Two structurally and kinetically distinct forms of *Wolinella succinogenes* nitrite reductase

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, U.K.

It is shown that the oxidized form of the hexa-haem nitrite reductase of *Wolinella succinogenes* exists in two structurally and functionally distinct forms, termed 'resting' and 'redox-cycled'. The nitrite reductase as initially isolated, termed 'resting', has five low-spin ferrihaem groups and one high-spin ferrihaem group. The reduction of these haem groups by $\text{Na}_2\text{S}_2\text{O}_4$ occurs in two kinetically and spectrally distinct phases. In the slower phase the haem groups are reduced by dithionite with a limiting rate of 4 s^{-1} . If the enzyme is re-oxidized after reduction with dithionite or with methyl viologen, the resulting ferric form, termed 'redox-cycled', possesses only low-spin haem centres and a rate of reduction in the slower phase that is no longer limited. In the resting form of the enzyme the high-spin ferrihaem group is weakly exchange-coupled to a low-spin haem group. It is proposed that in the redox-cycled form the exchange coupling occurs between two low-spin ferric haem groups. This change in spin state allows a more rapid rate of electron transfer to the coupled pair.

INTRODUCTION

The anaerobic bacterium *Wolinella succinogenes* is capable of growth on medium containing formate as the oxidizable substrate and nitrate as the terminal electron acceptor (Bokranz *et al.*, 1983). Under these conditions nitrate is reduced to ammonia in a dissimilatory process by two enzymes, a nitrate reductase to reduce nitrate to nitrite, and a nitrite reductase that reduces nitrite to ammonia (Bokranz *et al.*, 1983). The first report of the latter enzyme from *W. succinogenes* was by Liu *et al.* (1983), who identified it as a haem-protein consisting of six haem groups covalently bound to a single polypeptide chain of M_r 63 000.

A study of the ligand-binding and kinetic properties of the enzyme (Blackmore *et al.*, 1986; Blackmore & Brittain, 1986) revealed two distinct sets of haem groups. One set had spectral characteristics typical of six-co-ordinate low-spin haem iron. The observed rate of reduction of these haem groups by dithionite showed a linear dependence on the square root of the dithionite concentration. The other haem group had characteristics of five-co-ordinate haem iron and was reduced more slowly by dithionite. This slower phase showed a hyperbolic dependence of rate on the square root of dithionite concentration, with a rate limit of 1.5 s^{-1} .

An initial study of the haem magnetic and ligation states by m.c.d. and e.p.r. spectroscopy (Blackmore *et al.*, 1987) showed that the fully oxidized enzyme contains low-spin ferric haem groups co-ordinated with histidine residues and a single high-spin ferric haem group exchange-coupled to one low-spin haem group. After prolonged reduction with dithionite one haem group remained in the ferric state and gave rise in the e.p.r. spectrum to a set of low-spin ferric g -values of 3.08, 2.22 and 1.27 that were not present in the e.p.r. spectrum of the oxidized form of the enzyme.

In the present work m.c.d. and e.p.r. spectroscopy were used with kinetic studies to characterize further the exchange-coupled haem pair and the conformational and functional changes that occur following the reduction and oxidation of this enzyme.

MATERIALS AND METHODS

All reagents were purchased from Sigma Chemical Co.

W. succinogenes was grown anaerobically in 150-litre batch culture employing the growth conditions previously described (Bokranz *et al.*, 1983). Cell harvesting was carried out with a Millipore Pellicon tangential-flow ultrafiltration device. Nitrite reductase purification was carried out as described previously (Blackmore *et al.*, 1986). Nitrite reductase as isolated by this method is termed 'resting', and enzyme that has been reduced and oxidized one or more times is referred to as 'redox-cycled'.

E.p.r. spectra were recorded with a Bruker ER 200D-SRC spectrometer with an Aspect 2000 computer. Temperature regulation of e.p.r. 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.

M.c.d. 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 (Eglinton *et al.*, 1980). 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.

