Characterization of the paramagnetic iron-containing redox centres of *Thiosphaera pantotropha* periplasmic nitrate reductase

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Abstract

Electron paramagnetic resonance spectroscopy signals attributable to low-spin haem c in the oxidised protein and [4Fe-4S]\(^{2+}\) in the dithionite-reduced protein were identified, at low temperature, in *Thiosphaera pantotropha* periplasmic nitrate reductase. Spin integration of these signals as well as elemental analysis suggest a stoichiometry of 1.3-1.6 c-haem and 1 [4Fe-4S] cluster per enzyme molecule. The E\(_{ox}\) (at pH 7.4) of the [4Fe-4S]\(^{2+}\) couple, -160 mV, means that it is unlikely to be physiologically reducible. Peptide sequences from the 90 kDa subunit indicate that the enzyme is a member of the family of molybdopterin guanine dinucleotide-binding polypeptides, the majority of which possess a putative [4Fe-4S] cluster binding sequence and thus may also bind a (low potential) iron-sulphur cluster.

Key words: Periplasmic nitrate reductase; Iron--sulphur protein; Cytochrome c-type; *Thiosphaera pantotropha*

1. Introduction

A periplasmic nitrate reductase linked to the electron transport chain is found in many species of bacteria. The enzyme from *Thiosphaera pantotropha* has been most extensively characterized [1,2] but the enzyme has also been purified from *Rhodobacter sphaeroides* f.sp. *denitrificans* [3], *Rhodobacter capsulatus* [4-6] and *Alcaligenes eutrophus* [7]. In *T. pantotropha*, the enzyme comprises a 90 kDa molybdopterin-binding catalytic subunit (NapA) and a 16 kDa cytochrome-c\(_{552}\) (NapB). Mass spectrometry and redox potentiometry suggested that NapB binds two haem groups [1]. In this paper we report the low temperature EPR characterisation of iron-containing centres in *T. pantotropha* periplasmic nitrate reductase.

2. Materials and methods

The periplasmic nitrate reductase was purified from *T. pantotropha* strain M6 (\(\Delta\)mutH::Trn(Kus)) [2] as previously described [1] except that the final gel filtration step was omitted. Protein concentration was estimated using the Bradford method [8]. Acid-labile sulphide was determined by the Methylene blue method of Fogo and Popowski [9] as standards [14]. Dithionite reduction of the enzyme was performed in the anaerobic glovebox by incubating the samples with excess dithionite for 15 minutes. Complete reduction was tested by establishing the development of a purple patch, indicative of reduced methylviologen (MV\(^{2-}\)), on applying an aliquot of the nitrate reductase sample onto a piece of filter paper impregnated with oxidised methylviologen (MV\(^{3+}\)). For amino acid sequence determinations, nitrate reductase preparations were reduced with 2-mercaptoethanol, resolved on a 10% SDS-PAGE gel and electroblotted onto a polyvinylidene difluoride-type membrane (ProBlott, Applied Biosystems Ltd., Warrington, UK) [54]. The nitrate reductase NapA polypeptide was excised and digested 'in situ' with trypsin (sequencing grade from Promega Ltd., Southampton, UK) essentially as described by Dutton [15]. Mediators (20 \(\mu\)M) were Methylene blue, Indigo carmine, anthraquinone-6,6-disulphonate, phenosafranine, benzylviologen, methylviologen and Neutral red. Samples were poised at room temperature then transferred to an EPR tube and frozen in liquid nitrogen. All potentials quoted are with respect to the normal hydrogen electrode.

3. Results

The 15K EPR spectrum of oxidised (as prepared) *T. pantotropha* periplasmic nitrate reductase is shown in Fig. 1. The rhombic signal, which we have assigned to low spin ferric haem, has \(g_x = 2.92\), \(g_y = 2.26\) and ...
$g_s = 1.5$. The presence of a small proportion of high spin haem is also indicated by the weak $g = 6.0$ resonance. The two derivatives at $g = 4.3$ and $g = 2.04$ are probably caused by, respectively, adventitiously bound FeIII and CuII. Integration of the $g$ component of the low-spin haem signal, of two different enzyme preparations, gave intensities corresponding to 1.3 and 1.6 spin/mol (based on an enzyme molecular weight of 110 kDa [1]). In the light of previous results [1], these figures are taken to indicate the presence of two c-haems per enzyme. Spectra acquired over a range of redox potentials show that the low-spin haem signal had maximal intensity above +125 mV and could not be detected at −100 mV. Such behaviour is consistent with the reduction potentials of the two c-haems determined previously by room temperature visible spectropotentiometry [1].

