PARAMAGNETIC CENTERS OF THE NITRITE OXIDIZING BACTERIUM *NITROBACTER*

W. John INGLEDEW and Peter J. HALLING
Johnson Research Foundation, Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, PA 19174, USA

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1. Introduction

Nitrobacter is a chemoautotrophic bacterium which oxidizes the weakly reducing inorganic ion nitrite with oxygen and obtains from this process energy for carbon dioxide fixation via a Calvin cycle [1] and growth. As the midpoint potential of the nitrite–nitrate couple is reported to be +420 mV at pH 7.0 [2] the reduction of NAD^+ by nitrite is a thermodynamically unfavorable process without the input of energy.

The EPR profile of *Nitrobacter* sub-cellular particles resembles, in its major constituents, that of the nitrate reductase enzyme of *Micrococcus denitrificans* [3] and of *E. coli* [4]. EPR absorptions suggestive of molybdenum V, an iron–sulfur center paramagnetic in the oxidized form similar to the 'HiPIP-type' iron–sulfur center in mitochondria [5] and ferredoxin-type iron–sulfur centers [6] have been resolved in this bacterium. Although the requirements of *Nitrobacter* and *Micrococcus denitrificans* are opposite in terms of their relationship to the nitrite–nitrate couple it is perhaps not surprising that a functional nitrate reductase should resemble a functional nitrite oxidase.

Potentiometric techniques, combined with e.p.r. (electron paramagnetic resonance) spectroscopy have been used to identify several paramagnetic centers in *Nitrobacter,* all of which exhibit relatively high midpoint potentials. An e.p.r. absorption is observed in oxidized samples at g = 1.97 at temperatures above 20°K. The E_{m7} of this g = 1.97 signal is approximately +340 mV and it is suggested that the signal may originate from Molybdenum V. A g = 1.94 ferredoxin-type signal titrated biphasically giving components with E_{m7} at +60 mV, and at +320 mV and the HiPIP-type iron–sulfur centers (iron–sulfur center paramagnetic in the oxidized form), which were also observed, had E_{m7}'s of +280 mV and +130 mV.

2. Materials and methods

Nitrobacter winogradski cells were grown in batch culture [7] and the media was supplemented with 'trace elements': $FeSO_4$ 60 μM, $ZnSO_4$ 11 μM, $MnCl_2$ 1.5 μM, H_3BO_3 1.5 M, $Co(NO_3)_2$ 150 nM, $(NH_4)_6$ Mo_7O_{24} 45 nM, $CuSO_4$ 18 nM. The cells were harvested by a Sharples continuous flow centrifuge. Cells were re-suspended in a medium containing 2 mM EDTA, 1 mM reduced glutathione, 50 mM KCl, 100 mM sucrose, and 10 mM Tricine pH 7.8. Cells were broken by glass beads in a Braun MSK vibration mill obtained from Bronwill Co. Rochester, N.Y. (total of one minute at maximum rev/min.) and centrifuged at 10 000 *g* for 15 min to remove unbroken cells. The supernatant was re-centrifuged at 100 000 *g* for 1 h. The supernatant was discarded.

Redox titrations were conducted as described by Dutton [8] and samples for EPR were taken anaerobically at intervals during a titration, frozen rapidly in an isopentane–cyclohexane freezing mixture and stored in liquid nitrogen until used. E.p.r. spectra were obtained as described in the figure captions on a Varian E 4 spectrometer. Temperature control of e.p.r. samples was achieved by means of a variable temperature cryostat (Air Products Model LTD-3-110). Temperature was measured with a carbon resistor installed just below the bottom of the e.p.r. sample tube.

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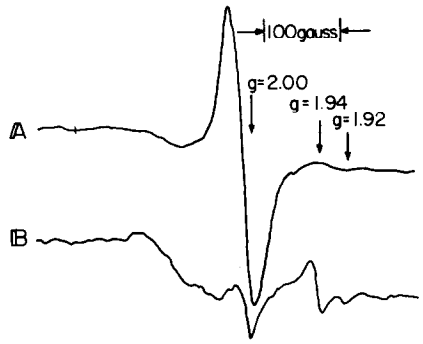

Fig.1. E.p.r. spectra of an oxidized and a reduced *Nitrobacter* membrane preparation. Spectra were taken at 7°K. The samples were either reduced with dithionite (B) or oxidized with ferricyanide (A). Medium was 50 mM KCl, 50 mM MOPS at pH 7.0. E.p.r. conditions: microwave power 5 mW, modulation amplitude 5 Gauss, scan time 250 Gauss per min, time constant 0.3 sec., modulation frequency 100 KHz, and microwave frequency 9.14 GHz.

