Volume 219, number 1, 244–248 FEB 04908 July 1987

Electron paramagnetic resonance and magnetic circular dichroism studies of a hexa-heme nitrite reductase from *Wolinella succinogenes*

Richard S. Blackmore, Thomas Brittain*, Paul M.A. Gadsby, Colin Greenwood and Andrew J. Thomson

School of Biological Sciences and School of Chemical Sciences, University of East Anglia, Norwich NR4 7TJ, England and **Dept of Biochemistry, University of Auckland, Private Bag, Auckland, New Zealand*

Received 4 May 1987

The nature of the heme centers in the hexa-heme dissimilatory nitrite reductase from the bacterium *Wolinella succinogenes* has been investigated with EPR and magnetic circular dichroism spectroscopy. The EPR spectrum of the ferric enzyme is complex showing, in addition to magnetically isolated low-spin ferric hemes with g values of 2.93, 2.3 and 1.48, two sets of signals at $g=10.3$, 3.7 and 4.8, 3.21, which we assign to two pairs of exchange coupled hemes. The MCD spectra show that the isolated hemes are bis-histidine coordinated and that there is one high-spin ferric heme. The exchange coupling is lost on treatment with SDS.

EPR; MCD; Heme-protein; Nitrite reductase; (*Wolinella succinogenes*)

1. INTRODUCTION

The anaerobic bacterium *Wolinella succinogenes* is capable of growth on medium containing formate as the oxidisable substrate and nitrate as the terminal electron acceptor [1]. Under these conditions reduction of nitrate to ammonia in a dissimilatory process is catalysed by two enzymes, namely nitrate reductase reducing nitrate to nitrite and nitrite reductase which reduces the nitrite to ammonia [1]. The first purification of the latter was reported by Liu et al. [2] who identified it as a heme-protein consisting of 6 hemes covalently bound to a single polypeptide chain of 63 kDa.

A study of the ligand binding and kinetic properties of the enzyme [3,4] revealed differences between the heme groups of the enzyme, two groups of hemes being identified. One group had spectral and ligand binding properties reminiscent of 5-coordinate iron, while the other group had characteristics of 6-coordinate iron. Experimental evidence has been presented that the latter group represents the site of electron donation to the enzyme [4] whereas the former sites may be the site of nitrite binding and reduction to ammonia.

The present work uses magnetic circular dichroism (MCD) and electron paramagnetic resonance (EPR) to characterise further the nature of the hemes present in this enzyme by determining the heme ligation and spin states and by investigating possible interactions between them.

Correspondence address: R. Blackmore, School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, England

2. MATERIALS AND METHODS

All reagents were purchased from Sigma.

W. succinogenes was grown anaerobically in 150 l batch culture employing the growth conditions described in [1]. Cell harvesting was carried out using a Millipore Pellicon tangential flow ultrafiltration device. Enzyme purification was carried out as in [3].

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

EPR spectra were recorded with a Bruker ER 200D-SRC spectrometer with an Aspect 2000 computer. Temperature regulation of EPR samples was by an Oxford Instruments ESR-900 continuous flow cryostat with a DTC-2 temperature controller and a gold + 0.03%-iron/chromel temperature sensor.

MCD measurements in the region 250–1050 nm were made with a Jasco J-500D spectropolarimeter, and in the region 800–2000 nm were recorded with a laboratory-built instrument described elsewhere [5]. The magnetic field for room-temperature samples was generated by an Oxford Instruments SM1 superconducting magnet with a maximum field of 6 T, and for low-temperature samples by an Oxford Instruments SM-4 split coil magnet with a maximum field of 5 T.

The cyanide complex of the enzyme was produced by addition of a 1000-fold molar excess of NaCN (pH 7.0) to a stoppered sample of the ferric enzyme. The enzyme required 48 h to equilibrate fully with the added cyanide as judged by absorption spectroscopy.

