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Electron paramagnetic resonance studies on the nature of hemoproteins in nitrite and nitric oxide reduction

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SUMMARY

Nitrite and nitric oxide reductases and *c*-type cytochromes were isolated from nitrate-grown *Pseudomonas perfectomarinus.*

EPR measurements indicate the direct participation of *c*-type cytochromes in the reduction of nitrite to nitric oxide. The EPR resonance of heme-NO complexes is observed with *g*-values at 2.03 and 2.10 with ^{14}N hyperfine splitting of 16.2 Gauss. Quantitative EPR measurements indicate that all the *c*-type cytochromes involved in nitrite reduction are in the heme-NO complex forms and that these complexes account for about 15% of the total nitric oxide observed.

A different *c*-type cytochrome was found to be involved in the reduction of NO to N_2O with the major *g*-value of the heme-NO complex at 2.06. A time-dependent broadening of EPR was noted.

Intermediates in the anaerobic respiratory reduction of nitrate to nitrogen (bacterial denitrification) are nitrite, nitric oxide and nitrous oxide[1]. Others may exist, but have not been identified. Enzyme fractions catalyzing the separate reduction of (i) nitrite to nitric oxide, (ii) nitric oxide to nitrous oxide, and (iii) nitrous oxide to nitrogen have recently been derived from crude cell-free extracts of the marine bacterium *Pseudomonas perfectomarinus* grown anaerobically at the expense of nitrate[2]. These enzymes are either absent or produced in minimal quantity when the bacteria are grown aerobically, but are synthesized within a few hours when fully aerobically grown cells are incubated anaerobically even in the absence of nitrate.

Unlike fermentation, which is characterized by flavin-mediated reduction of the organic compounds that serve as terminal electron acceptors, anaerobic respiration of nitrate involves cytochromes in the terminal electron transport events[3]. Two *c*-type cytochromes that carry out nitrite and nitric oxide reduction have now been separated from *P. perfectomarinus* enzyme fractions. Removal of these cytochromes resulted in

partial loss of enzyme activity, and adding each back to the enzyme fraction from which it was derived restored reductive activity. It thus seemed possible that cytochrome-nitrite and cytochrome-nitric oxide interactions could be demonstrated with these preparations. The purpose of this paper is to describe the use of EPR measurements to demonstrate reduction of nitrite and binding of nitric oxide by these cytochromes.

Cells of *P. perfectomarinus*[4] were grown anaerobically at 25° with magnetic stirring in 40-l quantities of medium containing tryptone, 0.5%; sea salt, 2%; $FeCl_3$, 0.002%; KNO_3, 0.1%. When gas release reached its maximum rate, the bacteria were centrifuged and washed twice in 0.02 M phosphate buffer, pH 7.0, containing 2% NaCl to prevent lysis during washing. The washed cells were suspended in buffer without added salt and ruptured by two passes through a French pressure cell at 16 000 lb/inch^2 at 0–4°. The extract was centrifuged at 37 500 × *g* for 2 h and the precipitate discarded. The supernatant was then centrifuged at 150 000 × *g* for 2 h more and the pellet discarded. Nucleic acid was precipitated from the supernatant with 0.5% streptomycin sulfate, and samples containing 200 mg protein were fractionated in the cold by reverse flow on Sephadex G-200 that had been equilibrated with 0.02 M phosphate buffer, pH 7.0 (1.0 mM with respect to 2-mercaptoethanol). Active protein eluates were loaded on a DEAE-cellulose column after overnight equilibration with 0.02 M phosphate buffer, pH 7.0 (1.0 mM with respect to 2-mercaptoethanol). The proteins (monitored at 280 nm) were eluted with a continuous 0 to 0.5 M NaCl gradient.

Cytochromes (monitored at 418 nm) eluted from the Sephadex G-200 columns were concentrated by ultrafiltration at 4° and fractionated by chromatography on DEAE-cellulose with a continuous NaCl gradient as well.

Optical spectra were obtained with a Cary Model 15 recording spectrophotometer in Hellma spectrophotometric cells of 1 ml volume and 1 cm light path.

EPR measurements were made as described previously[4] with further experimental details recorded in the figure legends. Quantitation of the heme-NO resonances was achieved by double integration with Cu(II) - EDTA as the standard and on the assumption that the signal was attributable to a doublet (*cf.* ref. 5).