EPR spectra of the dithionite-reduced enzyme recorded at 18K and two different microwave powers are shown in Fig. 2a and b. The dominant rhombic spectrum ($g_1 = 2.03$, $g_2 = 1.94$ and $g_3 = 1.89$) is very intense and must therefore represent a major paramagnetic species in the sample. Variable temperature studies at 2 mW microwave power showed that this signal could be observed up to 40K and to be almost completely saturated below 10K. The optimal temperature for detecting the signal was 18K. At this temperature the estimated half-saturation power was around 10 mW. The spectra and relaxation characteristics of the major signal are typical of a [4Fe-4S]$^{1+}$ cluster. Accurate spin integration of the [4Fe-4S]$^{1+}$ signal is difficult because of additional signals in the $g$=2 area. These signals are a three-peak signal at $g = 2$, previously observed with the E. coli membrane-bound nitrate reductase and attributed to a 5-coordinate Fe–NO adduct [18-20], and an axial signal ($g_{\perp} = 1.98$ and $g_\parallel = 1.95$) visible at temperatures up to 150K, which could arise from Mo(V), possibly in a glycerol-inhibited form [21]. Integration of the main 18K signal, in three different enzyme preparations, after correction for the underlying species gave = 1.0 spin/mol enzyme. Mediated redox potentiometry established that the $g = 1.89$ peak of the iron sulphur signal titrated as a Nernstian $n = 1$ component with $E_m$ (at pH 7.4) = −160 mV (Fig. 3).

T. pantotropha periplasmic nitrate reductase was previously reported to contain non-haem iron but no acid-labile sulphide [1]. In the light of the EPR experiments we have reexamined the iron and acid-labile sulphide analyses using alternative analytical procedures and employing Desulfovibrio africanus ferredoxin I, which contains a single [4Fe-4S] centre [21], as a control. When normalised to the D. africanus ferredoxin analysis and taking into account the expected haem iron quantitation (2 mol per mol enzyme, as determined by mass spectrometry [1]) the data in Table 1 indicate that each periplasmic

![Fig. 2](image-url)
The low temperature EPR spectrum of the iron–sulphur cluster and its relaxation characteristics are typical of a $[4\text{Fe}-4\text{S}]^+$ cluster although there are examples of $[2\text{Fe}-2\text{S}]^+$ clusters with similar properties (e.g. in xanthine oxidase [38]). Comparison of the iron and acid-labile sulphide analysis (4 mol iron/4 mol sulphide/1 mol protein) with the spin count (1 spin/1 mol protein) supports the $[4\text{Fe}-4\text{S}]$ cluster identification as does the presence of a $[4\text{Fe}-4\text{S}]$ cluster consensus binding motif in NapA from other organisms (Fig. 4b).

The $[4\text{Fe}-4\text{S}]$ cluster is probably bound by the four cysteine residues at the N-terminus of the NapA polypeptide (Fig. 4b). This motif is conserved in the sequences of most other MGD-binding proteins suggesting that these polypeptides also ligand a $[4\text{Fe}4\text{S}]$ cluster (Fig. 4b). While such a cluster has previously been suggested from sequence analysis in some cases [7,27–29,31,36] biochemical evidence for such a cluster is limited to the finding of 3–4 atoms of non-haem iron in FdhF [38] and the possible association of iron sulphur clusters with NarG and DmsA after fragmentation of the enzyme complexes of which they are components [40,41]. An iron–sulphur cluster(s) is clearly present in three prokaryotic assimilatory nitrate reductases [42–44] which are expected to be structurally related to those of known sequence [25,26] (Fig. 4b). The type, number and redox behaviour of the iron sulphur cluster(s) in these assimilatory nitrate reductases is uncertain. No iron–sulphur centres have been detected biochemically in TorA [45,46] consistent with the absence of the cysteine motif in this enzyme (Fig. 4b). Most interestingly the sequence comparisons shown in Fig. 4b suggest that if an iron–sulphur cluster is liganded by the MGD-binding subunit of the membrane-bound nitrate reductases of E. coli then one of the cluster ligands is likely to be a histidine. Such ligation is unprecedented for a $[4\text{Fe}-4\text{S}]$ cluster.