3. RESULTS

3.1. *EPR spectra*

The EPR spectra of the fully oxidised form of nitrite reductase, its adduct with CN^- and in the presence of SDS (1%, w/v) are shown as fig.1a,b and c, respectively. Although the spectrum of the fully oxidised state of the enzyme is very complex, unambiguous assignment of the signals to heme centers is possible by tracing the changes induced by CN^- binding and by treatment with limited amounts of SDS. The spectrum of the SDS-treated form (fig.1c) shows some high-spin ferric heme at $g = 6.11$ and a set of g values at 2.96, 2.26 and 1.48 that can be attributed to the low-spin ferric form of heme. The same low-spin signals are also present in the cyanide-treated form. Using metmyoglobin cyanide as a standard the spin intensities of the signal at $g = 2.96$ in the SDS-treated form and at $g = 2.93$ in the cyanide adduct were found to be 2.5 and 1.7 spins per molecule of enzyme, respectively. It is clear that the low-spin heme signals at 2.9(2), 2.3 and 1.48 are also present in the oxidised enzyme. An attempt to estimate the amount present was made by integration of the

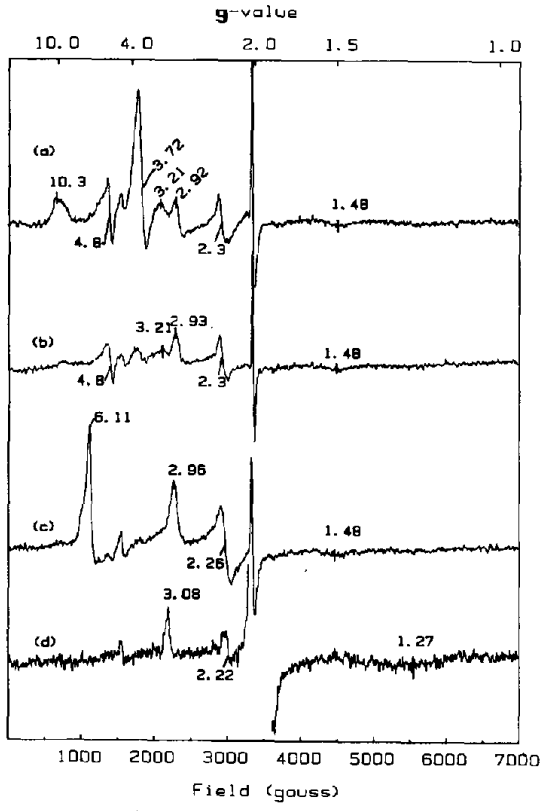

Fig.1. EPR spectra of nitrite reductase. Enzyme concentration was 60 μM in 100 mM Hepes buffer (pH 7.5) with 0.05% Triton X-100 (v/v). Temperature, 10 K; microwave frequency, 9.4 GHz; power, 2.01 mW. (a) Ferric enzyme as isolated; gain, 8×10^5. (b) Cyanide complex of ferric enzyme; cyanide concentration, 100 mM; gain, 8×10^5. (c) SDS-treated ferric enzyme; SDS concentration, 1% (w/v); gain, 8×10^5. (d) Dithionite-treated enzyme; dithionite concentration, 10 mM; gain, 1.6×10^6.

signal at $g = 2.92$ after subtraction of the underlying background from the feature at $g = 3.21$. This estimate gave 1.7 spins, the intensity being the same as for the cyanide adduct. The additional signals in the oxidised enzyme at $g = 10.3$, 4.8, 3.72 and 3.21 appear to arise from two different centers. On treatment with cyanide the prominent signals at $g = 10.3$ and 3.72 are lost virtually completely whereas the features at $g = 4.8$ and 3.21 persist although becoming weaker. These sets of g values are most unusual for heme-proteins but a similar spectrum with signals at $g = 10.13$ and 3.31 has been observed in another multi-heme protein,

