Arch Microbiol (1984) 137:79–84

Archives of
Microbiology

A dissimilatory nitrite reductase in *Paracoccus halodenitrificans*

Michael A. Grant* and L. I. Hochstein

Extraterrestrial Research Division, NASA Ames Research Center, Moffett Field, CA 94035, USA

Abstract. *Paracoccus halodenitrificans* produced a membrane-associated nitrite reductase. Spectrophotometric analysis showed it to be associated with a cd-cytochrome and located on the inner side of the cytoplasmic membrane. When supplied with nitrite, membrane preparations produced nitrous oxide and nitric oxide in different ratios depending on the electron donor employed. The nitrite reductase was maximally active at relatively low concentrations of sodium chloride and remained attached to the membranes at 100 mM sodium chloride.

Key words: Denitrification – Halophile – Nitrite reductase – *Paracoccus halodenitrificans* – cd-Cytochrome

Paracoccus halodenitrificans is a moderately halophilic bacterium which produces two nitrite reductases under denitrifying conditions. One, a soluble enzyme, produces ammonia from nitrite (Hochstein and Cronin, manuscript in preparation), whereas the other is membrane-bound and produces nitrous oxide. The soluble enzyme is associated with a cd-type cytochrome and requires a mediator such as methyl viologen in addition to a reducing agent for activity. The membrane-bound enzyme requires only a reducing agent such as dithionite, and initial spectral studies failed to reveal any features resembling a cd-cytochrome. In this paper[1], we will describe some of the properties of the membrane-bound enzyme and show that in spite of the absence of obvious spectral features, the membrane-bound enzyme is indeed a cd-type cytochrome nitrite reductase.

Materials and methods

Microorganism and growth conditions

Paracoccus halodenitrificans (ATCC 13511) was grown in the complex medium described by Sadler et al. (1980), except that the glycerol was replaced with 0.5% sodium lactate. Sterile nitrate (1% wt/v) or nitrite (0.25% wt/v) was added to the medium after it had been autoclaved and cooled. The inoculum was prepared as follows. First, the cells were allowed to grow aerobically at 30° C in 50 ml of medium containing 1% nitrate. A second flask, containing the same medium, was inoculated with cells from the aerobic culture, and incubated anaerobically for 24 h. A 1% inoculum from this culture was used to inoculate 200 ml of complex medium containing 0.25% nitrite, and was incubated anaerobically at 30° C. After 24 h, the entire culture was used to inoculate 6 l of 0.25% nitrite-containing medium, which had been autoclaved on the day of inoculation. The cells were harvested by centrifugation at 9,000 × *g* for 30 min at 4° C after the culture had entered the early stationary phase. No nitrite was detectable in the growth medium at this time. The usual cell yield was 2.5 g wet wt/l medium.

Preparation of the membrane fraction

The cells were suspended in 50 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) buffer (pH 7.4) which contained 1 M NaCl, 10 mM $MgCl_2$, and 100 μm dithiothreitol (HD buffer). The cell-buffer ratio was adjusted to 1:3 (wet wt/v) and the cells were homogenized at 4° C in a Sorvall Omni-Mixer for about 20 s. The homogenized cells were passed through a chilled French pressure cell operated at approximately 15,000 psi (1×10^5 kPa). Intact cells and debris were removed by centrifugation at 9,000 × *g* for 30 min and the resulting supernatant fraction was centrifuged for 1 h at 175,000 × *g*. The supernatant from this centrifugation (S_2) was decanted (and used as the source of the soluble nitrite reductase), whereas the sedimented material was suspended in HD buffer and recentrifuged for 1 h at 175,000 × *g*. The resulting supernatant was discarded and the pellet was suspended in HD buffer to a final protein concentration of about 15 to 20 mg · ml^{-1} and designated as the P_3 fraction. It was stored at −19° C and lost less than 25% of its initial activity over a period of 2 months.