<table>
<thead>
<tr>
<th>Material analysed:</th>
<th>Iron (mol/mol protein)</th>
<th>Sulphide</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. africanus ferredoxin I</td>
<td>3.5</td>
<td>2.7 (1.7)*</td>
</tr>
<tr>
<td>T. pantotropha periplasmic nitrate reductase</td>
<td>5.5</td>
<td>4.1 (1.8)*</td>
</tr>
</tbody>
</table>

*For sulphide, the unbracketed numbers are those obtained from samples assayed with internal standards (S$^2$–; standard addition) whilst the bracketed numbers are those obtained by comparison to an external standard curve. The standard addition procedure is more reliable as it takes into account the effect of the polypeptide in the assay system, which tends to lower the S$^2$– recovery efficiency. This efficiency was estimated to be ~80% for Ferredoxin I and ~50% for the nitrate reductase.

The data shown for nitrate reductase are for a single enzyme preparation. Analysis of two different nitrate reductase preparations and replicates of the ferredoxin sample indicate errors in the analysis of ±10%.

4. Discussion

The analysis of T. pantotropha periplasmic nitrate reductase presented in this paper demonstrates that the enzyme contains an iron–sulphur cluster. This redox centre has not previously been identified in a periplasmic nitrate reductase although the presence of some non-haem iron and a low sensitivity to the iron chelator bathophenanthroline had been observed [1,3].

![Graph showing EPR potentiometric titration of the periplasmic nitrate reductase iron–sulphur cluster.](image-url)
but two histidine ligands are indicated for Rieske-type [2Fe–2S] clusters ([47] and references therein). Although the reduction potential of the MGD-subunit iron–sulphur clusters in enzymes other than the periplasmic nitrate reductase is unknown, convincing assignment of both the high potential iron–sulphur centres of the E. coli major membrane-bound nitrate reductase to clusters in a subunit other than MGD-binding NarG [19,20,48] suggests that the NarG cluster, like that of NapA, is probably of relatively low reduction potential.

The periplasmic nitrate reductase pathway is linked to the respiratory electron transport chain at the level of the quinol pool [6] ($E_n$ approx +80mV). It is thus highly unlikely that the iron–sulphur cluster ($E_n = −160 mV$) in the periplasmic nitrate reductase could be reduced by the enzyme’s physiological electron donor. Such apparently physiologically irreducible redox centres are now recognised to occur in many electron transport systems e.g. the membrane-bound nitrate and DMSO reductases [19,20,41,48] and the tetrahaem cytochrome c subunit of the Rhodopseudomonas viridis reaction centre [49]. The function of such centres is obscure. The periplasmic nitrate reductase iron–sulphur cluster identified here might have a purely structural function for which a lack of physiologically accessible redox reactions would be an advantage. The reduction potential of the cluster (~160mV) is, however, considerably higher than clusters thought to have this function in cytoplasmic proteins [50-52]. A regulatory function for the cluster seems unlikely if the cluster is unable to change its reduction state in vivo. We suggest that the cluster may assist in electrons transfer between the c-haems in the NapB subunit and the MGD cofactor in NapA, acting not as a centre thought to have this function in cytoplasmic proteins [50-52]. A regulatory function for the cluster seems unlikely if the cluster is unable to change its reduction state in vivo. We suggest that the cluster may assist in electrons transfer between the c-haems in the NapB subunit and the MGD cofactor in NapA, acting not as a centre thought to have this function in cytoplasmic proteins [50-52]. A regulatory function for the cluster seems unlikely if the cluster is unable to change its reduction state in vivo. We suggest that the cluster may assist in electrons transfer between the c-haems in the NapB subunit and the MGD cofactor in NapA, acting not as a centre thought to have this function in cytoplasmic proteins [50-52].

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References