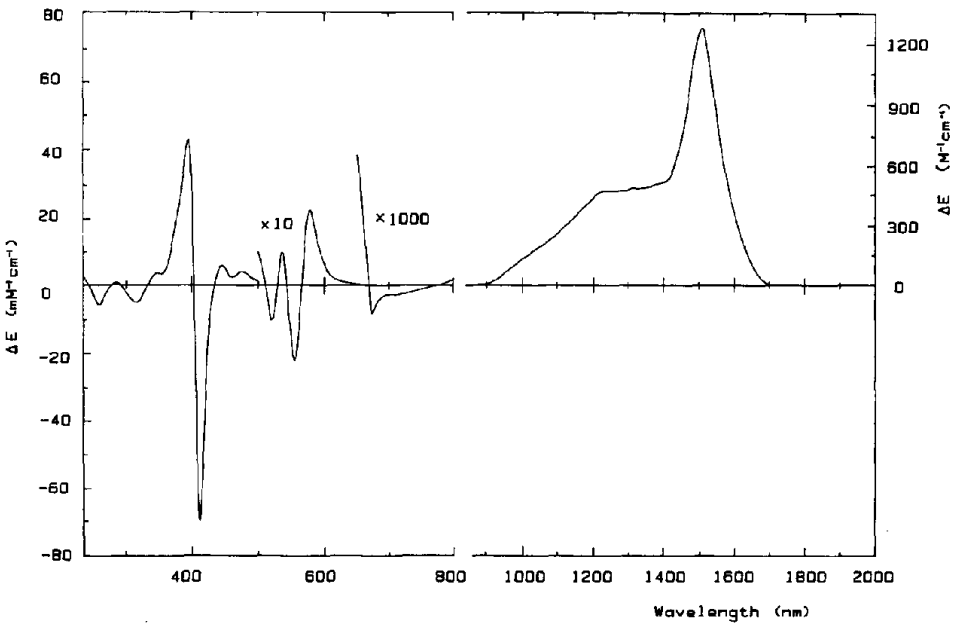

Fig.2. Near-UV/visible/near-IR MCD spectrum of ferric nitrite reductase at 4.2 K and 5 T. Enzyme concentration was 83 μM (8.3 μM for near-UV region) in 25 mM Hepes buffer (p*D* 7.5) containing 50% glycerol (v/v) with 0.05% Triton X-100 (v/v). Path length, 1.5 mm.

namely cytochrome *c*-554 from *Nitrosomonas europaea* [8]. These have been assigned on the basis of multi-frequency EPR and Mössbauer experiments to a pair of hemes weakly coupled magnetically.

On reduction of the enzyme with 10 mM dithionite all of the EPR signals in the spectrum of the ferric form were lost and a new set of low-spin heme signals appeared at g = 3.08, 2.22 and 1.27 (fig.1d). Spin integration showed that the signals account for one of the six hemes in the enzyme. These signals persisted in the presence of a 1000-fold excess of dithionite but were lost in the presence of 1% SDS (w/v). The sharp signal at g = 2.0 arises from a contaminating iron-sulfur protein.

3.2. *MCD spectra*

The spectrum of the fully oxidised form of nitrite reductase over the wavelength range 280–2000 nm at 4.2 K is characterised by features arising from low-spin ferric heme. The peak in the near-infrared at 1510 nm is characteristic of low-spin ferric heme. High-spin ferric MCD are obscured at 4.2 K by the presence of low-spin ferric heme except in the region 700–1000 nm where the latter has no absorption bands. The room-temperature MCD spectrum in this region (fig.4) shows clearly features with an intensity corresponding to about one heme per molecule of enzyme which arises from high-spin ferric heme. These signals were abolished in the presence of 100 mM CN^-.

The MCD spectrum of the dithionite-reduced form of the enzyme (fig.3) has features from reduced low-spin heme between 500 and 550 nm which are temperature-independent and hence arise from a diamagnetic heme. However, there are also temperature-dependent peaks with the form of low-spin ferric heme amounting to about 10% of the total heme content. These signals can therefore be assigned to the heme having the EPR g values at 3.08, 2.22 and 1.27. Increasing the dithionite concentration or addition of methyl viologen did not decrease the intensity of the ferric signals in the EPR or MCD spectra. There is no in-