Preparation of spheroplasts

Cells were suspended in 1 M NaCl, 50 mM [N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] (TES), 100 μm dithiothreitol (pH 8.0) buffer to a cell:buffer ratio of 0.3 and sedimented by centrifugation at 6,000 × *g* for 10 min. This was repeated for a total of three washes, after which the cells were suspended in the same buffer to a wet wt cell concentration of 110 mg · ml^{-1}. The cell suspension was gently stirred and made 10 mM with respect to Na_4EDTA and 0.1% (wt/v) with respect to egg-white lysozyme. After incubating for 10 min at room temperature, spheroplast formation was complete and they were sedimented by centrif-

Offprint requests to: L. I. Hochstein

* M. A. Grant is a National Research Council research associate

1 Portions of this paper were presented at the 1983 Annual Meeting of the American Society for Microbiology, New Orleans, Louisiana, March 1983

ugation for 10 min at 6,000 × *g*. The supernatant was designated as the "periplasmic space" fraction. The pellet was gently suspended in 1 M NaCl/10 mM $MgCl_2$/50 mM TES-100 μM dithiothreitol (pH 8.0), centrifuged, and the pellet was made up in the same buffer.

Assays

Unless otherwise noted, nitrite reductase activity was assayed by gas chromatographic analysis in reaction mixtures which contained the following additions in a total volume of 1 ml: phosphate buffer (pH 7.0, 160 μmol); $NaNO_2$ (5 μmol); an appropriate electron donor as indicated; and the P_3 fraction (from 0.5 to 0.9 mg protein). The reaction was carried out at 30° C in 5-ml vials which were sealed with trilayer silicone septa. The phosphate buffer was degassed by boiling it for about 30 s and then saturating it with helium; all of the reagents to be added to the vials were made using this buffer. The head space was filled with a gas mixture consisting of 1 % krypton in helium. The former gas served as an internal standard. Gas samples were withdrawn from the reaction vials using a Pressure-Lok syringe and injected onto a Poropak Q column (25 ft by 0.042 in.). The column was operated at 18° C at a flow rate of 18 ml He/min. A Carle Model 1100 microdetector and a Hewlett-Packard 3380A integrator were used to quantitate gas production. Under these conditions, nitrogen appeared after 1.9 min, nitric oxide after 2.36 min, and nitrous oxide after 11.83 min. The solubility of nitrous oxide and nitric oxide in the reaction mixture was determined empirically and found to be 0.43 ml nitrous oxide and 0.03 ml nitric oxide per ml of reaction mixture. Residual nitrite in the reaction mixture was measured by the diazotization method of Showe and DeMoss (1968).

Nitrate reductase activity was assayed at 30° C by determining nitrite formation in reaction mixtures which contained the following additions in a total volume of 3 ml: NaTES (pH 7.0, 50 μmol); methyl viologen (600 nmol); KNO_3 (15 μmol); $Na_2S_2O_6$ (6 μmol); and the membrane fraction, containing no more than 21 μg/ml protein. The reaction was started by adding dithionite. At appropriate times, aliquots were removed and assayed for nitrite by the diazotization reaction.

Proteins were determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. The membranes were solubilized by the addition of sodium dodecyl sulfate to a final concentration of 0.1 %.

Spectral Studies

Spectra were obtained using an Aminco DW-2A spectrophotometer. The spectra were stored in a MIDAN T microprocessor for further analysis. The so-called fourth-derivative spectra were obtained by sequential differentiation, as described by the manufacturer. Unfortunately, the manufacturer cannot document how such spectra are obtained and hence we are unable to supply details concerning the differencing intervals. When dual-wavelength kinetics were employed, the reaction was carried out in cuvettes which were sealed with a serum cap and whose atmosphere was replaced with helium. The isosbestic points for these experiments were obtained using the soluble cd-cytochrome which had been purified to homogeneity (Hochstein and Cronin, manuscript in preparation).

Table 1. Requirements for membrane-bound nitrite reductase activity

Reaction mixture	Specific activity[a]
Complete[b]	83
+ methyl viologen[c]	32
+ benzyl viologen	26
− enzyme control	0
− nitrite control	0
− dithionite control	0

[a] Units are nmol nitrous oxide formed · min^{-1} · mg $protein^{-1}$

[b] The complete reaction mixture was that described in the Materials and methods section, and included 0.7 mg of membrane protein added as the P_3 fraction

[c] The concentration of the viologen dyes was 4 mM

Chemicals

Sodium ascorbate, phenazine methosulfate, NADH, NADPH, and bovine serum albumin were obtained from the Sigma Chemical Company. FAD and FMN were obtained from Boehringer Mannheim. Egg-white lysozyme was obtained from Miles-Seravac.

Results

Properties of the P_3 fraction

When dithionite was the reductant, nitrous oxide was the only product of nitrite reduction. As shown in Table 1, inclusion of a mediator such as methyl or benzyl viologen resulted in a significant reduction of activity. No gas formation was observed in the absence of membrane preparation, nitrite, or dithionite.

