Identification of periplasmic nitrate reductase Mo(V) EPR signals in intact cells of *Paracoccus denitrificans*

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EPR spectroscopy has been successfully used to detect signals due to molybdenum (V) and ferric iron in intact cells of aerobically grown *Paracoccus denitrificans*. The signals are ascribed to the catalytic molybdenum centre and to the haem iron of the periplasmic nitrate reductase. These signals are absent

from a mutant strain deficient in this enzyme. The Mo(V) signal is due to the High-*g* Split species which has been well characterized in the purified enzyme. This confirms that the High-*g* Split is the physiologically relevant signal of a number observed in the previous work on the purified enzyme.

INTRODUCTION

The denitrifying bacterium *Paracoccus denitrificans* can express both membrane-bound and periplasmic respiratory nitrate reductases [1]. The membrane-bound enzyme has been studied by Mo(V) EPR [2] and the molybdenum centre appears to be very similar to that of the extensively characterized nitrate reductases A and Z from *Escherichia coli* [3–7]. The periplasmic nitrate reductase is an $\alpha\beta$ heterodimeric enzyme. The α subunit binds a molybdenum cofactor–guanidine mononucleotide moiety and a [4Fe-4S] cluster. The β subunit is a di-*c*-haem cytochrome [8,9]. The Mo(V) EPR signals from this enzyme have recently been described [10] and are quite distinct from those from the membrane-bound enzymes of *P. denitrificans* and *E. coli*. In *P. denitrificans*, the two nitrate reductases are differentially expressed: the membrane-bound enzyme is present in anaerobically grown cells and the periplasmic enzyme is present in aerobically grown cells [1].

An EPR study of the periplasmic nitrate reductase has identified three sets of signals, the High-*g* signals, the Very High-*g* signals and low *g*-value molybdenum hydroxylase-like signals [10] (note that by convention [11] capital letters are used to denote specific Mo(V) EPR signals with well-defined parameters). It was argued in the previous work that the High-*g* Split signal(s) is the only one likely to have any catalytic relevance. A Very High-*g* Mo(V) EPR signal has also been identified in the assimilatory nitrate reductase of *Azotobacter vinelandii* [12], a member of a group of assimilatory nitrate reductases that are closely related to the periplasmic nitrate reductases [13]. This signal was only observed in the periplasmic enzyme of *P. denitrificans* after treatment of the enzyme with cyanide, and intramolecular electron transport was found to be inhibited by this treatment. It was therefore proposed that the Very High-*g* Split signal in the *A. vinelandii* assimilatory enzyme was due to an inhibited or deactivated form of the enzyme [10].

A Mo(V) EPR study of intact cells of *P. denitrificans* was carried out with the aim of detecting which, if any, of the previously characterized Mo(V) EPR signal-giving states the molybdenum centre of the enzyme can adopt in the cell under anaerobic reducing conditions. Mo(V) EPR also has the potential for identifying inhibited or deactivated forms of the enzyme since

inactive forms of many oxomolybdenum enzymes, including the Very High-*g* species of periplasmic nitrate reductase, are known to exhibit Mo(V) EPR signals [10,11]. EPR was also carried out at liquid helium temperatures in order to detect signals attributable to the ferric *c*-type haems of the enzyme.

EXPERIMENTAL

P. denitrificans strains LMD 22.21 and PD 1222 were obtained from Dr. L. Robertson (Delft) and Dr. N Harms (Amsterdam) respectively. Strain H1 is a periplasmic nitrate reductase mutant constructed by disrupting the *napA* gene of PD 1222 with the Ω fragment ([14]; H. J. Sears, unpublished work). Cultures were grown aerobically with butyrate as sole carbon source and ammonia as sole nitrogen source as described elsewhere [1]. Cells were harvested by centrifugation and washed in phosphate buffer (10 mM, pH 7.5). Assays of the nitrate reductase activity of whole cells and Mo(V) EPR were carried out as described in earlier work [1,8,10]. Cell paste was centrifuged in quartz EPR tubes (30 min) and then rapidly frozen in liquid nitrogen (2 min) to provide samples. The viable counts of cells before and after a cycle of freeze/thawing were similar. Purified periplasmic nitrate reductase was prepared and characterized as in earlier work [8,12]. EPR spectroscopy was performed at 9.4 GHz as described previously [10]. Fourier filtering and least-squares fit third-order polynomial subtraction were employed only where stated using Bruker ER200-SH software.

