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Redox-linked conformational changes of a multiheme cytochrome from *Geobacter sulfurreducens*

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Abstract

Multiheme *c*-type cytochromes from members of the *Desulfovibrionacea* and *Geobactereacea* families play crucial roles in the bioenergetics of these microorganisms. Thermodynamic studies using NMR and visible spectroscopic techniques on tetraheme cytochromes c_3 isolated from *Desulfovibrio* spp. and more recently on a triheme cytochrome from *Geobacter sulfurreducens* showed that the properties of each redox centre are modulated by the neighbouring redox centres enabling these proteins to perform energy transduction and thus contributing to cellular energy conservation. Electron/proton transfer coupling relies on redox-linked conformational changes that were addressed for some multiheme cytochromes from the comparison of protein structure of fully reduced and fully oxidised forms. In this work, we identify for the first time in a multiheme cytochrome the simultaneous presence of two different conformations in solution. This was achieved by probing the different oxidation stages of a triheme cytochrome isolated from *G. sulfurreducens* using 2D-NMR techniques. The results presented here will be the foundations to evaluate the modulation of the redox centres properties by conformational changes that occur during the reoxidation of a multiheme protein.

Keywords: Multiheme cytochromes; NMR; Redox-linked conformations; *Geobacter*

Multiheme cytochromes have been found in several bacteria with particular profusion in the *Desulfovibrionacea*, *Geobactereacea,* and *Shewanellenacea* families, where they play important roles in the bioenergetic pathways [1–4]. Due to their intrinsic properties, namely small molecular weight (9–12 kDa) and low-spin heme groups, small multiheme cytochromes containing up to four heme redox centres are by far the best studied. This is the case of tetraheme cytochromes c_3 isolated from *Desulfovirionacea* bacteria [for a Revision see 1]. These cytochromes mediate electron transfer between the periplasmic hydrogenases and transmembrane electron transfer complexes coupling electron and proton transfer [5–9]. Though not observed for all multiheme proteins, this ability to perform energy transduction lays on redox (modulation of the reduction potential of one heme by the oxidation state of a neighbouring one) and redox-Bohr (influence of the solution pH in the reduction potentials of the heme groups) interactions that are displayed among the closely disposed redox centres. Indeed, the existence of positive and negative redox cooperativities between heme groups of a protein shows that the thermodynamic properties of a particular redox centre modulates (being modulated as well) those of a neighbouring one [5–13].

This ability to perform energy transduction is not exclusive of the *Desulfovibrionacea* tetraheme cytochromes and recently we described it for a triheme cytochrome isolated from *Geobacter sulfurreducens* [4]. In fact, the

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Abbreviations: PpcA, *Geobacter sulfurreducens* triheme cytochrome.

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thermodynamic characterization of the redox centres of this cytochrome, hereafter PpcA, showed that this protein contributes to the proton electrochemical potential gradient across the bacterium periplasmic membrane that drives ATP synthesis [4]. Under particular environmental conditions, PpcA (GSU0612) can function in a concerted fashion with four additional periplasmic triheme cytochromes designated by PpcB (GSU0364), PpcC (GSU0365), PpcD (GSU1760), and PpcE (GSU1024) which share more than 50% sequence identity [14,15].

Coupling thermodynamically the transfer of electrons and protons, cytochromes c_3 and PpcA show functional properties that can be phenomenological described in similar terms to those of the transmembrane proton pumps that contribute to the proton electrochemical gradient formation and thus may also undergo conformational changes linked to the transfer of electrons. Indeed, comparison between fully oxidised and fully reduced structures obtained for cytochromes c_3 evidenced redox-linked structural changes in some of these proteins [12,16–19]. However, in the case of multiheme proteins, which display several oxidation stages (see below), structural information obtained for fully reduced and oxidised proteins does not correlate directly with the particular intermediate oxidation stage(s) that triggered the redox-linked conformational change. Consequently, the evaluation of the effect that redox-linked conformational changes play on the redox properties of the heme groups (e.g. reduction potentials) and thus, on the functional interaction networks is not straightforward. In this work, we describe for the first time the co-existence of two conformational forms in solution of a multiheme cytochrome that were detected in intermediate oxidation stages of *G. sulfurreducens* PpcC.

