORP. The spectrum was acquired with a 700 MHz spectrometer, equipped with a TCI cryoprobe optimised for direct ^{13}C-direct detection. The peaks are the correlation/cross-peak between the nitrogen of a residue and the carbonyl of the previous one. In this region, the spectrum shows the cross-peaks corresponding to the residues of the 52–55 loop, which amide proton resonances could not be detected in the ^1H-^{15}N HSQC. (**B**) [^1H, ^{15}N] HSQC spectrum of the 1 mM apo-ORP. The spectrum was acquired with a 600 MHz spectrometer equipped with a TXI probe, at 298 K

were performed (Ferentz et al. 2000). The ^{1}H spin systems of the aromatic rings of His residues were identified using a 2D ^{1}H-^{15}N HSQC type spectrum with a INEPT constant optimised for ^{2}J detection. 2D ^{13}C-detected CON, CACO, CBCACO and COSY (Bermel et al. 2006) were performed to extend the assignment of backbone C/N and Cβ resonances of residues which NH were not detected in the [^{15}N, ^{1}H]–HSQC, and to confirm the assignments obtained with standard triple-resonance experiments.

Assignments and data deposition

The polypeptide chain of ORP is composed of 117 residues, 5 are prolines, plus 3 additional residues (ASH) that were added at the N-terminal due to the cloning procedure. Only 106 from the expected 114 amide proton resonances were observed in the ^{1}H–^{15}N HSQC, probably due to exchange with the bulk solution (Fig. 1B). After the complete sequential assignment of these NH resonances it was possible to locate the missing amide proton resonances in three regions of the protein: 3 residues at the N-terminal, and 4 residues in a loop region that compromises the residues 52–55 and Lys79. Similarly, in other homologous proteins, the amide proton resonances of the residues of a loop located in this same region was poorly defined (Etezady-Esfarjani et al. 2004). However, in our case, the use of ^{13}C detection experiments enabled the extension of the sequential assignment. The CON spectrum in combination with the CACO spectrum was used to complete and also confirm the previous sequential assignment. The CACO and CBCACO spectra with the (H)CCH TOCSY were used in the side-chain assignment. The assignment was established for all the residues, including the 5 prolines and the first residue. The total extent of the assignment for the ^{1}H, ^{13}C and ^{15}N is 95.5, 99.4 and 99.3%, respectively. The ^{1}H, ^{13}C and ^{15}N chemical shifts have been deposit in the BioMagResBank (http://www.bmrm.wisc.edu) under BMRB accession number 15200.

Acknowledgements This work was supported by a research project from the Fundação para a Ciência e Tecnologia (POCI/QUI/55350/2004). SRP, AGD and MSC would like to thank the Fundação para a Ciência e Tecnologia for financial support (POCI/SFRH/BPD/14067/2003, POCI/QUI/55350/2004, POCI/SFRH/BPD/24150/2005, respectively). NMR experiments were run at the CERM EU-NMR Research Infrastructure (Contract n° 026145), and SRP would like to thank the EU-NMR for the financial support during her visits to CERM.

References

Bermel W, Bertini I, Felli IC, Piccioli M, Pierattelli R (2006) ^{13}C-detected protonless NMR spectroscopy of proteins in solution. Prog NMR Spectrosc 48:25–45

Bursakov SA, Gavel OY, Di Rocco G, Lampreia J, Calvete J, Pereira AS, Moura JJG, Moura I (2004) Antagonists Mo and Cu in a heterometallic cluster present on a novel protein (orange protein) isolated from *Desulfovibrio gigas*. J Inorg Biochem 98:833–884

Cort JR, Yee A, Edwards AM, Arrowsmith CH, Kennedy MA (2000) NMR structure determination and structure-based functional characterization of conserved hypothetical protein MTH1175 from *Methanobacterium thermoautotrophicum*. J Struct Funct Genomics 1:15–25

Etezady-Esfarjani T, Herrmann T, Peti W, Klock HE, Lesley SA, Wüthrich K (2004) NMR structure determination of the hypothetical protein TM1290 from *Thermotoga maritima* using automated NOESY analysis. J Biomol NMR 29:403–406

Ferentz AE, Wagner G (2000) NMR spectroscopy: a multifaceted approach to macromolecular structure. Q Rev Biophys 33:29–65

George GN, Pickering IJ, Yu EY, Prince RC, Bursakov SA, Gavel OY, Moura I, Moura JJG (2000) A novel protein-bound copper-molybdenum cluster. JACS 122:8321–8322

Scholten JC, Culley DE, Brockman FJ, Wu G, Zhang W (2007) Evolution of the syntrophic interaction between *Desulfovibrio vulgaris* and *Methanosarcina barkeri*: Involvement of an ancient horizontal gene transfer. Biochem Biophys Res Commun 352:48–54