RESULTS

Mo(V) EPR spectra were acquired from purified periplasmic nitrate reductase (40 μ mol of reduced Methyl Viologen radical (MV⁺) oxidized/min per mg of protein) and three strains of *P. denitrificans* (specific activities in parentheses): PD 1222 (2.8 μ mol of MV⁺ oxidized/min per mg of whole-cell protein); LMD 22.21 (1.0 μ mol of MV⁺ oxidized/min per mg of whole-cell protein); H1 (0 μ mol of MV⁺ oxidized/min per mg of whole-cell protein). The spectra from whole cells (Figure 1) are complicated by the presence of intense broad overlapping features which are due principally to Mn²⁺, but may also include some contribution

Abbreviation used: MV⁺, reduced Methyl Viologen radical.

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Figure 1 Mo(V) EPR signals from purified nitrate reductase and intact cells of *P. denitrificans*

The spectra are from (a) purified periplasmic nitrate reductase, (b) intact cells of *P. denitrificans* strain LMD 22.21, (c) intact cells of *P. denitrificans* strain PD 1222 and (d) intact cells of *P. denitrificans* H1. Spectra were recorded at 90 K, 20 mW microwave power. The arrowed lines between spectra identify local maxima and minima in spectra b and c corresponding to features in the High-*g* Split signal shown in spectrum a. In this and succeeding Figures, B/G means magnetic field strength in Gauss. (Note $1\text{ G} \equiv 10^{-4}\text{ T}$.)

from iron signals. However, the lines drawn between the spectra shown in Figure 1 clearly point out the features in *P. denitrificans* LMD 22.21 (spectrum b) and PD 1222 (spectrum c) that correspond to those of the High-*g* Split Mo(V) signal [10] from the purified enzyme (spectrum a). Better resolution of these features can be observed in the spectra presented as the second derivative of EPR absorption in Figure 2, although the signal-to-noise ratio of these higher-derivative spectra is clearly poor, despite signal-acquisition times of a number of hours. However, spectral resolution could alternatively be enhanced by computer methods, and Figure 3 shows examples of spectra that have been either Fourier-filtered to remove broad underlying features or have had a third-order polynomial subtracted. From Figures 1–3, then, it can be clearly seen that intact cells of *P. denitrificans* strains LMD 22.21 and PD 1222 do contain periplasmic nitrate reductase exhibiting the Mo(V) High-*g* Split signal whereas *P.*

Figure 2 Second-derivative Mo(V) EPR signals from purified nitrate reductase and intact cells of *P. denitrificans*

Spectra a–d are the second derivative of absorption ($\partial\chi/\partial B$) spectra corresponding to the first derivative ($\partial^2\chi/\partial B^2$) spectra a–d of Figure 1. Details of the samples and recording conditions are given in the caption to Figure 1.

denitrificans H1 (*napA::Ω*) is devoid of Mo(V) EPR signals. Alignment of the spectra (not shown) with the other classes of Mo(V) EPR signals exhibited by the purified enzyme and with signals [2] from the membrane-bound enzyme demonstrated the lack of any features attributable to either the Very High-*g* or the molybdenum hydroxylase-like Pseudo-Rapid signals of the periplasmic enzyme or to signals from the membrane-bound enzyme in any of the *P. denitrificans* strains examined.

In addition to Mo(V) EPR signals, the signals due to cytochromes associated with the periplasmic nitrate reductase

Figure 3 Mo(V) EPR spectra of purified nitrate reductase and of *P. denitrificans* LMD 22.21 and H1 after Fourier filtering and after polynomial subtraction

Spectrum a is of purified periplasmic nitrate reductase, spectra b and c are from *P. denitrificans* strain LMD 22.21 and spectra d and e are from intact cells of *P. denitrificans* H1. Spectra b and d were Fourier-filtered using an identical procedure in each case. Spectra c and e had a third-order polynomial subtracted from them. The raw data are presented in Figure 1 [spectra b (LMD 22.21) and d (H1)]. Spectra were recorded at 90 K, 20 mW microwave power.

system were also examined in the intact cells (Figure 4). The feature in the low-temperature spectrum of the enzyme (spectrum a) at $g \sim 2.9$ (i.e. ~ 2260 G, 226 mT) is due to the oxidized *c*-haem groups present in the as-prepared enzyme [8]. These centres are readily reduced by dithionite, with resultant loss in the EPR signal. This feature was found in spectra of intact cells of *P. denitrificans* strains LMD 22.21 and PD 1222 and the dithionite-dependent disappearance of this signal with time is shown by the spectra b, c and d of Figure 4 for *P. denitrificans* strain LMD 22.21. This feature, however, was entirely lacking in the spectrum from *P. denitrificans* H1 (Figure 4 spectrum e).