It was not possible to demonstrate an absolute monovalent-cation requirement because the various components of the reaction mixture contributed cations. However, V_{max} (extrapolated to saturating nitrite concentrations) was stimulated by NaCl, reaching a maximum value at a monovalent-cation concentration of about 200 mM for either Na^+ or K^+. The apparent K_m for nitrite (270 μM) was relatively high for a dissimilatory nitrite reductase (Firestone 1982) and was unaffected by the NaCl concentration when it was varied from 106 to 600 mM[2]. These observations are consistent with the earlier observation (Sadler et al. 1980) that various enzymes from *P. halodenitrificans* were maximally active at relatively low concentrations of NaCl (< 250 mM). The membrane-bound nitrite reductase was stable in HD buffer containing 100 mM NaCl. After 4 days at 4° C only 15 % of the initial activity was lost. Furthermore, following this incubation period enzyme activity was still sedimented after centrifugation at 100,000 × *g* for 1 h.

The location of the nitrite reductase was determined using EDTA/lysozyme to prepare spheroplasts. As shown in Table 2, spheroplasts failed to cause the disappearance of nitrite. We cannot claim that nitrite never entered the cell, because washed resting cells produced nitrous oxide in the presence of nitrite (data not shown). Spheroplasts also failed

2 For these experiments, the phosphate buffer was replaced with the sodium salt of TES. In the absence of added NaCl, the Na^+ concentration was 106 mM

Table 2. Localization of nitrite reductase activity in *Paracoccus halodenitrificans*

Fraction	Total units[a]		
	Nitrite reductase[b]	NADH oxidase[c]	Nitrate reductase
Spheroplasts	0	0	96
Membrane vesicles (P_2)	1.2	19.7	332

[a] Units are $\mu mol \cdot min^{-1}$
[b] The nitrite reductase was assayed colorimetrically by the disappearance of nitrite in a reaction mixture containing 6 µmol dithionite
[c] The NADH oxidase was assayed as described in Sadler et al. (1980)

Table 3. Products of nitrite reduction with various electron donors

Electron donor[a]	Product[b, c]	
	Nitric oxide	Nitrous oxide
None	0	0
Formate	11	5
Succinate	15	5
Ascorbate/PMS	0	380
NADH	21	34
NADH + FMN	0	130
NADH + FAD	0	150
NADPH	0	0
NADPH + FMN	0	26
NADPH + FAD	0	5
Dithionite	0	75

[a] Electron donors were present in the following quantities: flavins, 50 nmol; PMS, 100 nmol; all others, 500 nmol
[b] Units are $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$
[c] No nitric oxide or nitrous oxide production was observed in the absence of the membrane fraction or nitrite

to exhibit NADH oxidase activity; this is consistent with the location of the oxidase on the inner side of the membrane. When the spheroplasts were passed through a French pressure cell, the resulting membrane vesicles exhibited NADH oxidase activity and catalyzed the disappearance of nitrite. These observations suggest that nitrite reductase was located on the cytoplasmic side of the membrane. The data with nitrate reductase activity were not as clear-cut. About 20 % of the nitrate reductase activity detected in the membrane fraction was found in the spheroplast preparations. This suggests that the nitrate reductase had a transmembranous distribution so that it was partially accessible, or that MV^+ was able to penetrate the membrane.

Gas production by membrane preparations

The products of nitrite disappearance were nitric and nitrous oxide (Table 3). Dinitrogen was not a product. This is consistent with the observation that cells which produced nitrogen while growing failed to do so after they were harvested. Nitric and nitrous oxide were detected when formate, succinate, or NADH were used as an electron donor (Table 3). However, only nitrous oxide was produced when either dithionite or ascorbate/phenazine methosulfate (PMS) was used as an electron donor. No gas formation was observed when ascorbate, NADPH, FAD, or FMN was added individually. However, when NADPH and either flavin were incubated together, nitrous oxide was produced. Flavins also increased the rate of nitrous oxide production from NADH.

