# Characterization of a nine heme cytochrome by NMR

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*Abstract: A multiheme cytochrome c was isolated from the periplasm of the sulphate reducing bacterium, Desulfovibrio desulfuricans ATCC 27774. This protein contains nine hemes with bis-histidinyl axial coordination and is part of a transmembrane complex which is involved in electron transfer across the cytoplasmic membrane for the reduction of sulphate. Nuclear magnetic resonance spectra in partially and fully oxidised samples allowed the preliminary assignment of the heme proton resonances. The chemical shifts of the heme methyl groups of the nine-heme cytochrome c are also predicted on the basis of the crystal structure.*

## Introduction

Anaerobic organisms contain multiple heme proteins that have an important role in many metabolic and biochemical pathways. They range from cytochromes as electron transfer proteins, with one or more hemes, to a vast array of other proteins, some with enzymatic activity, containing only heme groups or hemes and other prosthetic groups.

A multiheme cytochrome of 37.8 kDa was first discovered in *Desulfovibrio desulfuricans* ATCC 27774 (*Dd*27774).[1] Although the original report suggested the presence of 12 hemes, later refinement showed the protein to contain 9 hemes.[2] This cytochrome (9Hcc) is a monomeric protein containing 292 aminoacid residues. The three dimensional structure is made up of two domains, each one consisting of a tetraheme cytochrome $c_3$ like region, connected by a polypeptide chain which binds the isolated heme group.[2] All the hemes have bis-histidinyl axial coordination with very negative midpoint redox-potentials. Kinetic experiments showed that 9Hcc can be reduced by the hydrogenase from *Dd*27774 and that this reduction is faster in the presence of tetraheme cytochrome $c_3$ from the same organism.[2] Molecular docking studies predicted that specific interaction between these two proteins occurs between heme IV of cytochrome $c_3$ and heme I and II of 9Hcc.[3] This observation, together with the fact that 9Hcc is part of a transmembrane redox complex,[4] led to the proposal that 9Hcc is involved in the transfer of electrons from the periplasmic hydrogen oxidation to the cytoplasmic reduction of sulphate. 9Hcc displays a redox-Bohr effect in the physiological pH range, where the macroscopic interactions between the acid-base and the redox centres appear to be dominated by electrostatic effects.[5]

NMR is an important tool for structural and functional studies of heme proteins, and can provide information about the orientation of heme axial ligands with respect to the heme,[6,7] as well as to characterise the ligands themselves. The octahedral coordination of the iron and the bis-His axial coordination of hemes favour the diamagnetic low-spin form (S=0) in the reduced state (Fe II). Also, NMR spectra have several resonances shifted by the strong ring currents of hemes. In the oxidised state Fe(III) the low-spin iron is paramagnetic (S=1/2) giving rise to hyperfine shifts for hemes and neighbouring nuclei.

**Experimental**

NMR experiments were performed in a Bruker DRX500 spectrometer equipped with an inverse detection 5 mm probe using the 9Hcc protein from *Dd*27774 purified from the soluble fraction, as previously described.[5]