Reaction kinetics were studied with a Gibson–Milnes stopped-flow apparatus with a 2 cm path length and a 3 ms dead time coupled to an Acorn BBC Master computer running a storage oscilloscope emulation program and eight-bit analogue to digital converter/amplifier system supplied by Purtrax Electronics (Norwich, U.K.). For each trace 256 data points were collected and stored on magnetic disc. Data analysis was performed on an Acorn BBC Master computer using exponential 'peeling' routines. Static absorption spectroscopic studies were performed with a Cary 118C spectrophotometer.

Dithionite solutions were prepared as previously described (Blackmore & Brittain, 1986). Resting nitrite reductase is the enzyme as isolated and was fully ferric. Redox-cycled enzyme was produced for kinetic studies by stoichiometrically reducing

Abbreviation used: m.c.d., magnetic circular dichroism.

‡ To whom correspondence should be addressed.

the enzyme with dithionite, then re-oxidizing it by addition of air-saturated buffer. The dithionite-concentration-dependence of the reduction rate for both forms of the enzyme was determined with the same dithionite sample on the same day.

RESULTS

If the enzyme is reduced from the oxidized state to the fully reduced state by dithionite and then fully re-oxidized by air, the resulting form (called the 'redox-cycled' form) has different spectral properties from that of the starting state. These changes were monitored in the e.p.r., absorption and m.c.d. spectra. Reduction with reduced methyl viologen followed by oxidation with nitrite also results in a form of the enzyme with a changed e.p.r. spectrum similar to that of the redox-cycled state.

E.p.r.

Fig. 1 compares the X-band e.p.r. spectra of the oxidized resting and oxidized redox-cycled states. There are marked changes in the low-field region of the spectrum, with the loss of the signals at $g = 10.4$ and 3.72 after a redox cycle. The signals at $g = 2.93$, 2.3 and 1.48 , which are a rhombic trio arising from low-spin ferric haem co-ordinated by histidine ligands, were found by integration to remain unchanged in intensity following redox-cycling. The signals with complex line shapes at $g = 4.8$ and 3.21 decrease in intensity but are retained. A weak feature at $g = 6.0$ appears. After re-reduction of the redox-cycled enzyme with dithionite all the e.p.r. signals characteristic of the oxidized

Fig. 1. E.p.r. spectra of (a) resting and (b) redox-cycled ferric nitrite reductase

The nitrite reductase concentration was $130 \mu\text{M}$ in 100 mM -Hepes buffer, pH 7.5, containing 0.05% (v/v) Triton X-100. The temperature was 10 K , the microwave frequency 9.4 GHz , the power 2.02 mW and the gain 4×10^5 .

Fig. 2. (a) Visible-region and (b) Soret-region absorption spectra of resting (—) and redox-cycled (---) ferric nitrite reductase

(a) Visible-region absorption spectra. The nitrite reductase concentration was $5 \mu\text{M}$ in 60 mM -potassium phosphate buffer, pH 7.6, containing 0.05% (v/v) Triton X-100. (b) Soret-region absorption spectra. The nitrite reductase concentration was $1 \mu\text{M}$ in 60 mM -potassium phosphate buffer, pH 7.6, containing 0.05% (v/v) Triton X-100.

state of the enzyme were lost and a new set of signals with g -values of 3.08 , 2.22 and 1.27 were observed. These are identical with the g -values of the so-called 'non-reducible' haem group reported earlier (Blackmore *et al.*, 1987).

Absorption spectroscopy

Fig. 2 compares the absorption spectra in the visible and Soret regions of the two oxidized states of the enzyme. Small but clear differences between them are evident. The weak broad band at 610 nm is lost from the visible spectrum and the Soret band shifts and broadens on the red side in the redox-cycled form. Re-reduction of the redox-cycled enzyme yields an absorption spectrum identical with that of the reduced enzyme.