Table 4. Reduction of nitric oxide to nitrous oxide by membrane preparations

Electron donors	Nitrous oxide[a, b]
None[c]	0
Formate	7
Succinate	7
Ascorbate/PMS	190
NADH	12
NADH + FMN	104
NADH + FAD	101
NADPH	9
NADPH + FMN	82
NADPH + FAD	52

[a] Units are $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$
[b] No nitrous oxide production was observed in the absence of the membrane fraction or nitric oxide
[c] Reaction mixtures contained 18 µmol of nitric oxide

The membrane preparations also produced nitrous oxide from nitric oxide when suitably supplemented (see Table 4). It is interesting to note that whereas NADPH alone was ineffective when nitrite was the oxidant, significant quantities of nitrous oxide were produced when nitric oxide was used as an oxidant. Furthermore, the addition of either flavin to NADPH stimulated nitrous oxide formation. No nitrous oxide was detected when ascorbate was present, but ascorbate in the presence of PMS was the most effective reductant. A considerable quantity of nitrous oxide was detected when dithionite was used. However, at least 85 % of the gas was produced in the absence of the membrane fraction, indicating that the bulk of the nitrous oxide was produced non-enzymatically.

Spectral studies

A room-temperature dithionite-reduced difference spectrum of the membrane preparation had an absorption band with a maximum at about 549 nm and a shoulder at about 555 to 560 nm. While no definite absorption features were detected in the 600- to 700-nm region of the spectrum, there was a suggestion of some structures at about 630 to 640 nm, indicating the presence of a cd-type cytochrome (Fig. 1 a). When the 540- to 565-nm region was reexamined by taking a fourth-order finite difference spectrum, the results shown in Fig. 1 b were obtained. The broad absorption band was separated into several components, two of which corresponded to bands expected for the absorption maxima for the c-component of a cd-cytochrome (549 and 553 nm).

The absorption band located at 549 nm underwent redox changes consistent with those expected for the c-component of the cd-cytochrome. In addition, it was possible to demonstrate analogous changes in the 600- to 700-nm region. Figure 2 represents data taken from a typical experiment in which spectral changes associated with nitrite reduction were monitored using dual-wavelength kinetics. The trace in Fig. 2a represents the redox changes associated with the c-component. In this experiment, reduction was associated

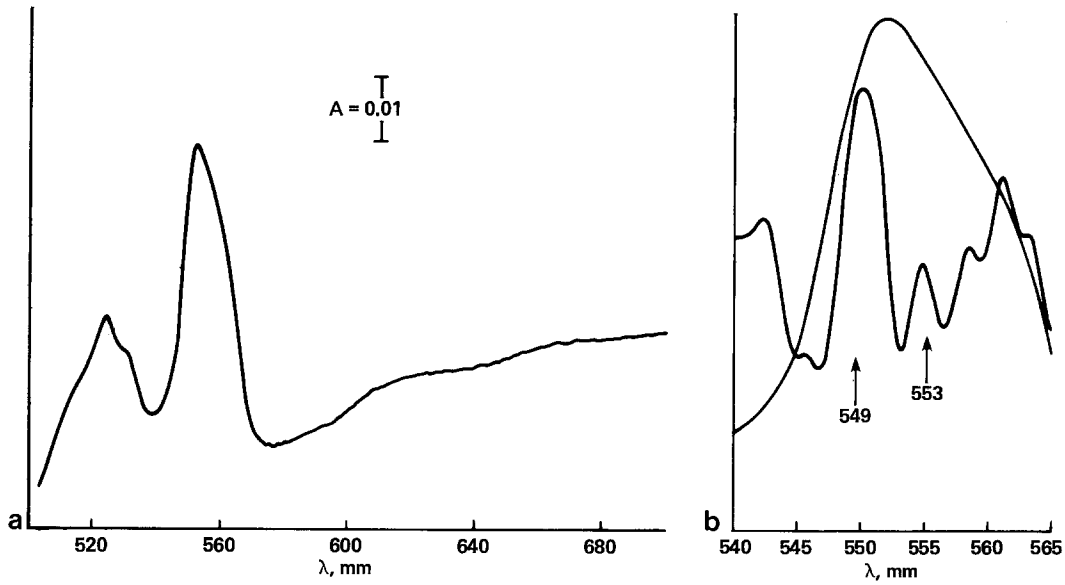

Fig. 1a, b. Reduced minus oxidized difference spectrum of the membrane fraction and its fourth-order finite difference spectrum. The membrane fraction, containing 18 mg · ml^{-1} protein, was diluted 1:15 with 1 M NaCl/50 mM HEPES/10 mM $MgCl_2$/pH 7.4 buffer. **a** The spectrum was obtained by oxidizing the reference cuvette with ferricyanide and reducing the sample cuvette with dithionite. The spectrum was scanned from 500 to 700 nm at 2 nm · s^{-1} and at a slit width of 1 nm. **b** The fourth-order finite difference spectrum was obtained by differentiating the region from 540 to 565 nm sequentially four times using a Midan T microprocessor