**Results and Discussion**

Extending experimental procedures for proteins containing up to 4 hemes[8-13] has already been demonstrated, and these data were used to assign signals from hemes. Even with paramagnetic shifts induced by the nine hemes in *Dd*27774 9Hcc, there is considerable overlap of many resonances (Figure 1).
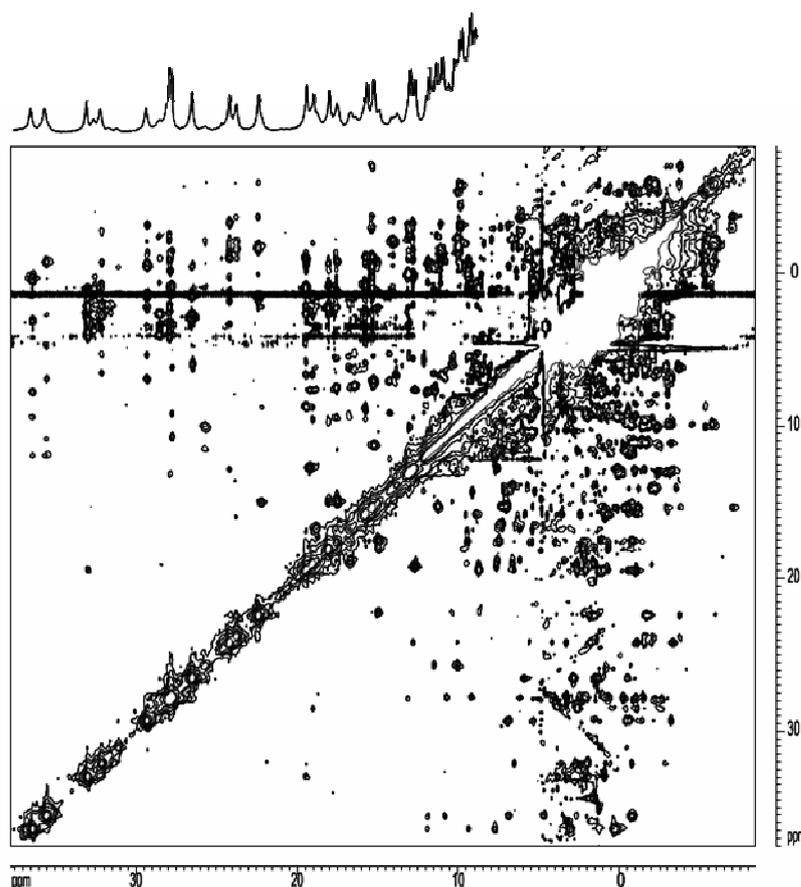


**Figure 1.** NOESY spectrum of *Dd*27774 9Hcc in the fully oxidised form at 303 K and pH 5.4

It was necessary to obtain spectra at different temperatures and pH values, in order to resolve more crowded regions. NOE experiments carried out with 25, 50 and 100 ms mixing times were then used for the preliminary assignment of heme proton resonances. The temperature dependence of ^{1}H signals arising from heme methyls (Figure 2) shows some deviations from the Curie Law.

This behaviour was also observed for other cytochromes,[15] and results from the partial occupation of thermally accessible excited states by the unpaired electron[6] were shown.
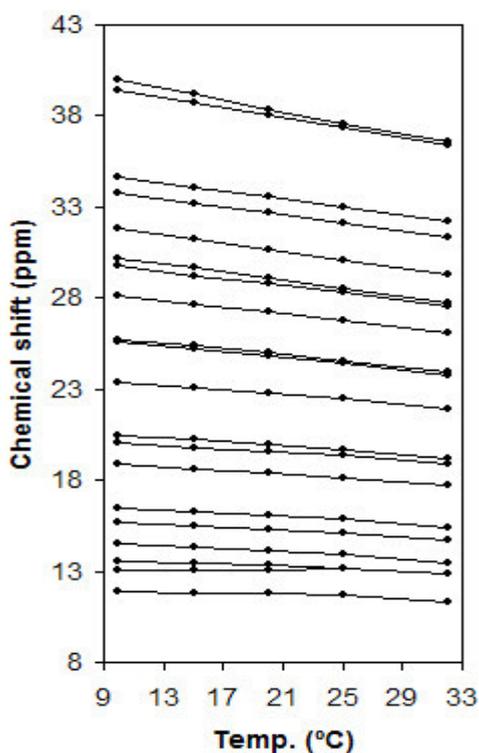


**Figure 2.** Temperature dependence of the downfield shifted heme methyl signals of *Dd*27774 9Hcc.

Using the geometry of the axial ligands of the hemes reported in the X-ray structure of 9Hcc (PDB entry 1OFW) ^{1}H chemical shifts of heme methyls groups at 298K can be predicted.[14] The pseudocontact shift induced by other hemes on a particular heme methyl group (extrinsic paramagnetic shifts) were also calculated as described in the literature.[10] They are listed in Table 1. Spectra of partially reduced samples were used in conditions where intermolecular electron exchange is slow on the NMR timescale and the intramolecular electron exchange is fast. This gives rise to a separate set of NMR resonances for the heme protons in each stage of oxidation, with shifts which depend on the relative populations of each oxidised heme.[8] The reoxidation profile of methyl signals depends on the redox potentials of the heme to which they are attached. Therefore, signals belonging to hemes with different redox potentials can be discriminated.