Materials and methods

Production and purification of PpcC. The DNA fragment coding for the mature sequence of cytochrome PpcC was amplified from the *G. sulfurreducens* genomic DNA, digested with restriction enzymes NotI and EcoRI, and cloned into vector pVA203 [20] digested with the same enzymes. The protein was expressed in *Escherichia coli* strain BL21(DE3) cotransformed with plasmid pEC86 harboring the *ccm* genes [21] and purified by cation exchange and gel filtration following the same protocol as was used for PpcA [14,22].

NMR sample preparation. For NMR experiments, the protein solution buffer was exchanged to 99.96% ^2H_2O using ultrafiltration methods (Amicon Ultra-4 with 5k cut-off) followed by one lyophilization step and ressuspended in NaCl/phosphate buffer solution prepared in 99.96% ^2H_2O to a final ionic strength of 250 mM. The final protein concentration was 70 μM. The reduced samples were obtained by adding gaseous hydrogen in the presence of catalytic amounts of Fe-hydrogenase isolated from *Desulfovibrio vulgaris* (Hildenborough). The partially oxidised samples were obtained by first removing the hydrogen from the reduced sample with nitrogen and then adding controlled amounts of air into the NMR tube with a Hamilton syringe. All the NMR spectra were recorded in a Bruker AV-800 spectrometer at 288K and ^1H chemical shifts were calibrated using the water signal as internal reference. 1D-^1H NMR spectra were recorded before and after protein lyophilization to check for protein integrity. 2D-NMR-EXSY experiments were recorded with 25 ms mixing-time collecting 2048 (t_2) × 256 (t_1) data points to cover a sweep width of 22.5 kHz, with 256 scans per increment.