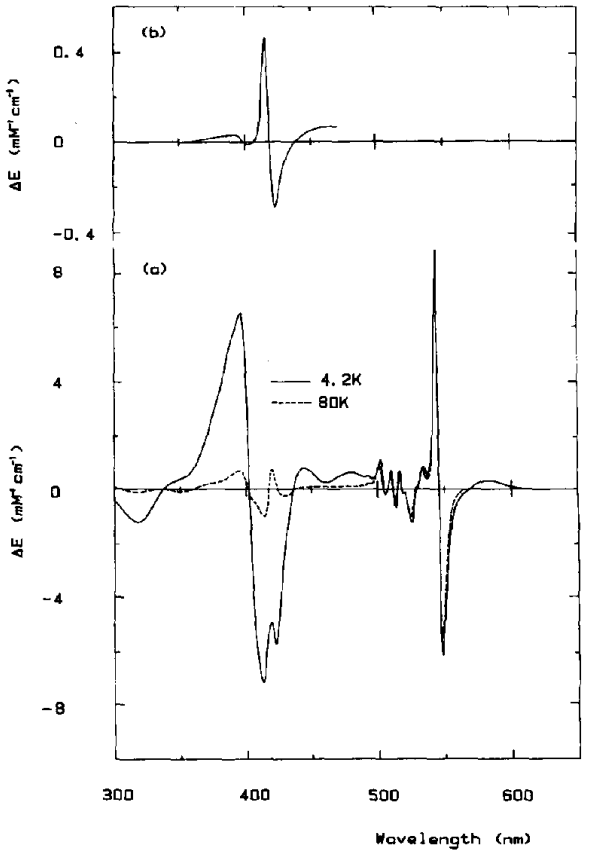

Fig.3. Near-UV/visible MCD and CD spectrum of dithionite-treated nitrite reductase at 4.2 K and 5 T in 25 mM Hepes (pH 7.5) with 50% glycerol (v/v) and 0.05% Triton X-100 (v/v). Enzyme concentration, 8.3 μM; dithionite concentration, 3 mM. (a) MCD spectrum at 4.2 K (——) and 80 K (– – –). (b) CD spectrum at 80 K.

dication of high-spin ferrous heme at either 4.2 K or room temperature [10]. The natural circular dichroism (CD) spectrum of the dithionite-reduced form of the enzyme in the Soret band region (fig.3b) is unusually intense compared with that of reduced *b* cytochromes [9]. This signal was lost in the presence of 1% SDS. These facts suggest splitting of the CD signal and its intensity may be a consequence of exciton coupling between one or more pairs of hemes in close proximity [9].

4. DISCUSSION

We consider first the oxidised form of the enzyme. The EPR signals at 2.96, 2.3 and 1.48, the visible-region MCD spectra and the near-IR MCD band at 1510 nm point to the presence of low-spin ferric heme co-ordinated by two histidine residues [6,7]. The spin integrations of the EPR signals in the oxidised and cyanide adducts giving 1.7 spins per molecule of enzyme suggest that two hemes have this co-ordination. They are magnetically isolated. The other four hemes are unusual in magnetic properties.

The EPR signals at $g = 10.3$ and 3.72 are similar to those found in the tetraheme cytochrome *c*-554 from *N. europaea* [8]. It was shown in this study that these signals arise from a low- and a high-spin heme being exchange coupled. By analogy, we propose that two of the hemes in the nitrite reductase are similarly a coupled low- and high-spin pair. The MCD at room temperature between 700 and 1000 nm gives direct evidence for one high-spin heme. This signal is lost on the addition of cyanide. Also the *g* values at 10.3 and 3.72 are lost. These facts support the assignment and suggest that binding of cyanide changes the coupling between the high- and low-spin hemes. Addition of SDS appears to break the coupling between the hemes allowing observation of the high-spin heme at $g = 6.11$ and the low-spin heme as an additional intensity at $g = 2.96$, 2.24 and 1.48 accounting for the increased low-spin intensity in the SDS-treated enzyme.

This accounts for four of the six hemes. It is likely that the third pair of hemes gives rise to the unusual EPR signals at $g = 4.8$ and 3.21. These values suggest that the hemes are weakly exchange coupled. However, we have no direct evidence for this.