M.c.d. spectra

Spectra were recorded both at room temperature and at 4.2 K in three regions, namely the Soret, the visible and the near i.r. Figs. 3 and 4 make a comparison between the room-temperature m.c.d. spectra in these three wavelength regions of the oxidized resting and redox-cycled forms of the enzyme. There are differences, most obviously (Fig. 3b) in the loss of the derivative shaped band at 850 nm on redox cycling. This band is characteristic of high-spin ferric haem and has previously been assigned to a single high-spin ferric haem group (Blackmore *et al.*, 1987). The peak at 1510 nm (Fig. 4) intensifies from 6 to $8 \text{ M}^{-1} \cdot \text{cm}^{-1} \cdot \text{T}^{-1}$ after redox cycling. This band probably arises from low-spin ferric haem co-ordinated by two histidine ligands, since this ligation state gives rise to characteristic charge-transfer transition at this wavelength (Gadsby & Thomson, 1990). Thus the natural interpretation of the loss of the high-spin ferric haem m.c.d. band and the intensity increase in the low-spin ferric m.c.d. band after one redox cycle is that the high-spin haem group has switched to

Fig. 3. (a) Room-temperature Soret-visible-region and (b) visible-region m.c.d. spectra of resting (—) and redox-cycled (---) ferric nitrite reductase

(a) Room-temperature Soret-visible-region m.c.d. spectra. The nitrite reductase concentration was $10\ \mu M$ in 100 mM-Hepes buffer, pH 7.5, containing 0.05% (v/v) Triton X-100. The path length was 2 mm and the magnetic field was 6 T. (b) Room-temperature visible-region m.c.d. spectra. The nitrite reductase concentration was $125\ \mu M$ in 100 mM-Hepes buffer, pH 7.5, containing 0.05% (v/v) Triton X-100. The path length was 2 mm and the magnetic field was 6 T.

low-spin by becoming bis-histidine-ligated. No distinction between any of the low-spin ferric haem group is possible by means of the peak at 1510 nm.

There are changes in the visible-region m.c.d. spectrum (Fig. 3) that parallel those observed in the absorption spectrum. The negative feature at 620 nm in the m.c.d. spectrum is lost on redox-cycling and a shoulder appears on the red edge of the Soret-band m.c.d. signal. Changes in the wavelength range 500–600 nm are not easily interpreted. The loss of the features at 620 nm probably corresponds to the loss of the high-spin ferric haem group. The low-spin ferric haem group thereby formed is presumed to be responsible for the new shoulder on the Soret band. Thus, although the low-spin ferric haem groups are not resolvable in the near-i.r. region, one can be distinguished in the Soret region.

Fig. 4. Room-temperature near-i.r. m.c.d. spectra of resting (—) and redox-cycled (---) ferric nitrite reductase

The nitrite reductase concentration was $125\ \mu M$ in 100 mM-Hepes buffer, pH 7.5, containing 0.05% (v/v) Triton X-100. The path length was 2 mm and the magnetic field was 6 T.

The m.c.d. spectra recorded at 4.2 K confirm these assignments. The spectra recorded before and after cycling are shown in Fig. 5 for the Soret and visible regions. The intensity of the high-spin ferric haem m.c.d. at low temperatures is several orders of magnitude less than that of the low-spin ferric haem. Hence the 4.2 K m.c.d. spectra have the form expected for bis-histidine-co-ordinated haem groups (Foote *et al.*, 1984). The Soret band reveals more clearly than the room-temperature spectra the appearance after redox cycling of the new low-spin ferric haem group, now as a distinct negative trough at 422 nm. The near-i.r. charge-transfer band (not shown) increases in intensity by $300\ M^{-1}\cdot cm^{-1}$ at 1470 nm (the wavelength that showed the greatest increase in $\Delta\epsilon$). The intensity of the near-i.r. charge-transfer bands can vary over a wide range depending upon the relative orientations of the two histidine planes (Thomson & Gadsby, 1990). $\Delta\epsilon$ varies between 175 and $400\ M^{-1}\cdot cm^{-1}$ at 4.2 K and 5 T. Hence the increase in $\Delta\epsilon$ at 1470 nm observed after redox cycling is consistent with an increase of one bis-histidine-co-ordinated haem group with a $\Delta\epsilon$ value of $300\ M^{-1}\cdot cm^{-1}$.