Nitrite reductase activity. This is a partially purified preparation of approx. mol. wt. 200 000 which requires free flavin (FMN + FAD), NADH and an added *c*-type cytochrome for reduction of nitrite to nitric oxide. NO formation was found to be proportional to the amount of added *c*-type cytochrome[6].

The absorption spectra of the reductase revealed in the oxidized state a maximum at 413 nm, which shifts on reduction to 417 nm, and an intensification of weak maxima at 548 nm and 522 nm. The reduced hemoprotein spectrum differed from that observed in the nitric oxide reductase. The added *c*-type cytochrome (of mol. wt. less than 17 000) revealed in the oxidized state a maximum at 406 nm and a broad maximum at about 520 nm. On reduction, maxima were observed at 415 nm, 520 nm and 548 nm.

In the absence of added reactants, the reductase displayed a weak EPR signal (Fig. 1A) presumably attributable to endogenous heme–NO complex formation. This EPR absorption increased slightly in the presence of added NADH, flavin and nitrite under helium probably as a result of additional small amounts of NO formed which reacted with the hemoprotein present in the reductase. When the *c*-type cytochrome was

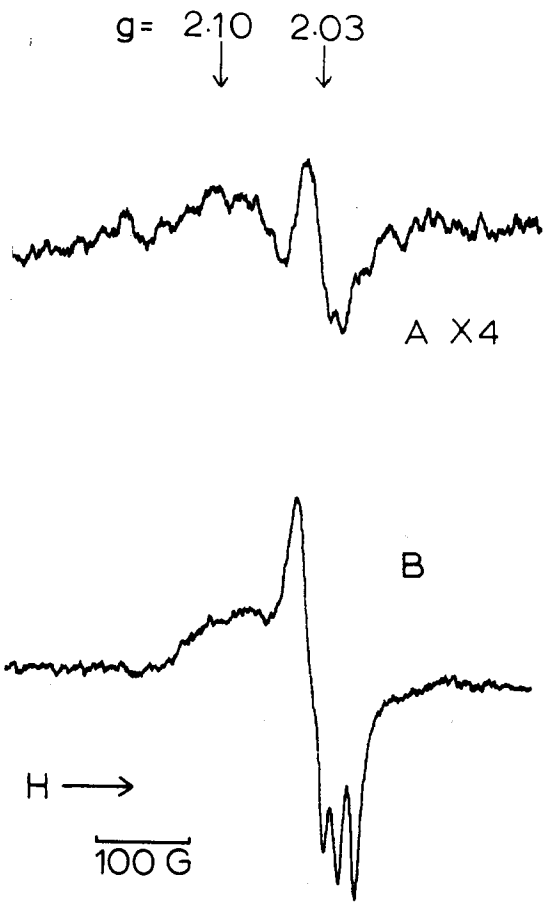

Fig. 1. EPR absorption of nitrite reductase. In these EPR studies all proteins and reactants were dissolved in 25 mM potassium phosphate buffer, pH 7.6. The anaerobic quartz tube as described in ref. 4 contained 4.8 mg of reductase in 250 μl of buffer. Based on a molar absorptivity of 28 000 (α peak, ferrous form), approx. 2.3 nmoles of *c*-type cytochrome were initially present (Trace A). The complete system contained in 250 μl of buffer: 4.8 mg reductase, 2.5 μmoles NADH, 1 μmole each of FMN and FAD, 10 μmoles of nitrite, 3.1 nmoles of added *c*-type cytochrome as described in the text (Trace B). EPR conditions: temperature, −191°; time constant, 0.3 sec; scanning rate, 400 Gauss/min; modulation amplitude, 5.9 Gauss; frequency, 9.239 GHz.

added to this system under helium, there was approximately a 2-fold increase in EPR absorption resulting in the type of spectrum observed in Fig. 1B. This absorption had *g*-values at 2.03 and 2.10 with a clearly resolved ^{14}N hyperfine splitting ($I=1$). The ^{14}N hyperfine splitting had a value of 16.2 Gauss. An EPR spectrum identical to Fig. 1B was observed under helium when the reductase was reacted directly with nitrite, NADH, free flavin and *c*-type cytochrome. The EPR absorption observed in Fig. 1B is due to a mixture of at least two heme-NO complexes arising when NO, the reduction product of nitrite, reacted strongly with the hemoprotein initially present and with the added *c*-type cytochrome. The EPR absorption did not change within 60 min even though NO formation was linear with respect to time, suggesting that there was a turnover of the

heme-NO system with release of NO from the active site of the reductase. This is supported by quantitation of the heme-NO resonances which account for approximately 15% of the total evolved NO.