Correlating all this information, it is possible to specifically assign some resonances. For example, the resonance at 38.82 ppm can be assigned to M18[1] due to the observation of cross-peaks between 12.59 ppm and 7.44 ppm belonging to P17[1] and P17[2], and to the observation of a cross-peak at 9.61 ppm that may be from M2[1] of the same heme. The presence of a cross-peak at -0.25 ppm for the two methyl groups in the NOESY spectra supports the assignment of this resonance, corresponding to the meso proton H20. The predicted ^{1}H chemical shifts (Table 1) show that these methyl groups can be specifically assigned to heme VI.

**Table 1**. Calculated chemical shifts of heme resonances and extrinsic paramagnetic shifts in *Dd*27774 9Hcc in the oxidised state at 298 K. Heme substituents chemical shifts were calculated using the empirical equation described in [14]

| Methyl | Heme | Chemical shift, *ppm* | Extrinsic paramagnetic shifts, *ppm* |
|--------|------|------|------|
| $12^1$ | V | 32.68 | -2.16 |
| $18^1$ | VI | 33.82 | -0.27 |
| $18^1$ | I | 33.60 | -0.13 |
| $18^1$ | II | 31.87 | -0.15 |
| $2^1$ | V | 31.28 | 0.05 |
| $12^1$ | IV | 29.62 | 0.78 |
| $2^1$ | IV | 28.22 | -0.02 |
| $7^1$ | IX | 28.78 | 0.93 |
| $2^1$ | VII | 23.78 | -1.97 |
| $7^1$ | II | 26.29 | 0.95 |
| $12^1$ | VII | 25.18 | 0.16 |
| $7^1$ | VI | 20.45 | -1.10 |
| $2^1$ | VIII | 18.32 | -1.76 |
| $12^1$ | VIII | 19.73 | -0.25 |
| $7^1$ | III | 16.90 | -2.55 |
| $18^1$ | IX | 18.81 | -0.10 |
| $2^1$ | III | 13.94 | -4.83 |
| $18^1$ | IV | 20.21 | 1.78 |
| $7^1$ | I | 17.76 | -0.14 |
| $12^1$ | I | 14.58 | -2.76 |
| $18^1$ | V | 16.76 | 0.05 |
| $12^1$ | III | 15.34 | -1.15 |
| $12^1$ | VI | 12.16 | -2.12 |
| $7^1$ | VIII | 12.41 | -1.58 |
| $2^1$ | I | 13.18 | 0.02 |
| $2^1$ | VI | 10.76 | -0.12 |
| $18^1$ | VII | 9.56 | -0.01 |
| $2^1$ | II | 4.93 | -2.34 |
| $12^1$ | II | 6.33 | 0.22 |
| $12^1$ | IX | 2.10 | -1.96 |
| $18^1$ | III | 1.68 | -1.30 |
| $7^1$ | VII | 3.50 | 0.88 |
| $2^1$ | IX | 0.70 | -0.06 |
| $7^1$ | IV | -0.65 | -0.30 |
| $18^1$ | VIII | -0.46 | -0.09 |
| $7^1$ | V | -0.35 | 0.53 |

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In order to confirm and extend the assignment proton-detected heteronuclear multiple-quantum coherence (HMQC), spectra should be taken to provide information on all the substituents directly attached to heme pyrrole N carbons.

**Conclusion**

The assignment of the heme proton resonances in the oxidised, intermediate and fully reduced stages can leads to the ordering of the nine hemes in terms of their midpoint redox potentials, making it possible to characterise this protein thermodynamically. This will be essential to understand the electron transfer properties of 9Hcc and to elucidate its role in the respiratory chain of *Dd*27774.

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