with an increase in the differential absorption measured at 549 minus 543 nm. The addition of ascorbate resulted in an increase in absorption (in other experiments this increase persisted for at least 30 min). The differential absorption increased when PMS was added and decreased when nitrite was added. At the end of the reaction, the residual absorption corresponded to the level of absorption initially produced by ascorbate. This was consistent with the failure to detect gas formation using ascorbate alone, whether nitrite or nitric oxide was used as the oxidant. The residual differential absorption at 549 minus 543 nm disappeared when oxygen was introduced into the cuvette. The redox changes associated with the putative d-component are shown in Fig. 2b. No spectral change was observed when ascorbate was added. This is consistent with the notion that the ascorbate-dependent change observed in Fig. 2a was not associated with the cd-cytochrome. Upon addition of PMS, a rapid reduction was observed (as shown by the decrease in differential absorption), which was reversed upon the addition of nitrite. The difference in the rate at which the d-component was reduced and oxidized reflected the fourfold difference in membrane concentration and the greater sensitivity in the assay for the d-type component. The changes in the differential absorption at 549 minus 543 nm could also be interpreted as reflecting the reduction and oxidation of a c-type cytochrome associated with the electron transport system and which donates electrons to the nitrite reductase. However, these changes were also consistent with the redox changes associated with the d-component of the putative cd-cytochrome nitrite reductase (Fig. 2b).

Discussion

Cytochrome-cd type nitrite reductases have been identified in *Thiobacillus denitrificans* (Le Gall et al. 1979; Sawhney and Nicholas 1978), *Alcaligenes faecalis* (Iwasaki and Matsubara 1971), *Pseudomonas aeruginosa* (Wharton and Weintraub 1980; Yamanaka and Okunuki 1963), *Pseudomonas perfectomarinus* (Zumft et al. 1979), *Pseudomonas stutzeri* (Kodama 1970), and *Paracoccus denitrificans* (Lam and Nicholas 1969). The product of nitrite reduction appears to be nitric oxide, although the nitrite reductase from *T. denitrificans* produces nitric oxide and nitrous oxide (Sawhney and Nicholas 1978).

The identification of the nitrite reductase in *P. halodenitrificans* as a cd-cytochrome was indirect. Membranes from nitrite-grown cells lacked spectral features that were clearly associated with a cd-cytochrome (i.e., a split reduced alpha peak located at 549 and 553 nm (cytochrome c) and the oxidized d peak around 635 nm). The possibility that the reductase was a copper enzyme was examined, but two experiments ruled out this possibility. First, we were unable to demonstrate an inhibition using the copper chelator diethyl dithiocarbamate (Iwasaki and Matsubara 1972). Second, dual-wavelength kinetics, comparing the spectral changes at 549 and 635 nm, were consistent with the operation of a cd-cytochrome.

There is some controversy concerning the location of the cd-cytochrome nitrite reductase. It has been described as a soluble enzyme and as a loosely membrane-associated enzyme (Payne 1981; Knowles 1982). The location of nitrite reductases on the membrane would be consistent with their role in energy conservation (Koike and Hattori 1975). The cd-cytochrome nitrite reductase in *T. denitrificans* is firmly membrane-associated (Sawhney and Nicholas 1978). Lam and Nicholas (1969) found about 30 % of the nitrite reductase activity in *P. denitrificans* remaining in the pellet fraction. Recently, Zumft and Vega (1979) described nitrite reductase activity in the membrane fraction of *Pseudomonas perfectomarious*, although the majority of nitrite reductase activity was previously shown to exist in the soluble fraction (Zumft et al. 1979). The dissimilatory enzyme is clearly

