

Assignment of haem ligands and detection of electronic absorption bands of molybdenum in the di-haem periplasmic nitrate reductase of *Paracoccus pantotrophus*

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Abstract The periplasmic nitrate reductase (NAP) from *Paracoccus pantotrophus* is a soluble two-subunit enzyme (NapAB) that binds two *c*-type haems, a [4Fe-4S] cluster and a bis-molybdopterin guanine dinucleotide cofactor that catalyses the reduction of nitrate to nitrite. In the present work the NapAB complex has been studied by magneto-optical spectroscopy to probe co-ordination of both the NapB haems and the NapA active site Mo. The absorption spectrum of the NapAB complex is dominated by features from the NapB *c*-type cytochromes. Using a combination of electron paramagnetic resonance spectroscopy and magnetic circular dichroism it was demonstrated that both haems are low-spin with bis-histidine axial ligation. In addition, a window between 600 and 800 nm was identified in which weak absorption features that may arise from Mo could be detected. The low-temperature MCD spectrum shows oppositely signed bands in this region (peak 648 nm, trough 714 nm) which have been assigned to S-to-Mo(V) charge transfer transitions. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Magnetic circular dichroism spectroscopy; Electron paramagnetic resonance spectroscopy; Heme ligand; Molybdoenzyme

1. Introduction

Nitrate reductases catalyse the reduction of nitrate (NO_3^-) to nitrite (NO_2^-). Bacteria express three distinct nitrate reductases, two are associated with energy-conserving respiratory electron transport pathways and the third with nitrate assimilation [1]. One of the respiratory enzymes is membrane-anchored with an active site in the cytoplasm and the other is a

water-soluble periplasmic enzyme [2]. The assimilatory enzymes are located in the cytoplasm [1]. Analysis of the amino acid sequences of many bacterial nitrate reductases suggests that they are all members of the sub-group of molybdoenzymes that bind the molybdopterin guanine dinucleotide (MGD) form of the molybdopterin cofactor [2]. The soluble periplasmic nitrate reductase (NAP) from *Paracoccus pantotrophus* (previously classified as *Thiosphaera pantotropha* [3] and *Paracoccus denitrificans* GB17 [4]) contains a 16 kDa di-haem *c*-type cytochrome subunit (NapB) and an 80 kDa catalytic subunit (NapA) that binds an N-terminal [4Fe-4S] cluster and the MGD cofactor [2,5–7]. A third component of the NAP system is NapC, a membrane-anchored tetra-haem *c*-type cytochrome that mediates electron flow between membrane quinols/quinones and the NapAB complex [8].

The crystal structure of monomeric NapA from *Desulfovibrio desulfuricans* has been reported recently [9] and has revealed the presence of two MGD moieties per polypeptide (bis-MGD). This is consistent with crystal structures of three other members of the MGD family, namely the soluble periplasmic dimethyl sulfoxide reductases (DMSOR) from *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*, the soluble formate dehydrogenase H (FdhH) from *Escherichia coli*, and the periplasmic trimethylamine *N*-oxide reductase from *Shewanella massilia* [10–14]. We have undertaken a detailed study of the Mo centre of NAP from *P. pantotrophus* using electron paramagnetic resonance (EPR) and EXAFS spectroscopies in order to determine Mo co-ordination during nitrate reduction [15,16]. Our observations suggest that Mo(VI) is a di-oxo species co-ordinated by five sulphur ligands, four provided by the two pterins at 2.43 Å and one provided by a cysteine ligand at 2.82 Å. Upon reduction with dithionite the Mo centre becomes a mono-oxo Mo(IV) species co-ordinated by three sulphur ligands at 2.35 Å. We have suggested that the loss of sulphur co-ordination, presumably by the displacement of one of the pterins, may play an important role in catalysis by allowing substrate to bind or modulating the redox potential.

Optical spectroscopy has not been widely applied to the study of molybdoenzymes since the presence of additional chromophores (haems and the iron-sulphur cluster) obscure the weak molybdenum transitions. In the present paper we report the first study of a periplasmic nitrate reductase (NapAB complex) using magneto-optical spectroscopy to probe both the NapB haems and the active site Mo.