The reduced form of the enzyme gives some support to the proposals. Thus, the intense natural CD which is abolished by 1% SDS is another piece of evidence in favour of heme-heme interaction in at least one pair of hemes. The presence of ferric heme in the MCD and EPR spectra of the dithionite-treated enzyme is unexpected. The loss of these signals on addition of SDS suggests that the protein structure is important in preventing the heme from becoming reduced. It may be that this is one of the exchange-coupled pair at $g = 10.3$ and 3.72. Possibly reduction of both hemes requires an unusually low potential.

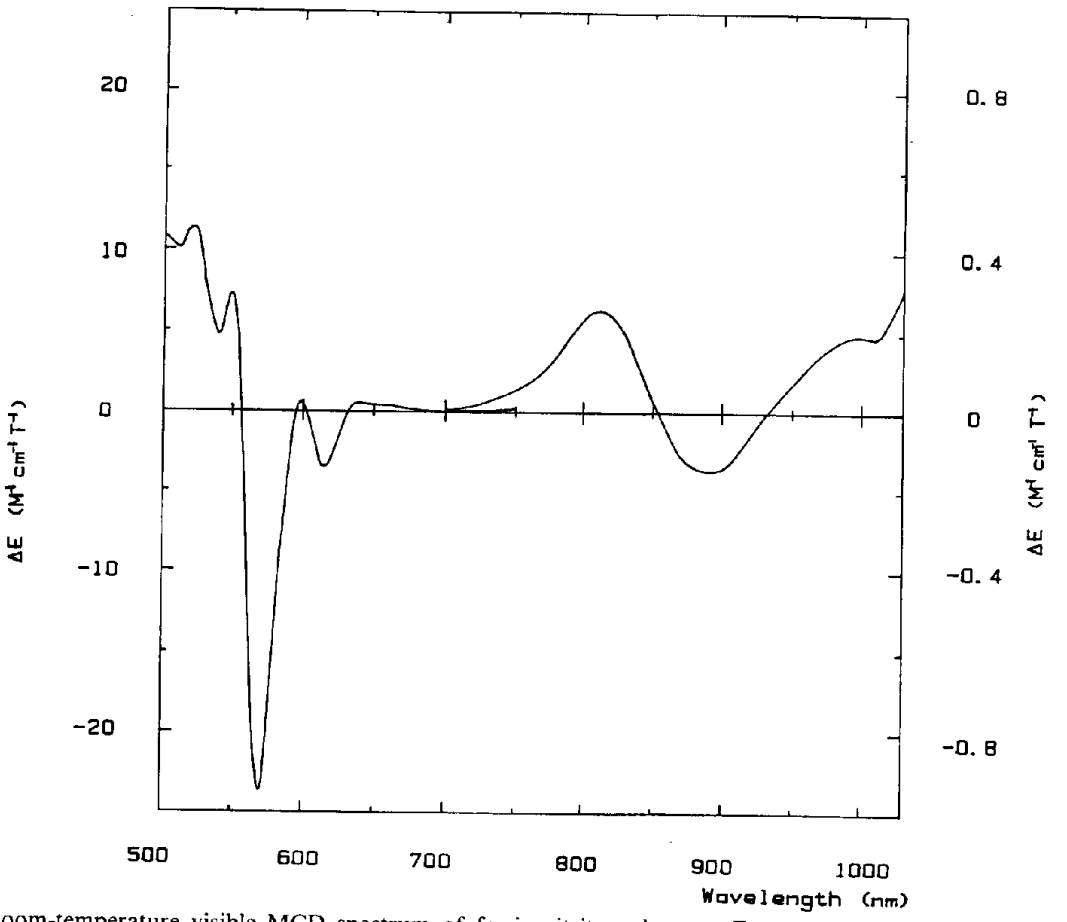

Fig.4. Room-temperature visible MCD spectrum of ferric nitrite reductase. Enzyme concentration was 125 μM in 100 mM Hepes (p*D* 7.9) with 0.05% Triton X-100 (v/v). Path length, 2 mm; magnetic field, 6 T.

ACKNOWLEDGEMENTS

R.S.B. thanks the Association of Commonwealth Universities for a scholarship, P.M.A.G. is an SERC-supported post-doctoral fellow. C.G. and A.J.T. wish to thank the SERC for support.

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