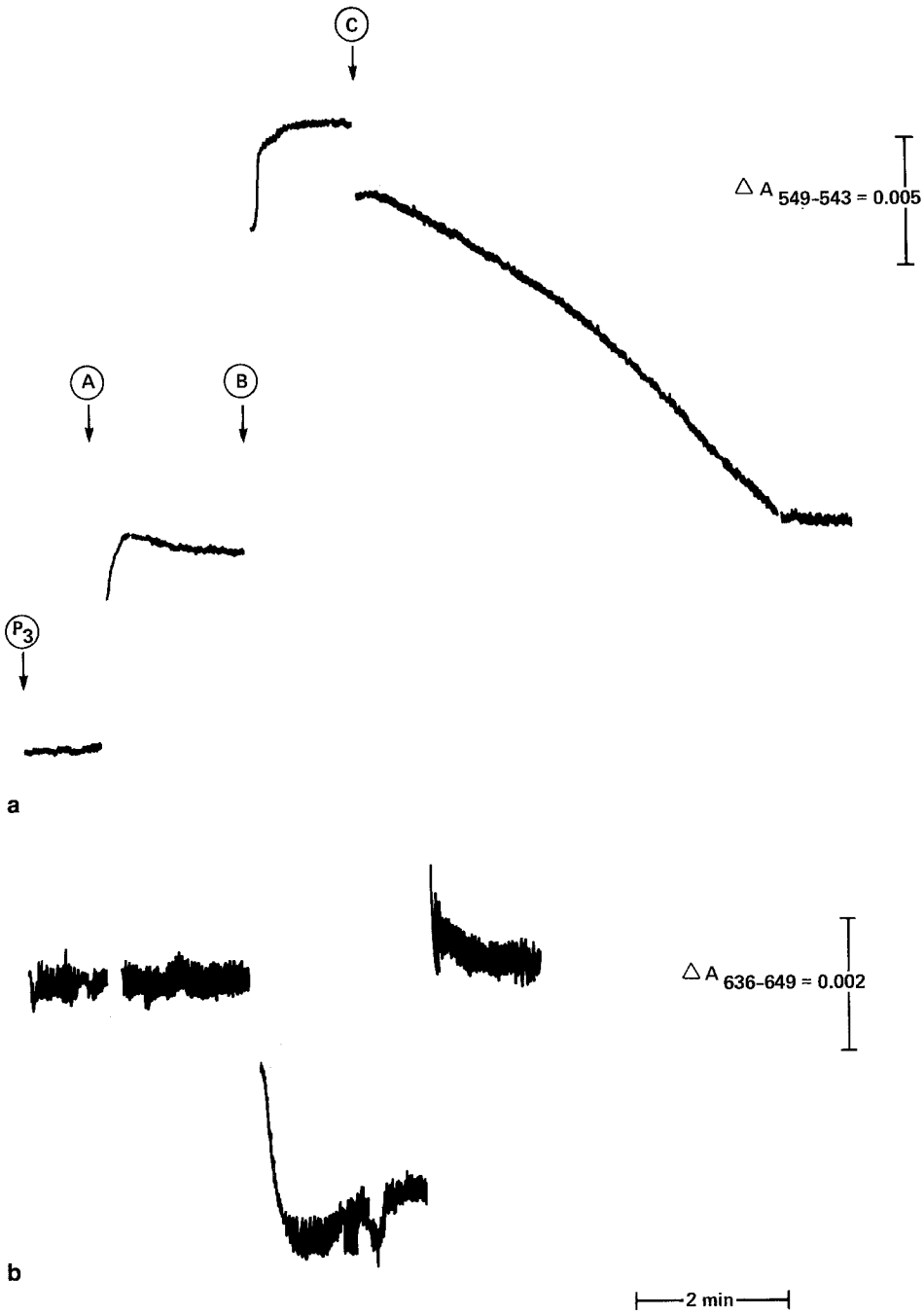

Fig. 2a and b
The kinetics of cytochrome reduction and oxidation as measured by dual wavelength kinetics. The membrane fraction was diluted in 400 mM/pH 7.0 phosphate buffer which had previously been saturated with helium. One milliliter of the diluted membrane was placed in a cuvette with a helium atmosphere and sealed with a serum stopper. Where indicated, 1 μmol of ascorbate (A), 100 nmol of PMS (B), and 5 μmol of nitrite (C) were sequentially added. In **a**, the membrane fraction was diluted to a final protein concentration of 0.9 $mg \cdot ml^{-1}$ and absorbance changes were monitored at 549 minus 543 nm. In **b**, the membranes were diluted to a protein concentration of 3.6 $mg \cdot ml^{-1}$ and absorbance changes were monitored at 636 minus 649 nm

membrane-bound in *P. halodenitrificans* and located on the same side of the membrane as NADH oxidase.