DISCUSSION

The data presented in this study represent the first successful detection of a defined Mo(V) signal from any nitrate reductase in intact bacterial cells. The use of intact cells inevitably results in low signal-to-noise ratios but the absence of the EPR signals in the nitrate reductase mutant provides clear evidence for our assignments. Detailed studies on the purified enzyme indicated that the molybdenum centre can adopt only three sets of EPR-distinguishable Mo(V) states for which putative structures were suggested [10]: (i) the High-*g* Split species; (ii) the molybdenum hydroxylase-like low-*g* Pseudo-Rapid species and (iii) the Very High-*g* species. The High-*g* Split was the only signal observed in

Figure 4 Low-temperature EPR signals from *P. denitrificans* and from purified nitrate reductase

Spectrum a is from as-prepared purified periplasmic nitrate reductase. Spectra b–d are from intact cells of *P. denitrificans* strain LMD 22.21; spectrum c was recorded after 2 min and d after 30 min incubation with 5 mM sodium dithionite. Spectrum e was recorded on intact cells of *P. denitrificans* H1 without treatment. Spectra were recorded at 15 K, 5 mW microwave power.

the present study of whole cells of *P. denitrificans*. The conclusion drawn from the earlier work [10], that, of the known Mo(V) species characterized by EPR, the High-*g* Split is the only catalytically relevant one, is supported by this finding [5]. The preparation of the bacteria for EPR analysis involved sedimenting cells in an EPR tube to a concentration of approx. 10^{11} /ml. At this cell density dissolved oxygen and any traces of nitrate would be rapidly exhausted. The metabolism of cellular carbon will increase the reduced state of the cytoplasmic pools of reductant (e.g. the nicotinamide nucleotide pool), providing a strong thermodynamic driving force for reduction of the respiratory electron-transport chain. Our data indicate that the driving force is not sufficiently reducing to reduce all of the molybdenum to the Mo(IV) state or all of the haems of the dihaem cytochrome *c*-552 to an EPR-silent state. We conclude that in this anaerobic state, in which the electron-transport system is reduced by physiological reductant, the nitrate reductase signals detected are likely to arise from a form of the enzyme in a physiologically relevant resting state.

The High-*g* Split EPR signal is indicative of the molybdopterin cofactor of the enzyme being in the dihydropterin rather than the tetrahydropterin state characterized by a low-*g* Pseudo-Rapid signal [10,15]. The reduction of the cofactor pterin from the dihydro to tetrahydro state requires reducing potentials much below the expected operating potentials of the physiological electron donors to the enzymes [10]. The present study confirms that even in the absence of respiratory electron acceptors, the conditions in the periplasm are not sufficiently reducing to produce the Pseudo-Rapid Mo(V) signal characteristic of the tetrahydropterin state. It is therefore unlikely that this species has any physiological relevance.

The third Mo(V) EPR signal detected in the periplasmic nitrate reductase, the Very High-*g* signal, was also not observed during the present study. This signal was reported to be present in preparations of *A. vinelandii* assimilatory nitrate reductase [12], although enzyme samples had been exposed to cyanate in the course of preparation. The signal could only be generated in the *P. denitrificans* enzyme using a procedure involving cyanide treatment [10]. Work on the purified enzyme also demonstrated that reduced haem and iron-sulphur centres of this form of the enzyme could not be reoxidized by treatment with nitrate. It should be noted that the generation of the Very High-*g* Split species in the nitrate reductase is not analogous to the generation of the desulpho forms of the molybdenum hydroxylases. The EPR parameters are quite different [10,16,17] and reaction of nitrate reductase with cyanide requires reducing conditions whereas molybdenum hydroxylases react at a sulphido group of Mo(VI) [10,16,17]. Failure to detect a Very High-*g* signal in whole cells of *P. denitrificans* is consistent with this inhibited form of the enzyme having no physiological relevance.

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