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Abbreviations: MGD, molybdopterin guanine dinucleotide; NAP, periplasmic nitrate reductase; DMSOR, dimethyl sulfoxide reductase; EPR, electron paramagnetic resonance; MCD, magnetic circular dichroism

2. Material and methods

P. pantotrophus M6 was grown anaerobically on nitrate and the NapAB complex was purified as described by Berks et al. [6]. Purified samples were prepared in 20 mM HEPES, pH 7.2 and concentrated to $\sim 300 \mu\text{M}$ for EPR and magnetic circular dichroism (MCD) analysis. Samples for MCD were exchanged into D_2O buffer containing 20 mM Na-HEPES, pH* 7.2. pH* is the apparent pH of the buffer in D_2O measured with a standard glass pH electrode.

EPR spectroscopy was performed on an X-band ER200-D spectrometer (Bruker Spectrospin) interfaced to an ESP1600 computer and fitted with a liquid helium flow cryostat (ESR-9; Oxford Instruments). Haem EPR spectra were recorded at 10 K, ca. 9.64 GHz microwave frequency, 2 mW microwave power and 1.0 mT field modulation amplitude. Mo(V) EPR spectra were recorded at 60 K, ca. 9.64 GHz microwave frequency, 2 mW microwave power and 0.1 mT field modulation amplitude. Spin concentration of samples was determined from integrations of their EPR absorption spectra by comparison to those of a 2 mM Cu^{II} -EDTA standard as in earlier work [16]. Electronic absorption spectra were recorded on a Hitachi UV3000 spectrophotometer. MCD spectra were measured using a split-coil superconducting solenoid, Oxford Instruments type SM-4, capable of generating a maximum magnetic field of 5 T. Spectropolarimeters Jasco J-500D and J-730 were employed for the wavelength ranges 240–1000 and 800–2000 nm, respectively.

3. Results and discussion

The room-temperature absorption spectrum of the air-oxidised NapAB complex is dominated by the spectrum of the NapB *c*-type cytochrome spectrum with bands at 408 nm and 525 nm (Fig. 1A). Low-temperature MCD spectra of air-oxidised NapAB were dominated by intense absorption bands over the wavelength range 300–600 nm (Fig. 1B). These are characteristic of low-spin ferric haem, which in the Soret region (~ 400 nm) of the MCD spectrum gives rise to a temperature-dependent derivative-shaped band with a peak to trough intensity of approximately $32\,000 \text{ M}^{-1} \text{ cm}^{-1}$ per haem at 4.2 K. The presence of low-spin ferric haem in the NapAB complex is also supported by the typical features and intensities of α,β -MCD bands at 550–600 nm. No features typical of high-spin ferric haem were apparent in the 600–650 nm region. The porphyrin $\rightarrow \text{Fe}(\text{III})$ charge transfer transition for low-spin ferric haem occurs in the near-IR region of the spectrum (800–2500 nm). This band is readily observed by MCD spectroscopy, with the peak wavelength being an excellent indicator of the axial ligands to the haem iron [17]. The near-IR MCD spectrum for NapAB is characteristic of a low-spin haem porphyrin $\rightarrow \text{Fe}(\text{III})$ charge transfer band and has a maximum peak at ~ 1480 nm (Fig. 1C). A band at this wavelength is in the range for haems with bis-His ligation. No intense absorbance band at ~ 1725 nm characteristic of His-Met ligation could be detected. It is recognised, however, that an absorbance band at 1480 nm is also characteristic of His-Lys co-ordination [17].