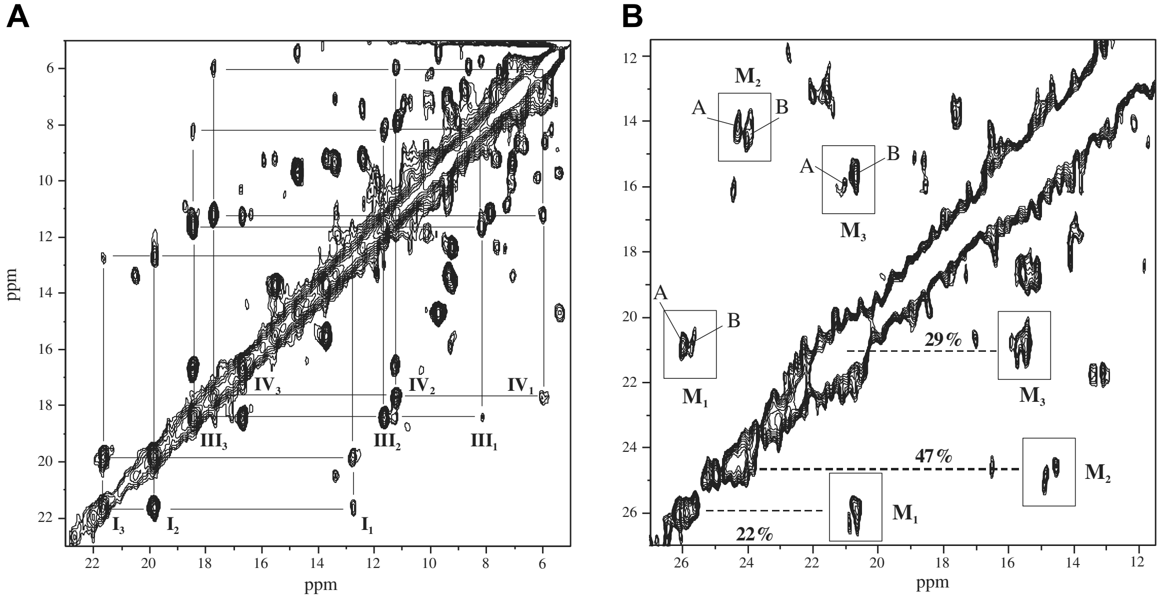

Fig. 1. (A) Portion of a 2D-EXSY NMR spectrum of partially oxidised PpcA (pH 6). Cross-peaks connecting the signals of the heme methyls belonging to hemes I, III, and IV in different oxidation stages (3, 2, and 1) are indicated by straight lines. The Roman and Arabic numbers indicate the heme groups and the oxidation stages, respectively. (B) Portion of a 2D-EXSY NMR spectrum of partially oxidised PpcC (pH 6). Cross-peaks connecting heme methyl signals in oxidation stages 2 and 3 are boxed. Each box emphasises the two components of the cross-peaks that relates with each of the two conformations in solution (A and B). The oxidation fractions for heme methyl signals M_1, M_2, and M_3 were calculated as previously described [24] and refer to the methyl group in the fully oxidised protein (see Fig. 2).

Results and discussion

As stated above, a multiheme protein can present different microstates in solution, which can be grouped according to the number of oxidised heme in macroscopic oxidation stages, linked by successive one-electron reductions [for a review see 4]. In conditions of fast intramolecular electron exchange (among the different microstates within the same oxidation stage) and slow intermolecular electron exchange (among different oxidation stages) on the NMR time scale, the individual hemes can be discriminated at different degrees of protein oxidation. This is typically achieved by following the position of the heme methyl signals, which shift towards low field, outside the protein envelope as the protein oxidation progresses, throughout 2D-EXSY NMR redox titrations [23,24]. A relevant portion of a 2D-EXSY NMR spectrum of PpcA obtained at a solution potential for which microstates belonging to oxidation stages 1 (one heme oxidised), 2 (two hemes oxidised), and 3 (three hemes oxidised) co-exist is presented in Fig. 1A. Single cross-peaks connecting one methyl signal of a particular heme group at oxidation stages 1–3 are indicated. Since the position of the heme methyl signals is approximately 3 ppm in the fully reduced protein, from the patterns indicated it is clear that heme I dominates the first oxidation step, followed by hemes IV and III. The analysis of the pH dependence of the heme methyl paramagnetic shifts together with data from visible titrations was used to determine the network of cooperativities of the redox centres for PpcA [4].

In contrast to that observed for PpcA, NMR redox titrations carried out for the PpcA homologue PpcC showed that it is not possible to observe, in a single experiment, heme methyl signals connecting simultaneously oxidation stages 1, 2, and 3, suggesting that the individual heme reduction potential are more separated in comparison to those of PpcA. As an example, a 2D-EXSY NMR spectrum of PpcC with heme methyl signals connecting oxidation stages 2 and 3 (M_1, M_2, and M_3) is shown in Fig. 1B. The oxidation fractions of heme groups monitored by signals M_1, M_2, and M_3 are 22%, 47%, and 29%, confirming that each signal belongs to a different heme.

However, the 2D-EXSY NMR spectrum obtained for PpcC showed clearly that heme methyl cross-peaks connecting different oxidation stages (e.g., M_1, M_2, and M_3) are clearly splitted in two components, a behaviour that was not observed for PpcA. This splitting is observed for signals M_1, M_2, and M_3 (see Fig. 1B, signal components A and B) and since each signal belongs to a different heme group, it can be deduced that each redox centre experiments different chemical environments during the protein redox cycle. Thus, for the first time in a multiheme protein, the results here described for PpcC showed that two different conformations (hereafter designated by A and B) coexist in solution during the protein redox cycle and that the effect of the redox-linked conformational change span to all protein redox centres. As stated before, the results obtained for PpcC clearly contrasts with that observed for PpcA since in this protein heme methyl signals connecting different oxidation stages have a single component (Fig. 1A) hence indicating that an exchange process associated with the presence of different conformations is not present or not detectable.