The heme-NO complex is analogous to heme-NO complexes previously reported[5,7,8]. The evolution of NO as determined by gas chromotography is proportional to the amount of added *c*-type cytochrome suggesting that the known avidity of

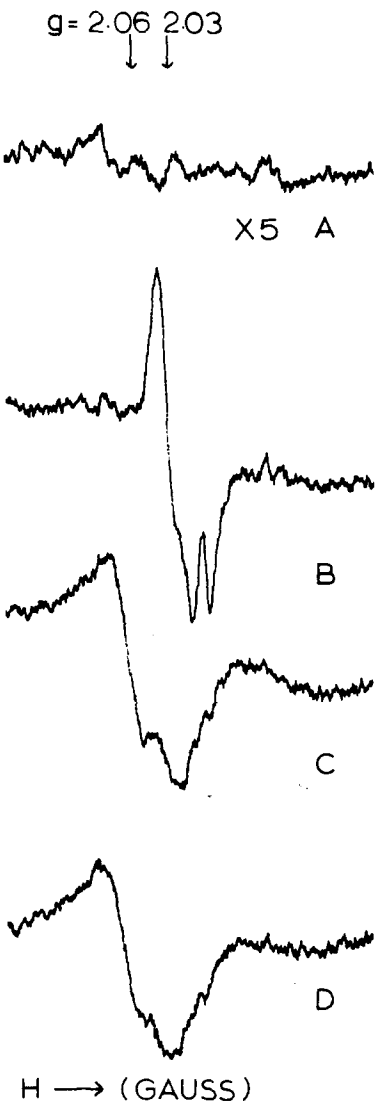

Fig. 2. Trace A: EPR absorption of nitric oxide reductase containing 27.0 mg protein per ml and 27.8 nmoles/ml of *c*-type cytochrome. Trace B: after exposure to nitric oxide *plus* 4 μmoles NADH (added in a volume of 2.5 μl to 250 μl of reductase) for 5 min at 25°. Trace C: as B but after 25 min at 25°. Trace D: after 60 min at 25°. EPR conditions as in Fig. 1 but at a frequency of 9.224 GHz.

hemoproteins for NO may be the driving force which shifts reduction equilibrium in the direction of NO formation.

Nitric oxide reductase. This is a partially purified preparation of approx. mol. wt. 800 000. The absorption spectra indicated a *c*-type cytochrome with a maximum in the oxidized state at 414 nm and maxima in the reduced state at 421 nm, 520 nm and 548 nm. NADH was required for reduction of NO to N_2O. In the absence of NO or NADH no EPR absorption was observed (Fig. 2A). When NADH was added under a nitric oxide atmosphere, there was an immediate reaction (within 30 sec), and a new EPR signal appeared at g=2.03 with a poorly resolved ^{14}N triplet (Fig. 2B). The signal is different from that observed in the nitrite reductase system suggesting that a different *c*-type cytochrome is involved. This presumption was supported by the different oxidized-reduced optical spectra that were seen. Quantitative EPR determinations indicated that essentially all the *c*-type cytochrome was in the heme-NO form.

Nitric oxide was in excess with respect to the *c*-type cytochrome. However, after 25 min (Fig. 2C) when N_2O formation was maximal (approx. 1.5 μmoles N_2O), the initial EPR absorption had broadened, decreased slightly in intensity and shifted in g-value to 2.06. In addition, the weak ^{14}N splitting was lost. Two likely explanations for the broadened EPR absorption are: (i) interaction of N_2O with NO at the active site and (ii) a conformational change in the heme-NO complex as a function of time. The latter possibility is supported by the observation that the reaction of N_2O with the reductase did not result in any detectable EPR absorption and that the reaction of NO with reductase in the absence of NADH resulted in a time-dependent broadening with the main g-value at 2.06, similar to Fig. 2D.

Although within 25 min no significant change occurred in the EPR heme-NO complex, there was very likely a turnover of the heme-NO system; for in this period there was linear evolution of N_2O as detected by gas chromatography.

These studies provide the first EPR evidence that the involvement of the *c*-type cytochromes in the formation of NO and N_2O is very likely in the form of heme-NO intermediates. Hemoproteins have been implicated by other workers in both nitrite and NO reductase activities[9-11]. The exact mechanism for nitrite or NO reduction remains to be established.

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