Inspection of a multiple sequence alignment of NapB reveals that the haem *c* binding motif CxxCH occurs twice in the sequence and two further His residues are also highly conserved at positions 79 and 114 (Fig. 2). The absence of highly conserved lysine residues suggests that both haems are bis-His-ligated. However, lysine residues are apparent in *P. pantotrophus* NapB and other NapB proteins (Fig. 2). It is dangerous to derive amino acid ligation solely from sequence analysis, as illustrated by studies on the cytochrome *cd*₁ nitrite reductases [18]. Consequently EPR spectroscopic evidence in support of the absence of a Lys ligand was sought. In the

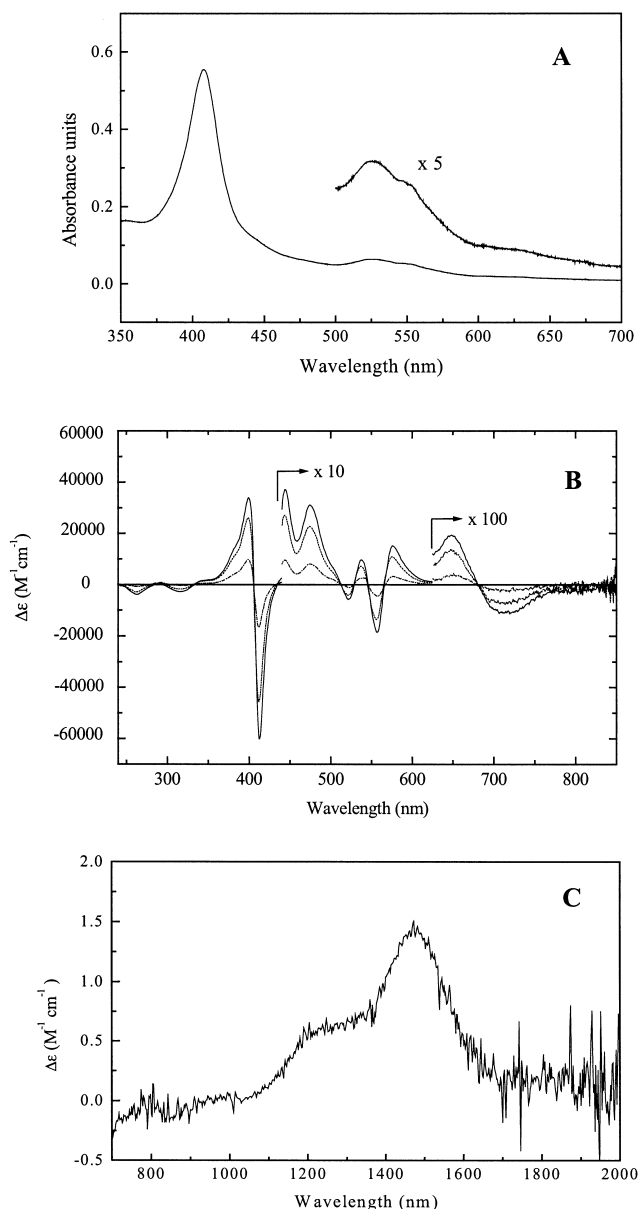


Fig. 1. Electronic absorption spectra of NapAB. A: Room-temperature absorption spectra of NapAB complex. B: Variable-temperature MCD spectra of the NapAB complex. Spectra were all collected at 5 T and recorded at 1.6 K (solid line), 4.2 K (dashed line) and 15.0 K (dot-dashed line). Enzyme was in 20 mM HEPES buffer, pH 7.2, diluted to 50% (v/v) with glycerol, at a final concentration of $293 \mu\text{M}$. C: Room-temperature near-IR MCD spectrum of air-oxidised NapAB complex. Enzyme was in 20 mM HEPES D_2O buffer, pH* 7.2, at a final concentration of $293 \mu\text{M}$.

EPR spectra recorded at 10 K a rhombic signal with *g*-values 2.9, 2.2 and a broad feature at 1.5 could be detected which is typical of a low-spin ferric haem ($S=1/2$) with two His ligands in which the imidazole planes are close to parallel (Fig. 3). Spin integration of the signal compared to a Cu^{II} standard revealed ~ 1.7 mol of spin/mol of NAP, thus predicting that both haems in NapAB complex contributed to this signal. In addition, signals at $g \approx 2.01$, $g \approx 5.9$ and $g \approx 4.3$ were detected. These arise from an oxidised $[3\text{Fe}-4\text{S}]$ cluster (~ 0.1 mol of spin/mol of NapAB; a product of the degradation of a small amount of $[4\text{Fe}-4\text{S}]$ cluster during

plication of VTMCD will prove useful for characterising these species further.

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