Fig. 5. Soret-visible-region m.c.d. spectra of resting (—) and redox-cycled (---) ferric nitrite reductase at 4.2 K

The nitrite reductase concentration was $8.3\ \mu M$ in 50 mM-Hepes buffer, pH 7.5, containing 50% (v/v) glycerol and 0.05% (v/v) Triton X-100. The path length was 1.5 mm and the magnetic field was 5 T.

M.c.d. magnetization measurements

The combined evidence of the m.c.d. and absorption spectra presented in the previous sections shows that on redox-cycling of the enzyme one haem group switches from a high-spin to a low-spin ferric state. This leads in the e.p.r. spectrum to the loss of the signals at $g = 10.4$ and 3.7 , earlier assigned as a coupled high-spin and low-spin pair of ferric haem groups (Blackmore *et al.*, 1987). However, no new signals arise in the e.p.r. spectrum. One possible inference is that the spin switch leads to an exchange-coupled pair of low-spin ferric haem groups that are e.p.r.-silent. The magnetic properties of this pair can be probed optically by means of the m.c.d. magnetization curves (Thomson & Johnson, 1980). It is necessary to use a wavelength region where the optical absorption of the haem pair is not overlapped by others. The wavelengths between 420 nm and 440 nm corresponding to the shoulder on the Soret band (Fig. 5) offer this possibility. Therefore the m.c.d. magnetization curves were recorded from the resting oxidized form of the enzyme at 422 nm and from the redox-cycled form at two wavelengths, 422 nm and 436 nm (Figs. 6a–6c). For the resting form the curves recorded at 422 nm and temperatures of 1.6, 4.2 and 10 K overlay one another almost perfectly, showing that the ground state of the paramagnet being probed is a spin $S = \frac{1}{2}$, with no excited states thermally accessible over the temperature range used. The curves can be fitted well to a set of g -values of 3.09, 2.20 and 1.47 and an x,y -polarized optical transition.

By contrast, magnetization curves recorded from the redox-cycled enzyme exhibit a 'nesting', that is the curves measured at 1.6, 4.2 and 10 K do not overlay one another. This shows that the ground state is not an isolated $S = \frac{1}{2}$ system. Since the evidence from the m.c.d. spectra leads to the conclusion that all the haem groups are low-spin after redox-cycling, the simplest model to account for the m.c.d. magnetization properties is one in which a pair of low-spin ferric haem groups are exchange-coupled. Two haem groups with spins $S_1 = S_2 = \frac{1}{2}$ give two resultant electronic states with total effective spins of $S = 0$ and 1 . If the coupling is antiferromagnetic, the state with $S = 0$ is lower in energy than that with $S = 1$. In this case, if the exchange coupling were greater than, say, 10 cm^{-1} the ground state would be $S = 0$ and the resulting m.c.d. magnetization curves would not become field-independent at low temperature and high field. The magnetization curves will be more nearly linear. This model clearly does not account for the data shown in Fig. 6. It is, however, possible to simulate a set of curves of the type shown in Fig. 6 by means of a ground state with a total spin $S = 1$ and an axial zero-field splitting parameter D between 1 and 2 cm^{-1} . An example is shown in Fig. 7. Such a ground state will arise if the coupling between the two haem groups is ferromagnetic, leaving a ground state of $S = 1$ and a higher excited state of $S = 0$ not thermally accessible over the temperature range used. This model is consistent with the data. However, it is also possible that the coupling between the two haem groups is weak and antiferromagnetic such that the $S = 1$ and $S = 0$ states are very close in energy, say less than $2\text{--}3 \text{ cm}^{-1}$, with a large axial zero-field splitting parameter. We have not attempted to simulate magnetization curves for this situation because of the large number of arbitrary fitting parameters that arise. Both of the situations outlined above could lead to the loss of the e.p.r. signals at the X-band. Indeed, it would need a rather special combination of parameters to enable the e.p.r. spectra to be observable.

