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Letter to the Editor: Sequential NMR assignment of the ferri-cytochrome c_3 from *Desulfovibrio vulgaris* Hildenborough

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Biological context

Cytochromes c_3 are low redox potential cytochromes involved in anaerobic metabolism. These periplasmic proteins contain four bi-histidinyl coordinated hemes. Multiheme cytochromes have been found in sulfate reducing bacteria of the genus *Desulfovibrio*. Preliminary analysis of *Desulfovibrio vulgaris* Hildenborough genome pointed out the existence of several putative genes encoding tetraheme cytochromes (http://www.tigr.org). Analysis of their genomic context showed that some of them are isolated (i.e., the gene encoding the well known soluble cytochrome c_3 (Mr 13,000)) while others are part of multienzymatic complexes (i.e., the tetraheme cytochrome subunit in formate dehydrogenase (Sebban et al., 1995)). The large diversity of the tetraheme cytochromes in this organism must be correlated with the great specificity of these molecules for their oxidoreduction partners. We have recently reported a new approach to study electron transfer complexes combining NMR spectroscopy and theoretical calculations. ^{1}H-^{15}N HSQC are performed on an ^{15}N-labelled redox partner, and we use the chemical shift variations induced upon complex formation to map the interacting site and to filter the *ab initio* models obtained by Bigger (Morelli et al., 2000). ^{1}H-^{15}N HSQC assignment is thus the first step of the functional study of cytochromes c_3. We have initiated our studies with the soluble cytochrome c_3 (Mr 13,000). The gene of this protein was cloned and sequenced (Voordouw et al., 1985) and the structure of this tetraheme cytochrome was solved by x-ray (Matias et al., 1993).

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We report in this paper for the first time the protocol for obtaining the ^{15}N labelling of the tetrahemic cytochrome c_3 and the challenging assignment of the 107 residues in the paramagnetic form. The expression of this tetraheme cytochrome in *E. coli* was insufficient for protein labelling (Herbaud et al., 2000) and requires the protein expression in another *Desulfovibrio* (Voordouw et al., 1990). We have performed the sequence specific assignment of the protein using heteronuclear NMR spectroscopy. The oxidized form of the cytochrome contains four hemes in a paramagnetic state, leading to a well-resolved ^{1}H-NMR spectrum, but inducing large effects on the proton chemical shifts and the proton-proton correlations.

Methods and results

Expression and isotopic enrichment

To produce labelled cytochrome c_3 (Mr 13,000), we grew *D. desulfuricans* G201 (pKJ800) in rich lactate-sulfate medium (Postgate, 1984) without Yeast extract and NH_4Cl, supplemented with $^{15}NH_4Cl$ (1 g l^{-1}), ^{15}N-Celtone powder from Martek (4 g l^{-1}), and 0.27 mM kanamycin (Morelli et al., 1999). From 3 l of growth medium, 7 mg of pure cytochrome c_3 were obtained using the purification protocol already reported (Voordouw et al., 1990).

NMR spectroscopy

The NMR sample was prepared in 10 mM phosphate buffer pH 5.9, 10% D_2O at a protein concentration of 1.2 mM. All NMR spectra were recorded on a 500 MHz DRX Bruker spectrometer, equipped with 5 mm triple resonance HCN probe with self-shielded triple axis gradients. The ^{1}H-^{15}N HSQC spectra

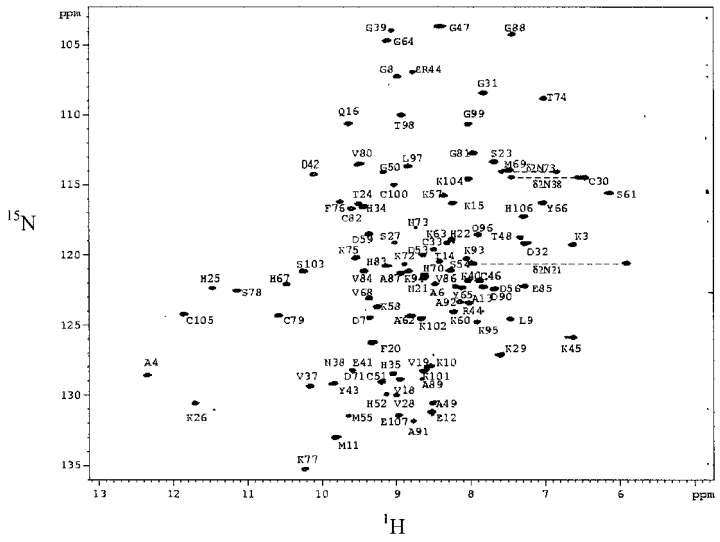

Figure 1. 2D ^{1}H-^{15}N HSQC spectrum of 1.2 mM ^{15}N-labelled cytochrome c_3 recorded on a 500 MHz Bruker spectrometer at 308 K, in 10 mM phosphate buffer (pH 5.9). NH_2 resonances of the side chains of asparagine and glutamine residues are connected.

recorded at 308 K exhibited 102 well-resolved correlation peaks for the 107 amino acids of cytochrome c_3. Due to the paramagnetic effect, the full assignment of spin systems for residues with long side chains (K, R, L and I) was not always possible using standard homonuclear or heteronuclear TOCSY experiments. The peculiar chemical shifts resulting from the paramagnetic effect were validated by COSY and HNHA experiments. Short spin systems (A, V and T) were confirmed by ^{1}H-^{13}C HSQC experiments, with ^{13}C in natural abundance. AMX spin systems of aromatics (Y and F) and N and Q spin systems were assigned from 3D NOESY-HSQC experiments (150 ms). AMX spin systems of the heme ligand cysteine (4×2) and histidine (4×2) residues were deduced from sequential assignment using short mixing time 3D NOESY-HSQC experiments (40 ms). Most of the sequential assignment was done by 3D NOESY-HSQC and 3D HSQC-NOESY-HSQC experiments. Additional experiments were done at 296 K and 318 K, to solve problems of signal overlap. 3D experiments were processed with XwinNMR from Bruker and analysed using Felix from Accelrys (http://www.accelrys.com) on an Silicon Graphics Indy workstation. Proton chemical shifts were calibrated with respect to the H_2O signal and reported relative to DSS and ^{15}N chemical shifts were referenced indirectly using the ^{1}H/^{15}N frequency ratio 0.101329118 (Wishart et al., 1995).

Extent of assignments and data deposition

All the amide groups (^{15}N and ^{1}H) were assigned in ^{1}H-^{15}N HSQC (Figure 1). The ^{1}H and ^{15}N chemical shifts have been deposited in the BioMagResBank database (http://www.brmb.wisc.edu) under accession number BMRB-5239.

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