3. Results

E.p.r. spectra of a sub-cellular preparation of *Nitrobacter* are shown in figs.1 and 2. At 7°K major differences between the e.p.r. spectra of oxidized and reduced samples can be seen at g = 1.94, 1.92 and 1.88 (paramagnetic in the reduced form) and at g = 2.01 (maximum at g = 2.02) paramagnetic in the oxidized form (HiPIP). At 20°K additional signals can be seen at g = 1.98, 1.96 (paramagnetic in the oxidized form). This signal is partially saturated at this temperature and is visible at liquid nitrogen temperatures (77°K). The HiPIP-type iron–sulfur center signal is not observed at temperatures above 20°K at a microwave power of 100 mW. The signals at g = 1.97 and 1.96 are found in the fully oxidized samples at 20°K (fig.2) and at 77°K. These two absorbances have been attributed to the same component because of their similar midpoint potential (about +340 mV, fig.3) and temperature dependence. The characteristics of this component are unusual and it is suggested that the signals are not due to a non-heme iron signal but to a signal from molybdenum V. Fig.3 is a plot of electrode potential versus signal height of the g = 1.97 signal, an E_{m7} of +340 mV was obtained although the curve does not adequately fit either an $n = 1$ or $n = 2$ line; the fit for the latter (drawn in fig.3) is the better.

In fig.1, an intensive absorption centered at g = 2.01 can be seen in oxidized samples. This absorption is only visible below about 15°K and resembles the mitochondrial HiPIP-center in line shape [10]. A

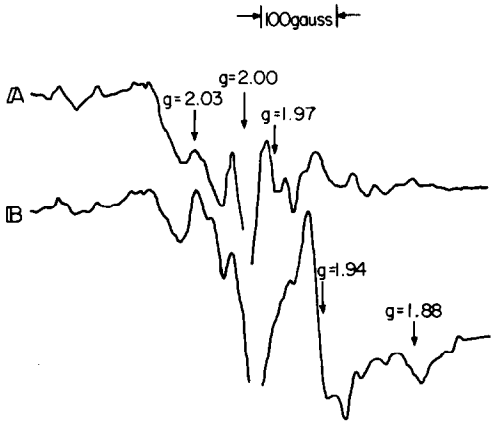

Fig.2. E.p.r. spectra of an oxidized (A) and a reduced (B) *Nitrobacter* membrane preparation. Spectra were taken at 20°K. All other conditions as in fig.1.

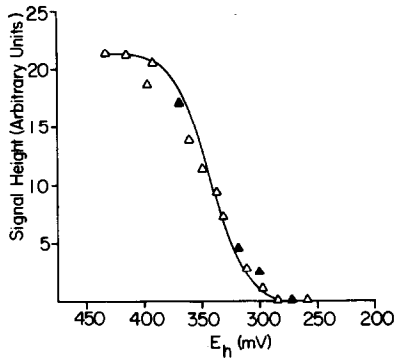

Fig.3. Amplitude of the g = 1.97 signal as a function of electrode potential. Samples were taken during a redox titration. *Nitrobacter* sub-cellular particles, 20 mg protein/ml, were suspended in 50 mM HEPES at pH 7.0 and 50 mM KCl. The redox mediators, ferricyanide, diaminodurol, phenazine methosulphate, phenazine ethosulphate, pyocyanine, duroquinone, indigo tetrasulphonate, 2-OH, 1,4 naphthoquinone, anthraquinone 2,6 disulphonate, anthraquinone 2 sulphonate, benzyl viologen and methyl viologen were added at various times during the titration at concentrations of between 25 and 75 μM. Aliquots of dithionite or ascorbate were used as reductant and ferricyanide was used as oxidant. Conditions for e.p.r. measurement were: temperature 20°K, microwave power 5 mW, modulation amplitude 10 Gauss, scan time 250 Gauss/min., time constant 0.3 sec., modulation frequency 100 KHz, and microwave frequency 9.14 GHz.

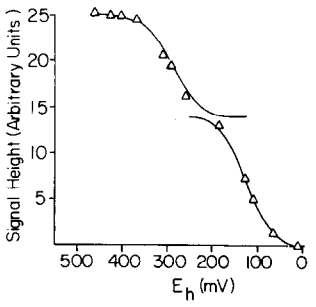

Fig.4. Signal amplitude of the 'HiPIP'-type absorption plotted as a function of electrode potential. Conditions are as described in fig.3 except that the temperature at which the spectra were taken was 7°K.

plot of the data from redox titrations of this component is shown in fig.4. Two components are resolved in the sub-cellular preparation; these are both $n = 1$ redox couples with E_{m7}'s at +280 mV and +130 mV.