The same effect was also observed for PpcC at other pH values in the range 6–8 as well as for heme methyl cross-peaks connecting oxidation stages 1 and 2 (data not shown) which foresight that the pH dependence of PpcC heme methyl paramagnetic shifts will allow to determine for each conformation the redox centre cooperativity networks and thus to evaluate the effect of this redox-linked conformational change on the modulation of the redox centres properties.

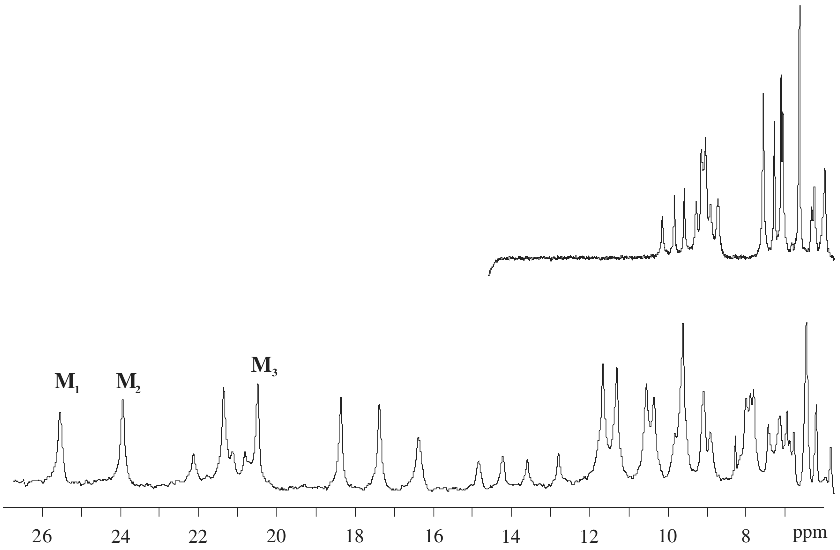

Fig. 2. 1D-^{1}H NMR spectrum of the fingerprint regions of PpcC in oxidised (lower spectrum) and reduced (upper spectrum) forms. Signals M_1, M_2, and M_3 correspond to those indicated in Fig. 1B.

To prove that the different conformations observed for PpcC are indeed redox-linked and not due to different forms already present in solution, i.e., in fully reduced or fully oxidised protein, both 1D-^{1}H NMR obtained in these pure oxidation stages were obtained (Fig. 2). As can be observed, both spectra are well resolved, being characteristic of low-spin proteins, with no evidence of multi-conformational forms. The two most downfield shifted signals of PpcC in the 1D-^{1}H NMR spectrum of the oxidised protein (signals M_1 and M_2 in Fig. 2) are quite illustrative. These two signals appeared in an empty region of the oxidised spectrum, being separated from other signals. However, for samples placed at intermediate levels of oxidation, both signals have two separate exchange cross-peaks (conformation A and B) connecting different oxidation stages showing that conformations A and B are in slow exchange at the experimental conditions used (Fig. 1B). If both conformations were present in the fully oxidised PpcC four signals between 23 and 26 ppm would be observed. The slow conformation interconversion rate observed for PpcA is most probably associated with significant movement of the protein loop regions and not by side-chain motions, which occur generally rather quickly [25]. Examination of the sequences of PpcA and PpcC [14] revealed that PpcC has a higher percentage of hydrophobic residues on the surface of the molecule, which might affect the interconversion of conformational states. In addition, an internal Tyr is located at position 6 in PpcC being stabilised by a hydrogen bond to a propionate of heme I [Pokkuluri et al., unpublished results] whereas in PpcA this residue is replaced by a leucine. Site specific mutagenesis experiments should reveal the underlying causes for this behaviour observed in PpcC.

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