Hence the m.c.d. magnetization curves give evidence for exchange coupling between two low-spin ferric haem groups but do not at this stage give a unique set of parameters such as the spin and the magnitude of the coupling constant J or the zero-field parameters D and E .

Fig. 6. Magnetization curves for ferric nitrite reductase: (a) resting enzyme at 422 nm, (b) redox-cycled enzyme at 422 nm and (c) redox-cycled enzyme at 436 nm

The nitrite reductase concentration was $83 \mu\text{M}$ in 50 mM-Hepes buffer, pH 7.5, containing 50% (v/v) glycerol and 0.05% (v/v) Triton X-100. The path length was 1.5 mm. The signal intensity is shown as a percentage of the most intense signal observed.

Kinetic studies

As is the case with resting nitrite reductase (Blackmore & Brittain, 1986), rapid mixing of anaerobic redox-cycled nitrite reductase with dithionite in the stopped-flow apparatus produced biphasic reaction curves at 427 nm. The dithionite-concentration-dependence of the observed rate constant of the slower process

Fig. 7. M.c.d. magnetization curve simulated for an electronic ground state $S = 1$ subject to an axial zero-field splitting, $D = -1.80 \text{ cm}^{-1}$, and zero rhombic distortion, $E/D = 0$.

The ground state g -value was taken to be 2.0 and the electronic transition was assumed to be x, y -polarized. Temperatures are 1.60, 4.20 and 10.00 K. The magnetic-field range 0–5 T.

of dithionite reduction of resting and redox-cycled forms of the enzyme is shown in Fig. 8. The square root of the dithionite concentration is shown, as the active reductant with this enzyme is $\text{SO}_2^{\cdot -}$ (Blackmore & Brittain, 1986), which is generated by the dissociation of dithionite in solution (Lambeth & Palmer, 1973). It can be seen that for the resting enzyme the slower process

Fig. 8. Concentration-dependence of the slow phase observed in the reduction of resting (●) and redox-cycled (□) nitrite reductase by dithionite

Deoxygenated nitrite reductase ($1 \mu\text{M}$) was allowed to react anaerobically with dithionite in 60 mM-potassium phosphate buffer, pH 7.6, containing 0.05% (v/v) Triton X-100 in the stopped-flow apparatus. The reaction was monitored at 427 nm.

observed has a hyperbolic dependence of rate on dithionite concentration. The double-reciprocal plot, shown as an insert in Fig. 8, gives a limiting rate of 4 s^{-1} . The dithionite-concentration-dependence of the observed lower rate in the redox-cycled form is a straight line and does not show a rate limit. The dithionite-concentration-dependence of the observed rate of the faster process of the redox-cycled enzyme was found to be identical with that of the resting enzyme.

In the presence of 4 mM-nitrite and 100 mM-dithionite, the enzyme enters a steady-state turnover phase that lasts for several minutes. From the visible-absorption spectrum (not shown) it is clear that the band at 610 nm, present in the resting ferric enzyme, is missing. The resting form of the enzyme is not present during turnover.

DISCUSSION

The evidence presented in this paper shows unambiguously that the fully oxidized state of the nitrite reductase from *W. succinogenes* can exist in one of two states, namely, the resting and the redox-cycled forms. The resting form contains five low-spin ferric haem groups and a single high-spin ferric haem group. All the low-spin ferric haem groups are co-ordinated by two histidine ligands, and at least two of the haem groups are magnetically isolated. One of the five low-spin haem groups interacts magnetically with the high-spin ferric haem group. The spectroscopic signatures of this pair are e.p.r. signals at $g = 10.4$ and 3.7, an absorption band at 610 nm and a charge-transfer band at 850 nm, detected in the m.c.d. spectrum, belonging to the high-spin ferric haem partner.