There are at least two components capable of undergoing redox changes in *Nitrobacter* sub-cellular preparations which give signals at g = 1.94. These components are paramagnetic in the reduced form. Fig.5 shows a plot of electrode potential versus the signal height of the g = 1.94 signal at 5.5°K and at 13.5°K. It can be seen that at 13.5°K there are two separate electron transfer components absorbing at g = 1.94, with midpoint potentials at approximately +320 mV and +60 mV. When measured at 5.5°K the

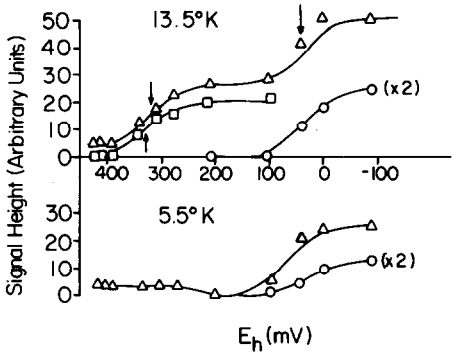

Fig.5. Plot of the electrode potential versus signal height for those signals from *Nitrobacter* which are paramagnetic in the reduced form. Redox titrations were carried out as in fig.3. Measurement was at 5.5°K and 13.5°K (△–△) g = 1.94, (■–■) g = 1.88 and (○–○) g = 1.92.

results are slightly simplified because the component with an E_{m7} at +320 mV is saturated and is not observed. It should be noted that at both temperatures there is an absorption at close to g = 1.94 (g = 1.93) even in the most oxidized samples. This signal is consistently observed but its function or meaning is not known.

Two other signals, paramagnetic in the reduced form, at g = 1.92 and g = 1.88 may be associated with the g = 1.94 components. The g = 1.88 signal may, on the other hand, be due to another component. The g = 1.92 signal has a midpoint close to that measured for the lower potential g = 1.94 signal. The temperature saturation profiles of the low potential g = 1.94 and g = 1.92 absorptions are similar. The g = 1.88 signal titrates with a midpoint of about +330 mV (fig.5) and this signal may belong to a third component, one with a temperature profile which differs slightly from that of the low potential g = 1.94 component.

4. Discussion

The respiratory chain of *Nitrobacter* has been reported to contain at least five cytochromes. These include a cytochrome *c* ($E_{m7.0}$, +274 mV), a cytochrome *a* ($E_{m7.0}$, +240 mV), a cytochrome a_3 ($E_{m7.0}$, +400 mV) and two a_1-type cytochromes ($E_{m7.0}$, +352 mV and +100 mV) [11,13]. The cytochromes *a* and a_3 are thought to constitute the terminal oxidase [11].

The midpoint potentials of all the detected components, are unusually high, having values in the region of +400 to +60 mV. Forget and Dervartanian [9] have reported e.p.r. studies on a NO_3^- reductase from *Micrococcus dentrificans* and have shown that when oxidized it exhibits signals at g = 1.98 and g = 2.045; these signals are attributable to Mo (V). In the same enzyme preparation there is a g = 2.01 signal 'due to Fe (III)' with a signal which resembles that of mitochondrial 'HiPIP' [10]. In addition to these two species there appears on reduction of the enzyme a ferredoxin type signal. Fig.6 speculates schematically on the data available concerning the redox properties of the *Nitrobacter* respiratory chain.

There are strong similarities between the signals of the high-potential components reported in the present

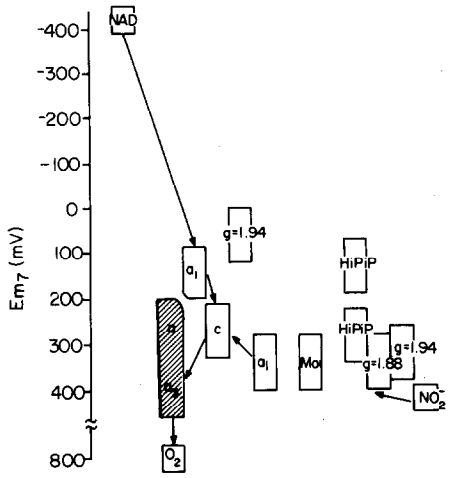

Fig.6. Thermodynamic profile of *Nitrobacter*. The cytochrome data is taken from reference [11]. The e.p.r. data is presented in the present work.

publication and those reported for the nitrate reductases. The essential differences between the *Nitrobacter* system and the nitrate reductase of *Micrococcus dentrificans* is that in *Nitrobacter* the physiological process is that of NO_2^- oxidation and the bacterium lives by coupling the oxidation of NO_2^- (E_m +420 mV) to the reduction of oxygen. The absence of e.p.r. signals from components with midpoint potentials more negative than zero is noteworthy. Preparations from this bacterium oxidize NADH rapidly and can couple this oxidation to ATP synthesis exhibiting respiratory control [1]. So some lower potential components would be expected. One of the reasons for the apparent absence of iron–sulfur centers of low potential (NADH segment) may be that they are present at concentrations much lower than those of the nitrite oxidase. For every electron passing up the electron chain from NO_2^- to NAD about seventy will be oxidized by oxygen [13].

One possible significance of the presence of molybdenum in *Nitrobacter* lies in the model proposed by Cobley [14] for oxidative phosphorylation in this bacterium. This scheme proposes that transmembrane hydride (H^-) transfer is involved in the respiratory chain in *Nitrobacter*, this scheme explains the differential effects of a membrane potential and a transmembrane pH gradient on the rate of nitrite oxidation. Hitherto the only biological hydride transfer demonstrated has been in the molybdenum containing enzyme, xanthine oxidase [15]. It is worth noting that molybdenum is an essential nutrient for *Nitrobacter* growth [16].

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