The redox-cycled species contains only low-spin ferric haem groups. The magnetically isolated haem groups have identical e.p.r., m.c.d. and optical-absorption parameters in this species and in the resting state. However, the high-spin haem group has switched to a low-spin state by gaining a sixth ligand, possibly a histidine residue. The increase in the intensity of the charge-transfer peak at 1510 nm may signify an increase in the amount of bis-histidine-co-ordinated haem. Thus all six haem groups may be co-ordinated by histidine ligands in the redox-cycled state, both at room temperature and at 4.2 K. The special pair of haem groups appear to be magnetically coupled low-spin ferric. Although no e.p.r. signals at the X-band attributable to this pair have been found, the near-i.r. and visible-region m.c.d. spectra show clearly that all the haem groups are low-spin ferric. Evidence is obtained from the m.c.d. magnetization properties that the low-spin ferric special haem pair are exchange-coupled, probably with a weak coupling $|J|$ of $< 1 \text{ cm}^{-1}$. At least one component of the special pair is observable with a Soret peak shifted to longer wavelengths.

Redox-cycling of nitrite reductase with a range of reductants and oxidants leads to a change in the spin state and ligation state of the special pair of haem groups. This suggests that only electron transfer is required to effect the spin-state transition. Allowing the redox-cycled enzyme to remain at room temperature leads to the regeneration of the resting state in a process with widely variant time scale. The process may be preparation-dependent.

In a recent study of the enzyme we have shown that reduction with dithionite causes a switch in the spin state of the high-spin ferric haem group to a low-spin ferrous form (Blackmore *et al.*, 1990). This is attributed to the binding by this haem, in the reduced state only, of a ligand X, which could be the dithionite anion itself or more probably a product of dithionite oxidation. Since stoichiometric amounts of dithionite lead to a low-spin ferrous haem, the species must bind tightly to the haem. The rate constant for the pulsed-to-resting transition is slow, approx.

10^{-6} s^{-1} , whereas the turnover rate of the enzyme is $> 500 \text{ s}^{-1}$. Hence the formation of haem $\text{Fe}^{\text{II}}\text{-X}$ is of no kinetic significance in nitrite turnover. The fact that spectrally identical forms of the pulsed enzyme are produced by redox-cycling either with methyl viologen or with dithionite eliminates the possibility that the pulsed enzyme contains haem $\text{Fe}^{\text{III}}\text{-X}$.

After redox-cycling the observed rate of reduction with dithionite of the slower phase changes. This links the slow reduction phase to the special haem pair. The redox-cycled enzyme no longer exhibits the rate limit on internal electron transfer previously reported (Blackmore & Brittain, 1986). We therefore propose that the role of the redox-cycled form of the enzyme is to allow a faster internal electron transfer during turnover. Since evidence has been presented that the resting form is absent during the turnover, presumably the redox-cycled form is the dominant species throughout the turnover reaction.

The presence of two distinct ferric forms of nitrite reductase is reminiscent of cytochrome oxidase, which has been shown to exist in two states with different spectroscopic and kinetic properties, a resting state with a slow internal electron-transfer rate and a pulsed state with a rapid internal electron-transfer rate (Antonini *et al.*, 1977). Wilson *et al.* (1981) have proposed a two-state model for cytochrome oxidase with resting and pulsed components in equilibrium in both the oxidized and the reduced forms of the enzyme, with the resting form dominant in the oxidized enzyme and pulsed form dominant in the reduced enzyme. We see a similar pattern to this in the ferric nitrite reductase, but have been unable to find either kinetic or spectroscopic evidence for an alternative structural form in the reduced enzyme. It is clear, however, that conformational changes occur in the nitrite reductase during turnover and that these have an important role in controlling electron transfer.

R. S. B. thanks the Association of Commonwealth Universities for a Scholarship. C. G. and A. J. T. thank the Science and Engineering Research Council for support.

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Received 4 April 1990; accepted 18 April 1990