The reactivity of the nitrogen oxides gives rise to nonenzymatic reactions with products similar to those formed during denitrification. Cresswell et al. (1965) reported that chemical reduction of nitrite occurred when 75% of the benzyl viologen was in the reduced form. Zumft and Frunzke (1982) observed that iron(II) ascorbate catalyzed the nonenzymatic reduction of nitric oxide to nitrous oxide and that phosphate or EDTA inhibited this reaction. The production of nitrous oxide by our membrane fractions was not inhibited by EDTA or stimulated by iron, thus precluding the possibility of substantial nonenzymatic activity in our phosphate-buffered reaction mixtures.

The relation between the membrane-bound reductase and the soluble cd-cytochrome-linked nitrite reductase is unclear at this time. We have attempted to solubilize the membrane-bound reductase for further identification but have been unsuccessful to date. It is not presently clear whether this is due to a strict requirement for membrane association or to problems in assaying the relatively small quantity of membrane-bound reductase. Recent improvements in our assay system may allow resolution of this question.

References

Cresswell CF, Hageman RH, Hewitt EJ, Hucklesby DP (1965) The reduction of nitrate, nitrite, and hydroxylamine to ammonia by enzymes from *Cucurbita pepo L.* in the presence of reduced benzyl viologen as electron donor. Biochem J 94:40–53

Firestone MK (1982) Biological denitrification. In: Stevenson FJ (ed) Nitrogen in agricultural soils. American Society for Agronomy, Madison, Wisconsin, pp 289–326

Iwasaki H, Matsubara T (1971) Cytochrome c-557 (551) and cytochrome cd of *Alcaligenes feacalis*. J Biochem (Tokyo) 69:847–857

Iwasaki H, Matsubara T (1972) A nitrite reductase from *Achromobacter cycloclastes*. J Biochem 71:645–652

Knowles R (1982) Denitrification. Microbiol Rev 46:43–70

Kodama T (1970) Effects of growth conditions on formation of cytochrome system of a denitrifying bacterium, *Pseudomonas stutzeri*. Plant Cell Physiol 11:231–239

Koike I, Hattori A (1975) Energy yield of denitrification: an estimate from growth yields in continuous culture of *Pseudomonas denitrificans* under nitrate, nitrite, and nitrous oxide-limited conditions. J Gen Microbiol 88:11–19

Lam Y, Nicholas DJD (1969) A nitrite reductase with cytochrome oxidase activity from *Micrococcus denitrificans*. Biochim Biophys Acta 180:459–472

Le Gall J, Payne WJ, Morgan V, Dervartanian D (1979) On the purification of nitrite reductase from *Thiobacillus denitrificans* and its reaction with nitrite under reducing conditions. Biochem Biophys Res Comm 87:335–362

Lowry O, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Payne WJ (1981) Denitrification. Wiley-Interscience, New York, NY

Sadler M, McAninch M, Alico R, Hochstein LI (1980) The intracellular Na^+ and K^+ composition of the moderately halophilic bacterium *Paracoccus halodenitrificans*. Can J Microbiol 26:496–502

Sawhney V, Nicholas DJD (1978) Sulphide-linked nitrite reductase from *Thiobacillus denitrificans* with cytochrome oxidase activity: purification and properties. J Gen Microbiol 106:119–128

Showe MK, DeMoss JA (1968) Localization and regulation of synthesis of nitrate reductase in *Escherichia coli*. J Bacteriol 95:1305–1313

Wharton DC, Weintraub ST (1980) Identification of nitric oxide and nitrous oxide as products of nitrite reduction by *Pseudomonas* cytochrome oxidase (nitrite reductase). Biochem Biophys Res Comm 97:236–242

Yamanaka T, Okunuki K (1963) Crystalline *Pseudomonas* cytochrome oxidase. I. Enzymatic properties with special reference to the biological specificity. Biochim Biophys Acta 67:366–378

Zumft WG, Sherr BF, Payne WJ (1979) A reappraisal of the nitric-oxide binding protein of denitrifying *Pseudomonas*. Biochem Biophys Res Comm 88:1230–1236

Zumft WG, Frunzke K (1982) Discrimination of ascorbate-dependent nonenzymatic and enzymatic, membrane-bound reduction of nitric oxide in denitrifying *Pseudomonas perfectomarinus*. Biochim Biophys Acta 681:459–468

Zumft WG, Vega JM (1979) Reduction of nitrite to nitrous oxide by a cytoplasmic membrane fraction from the marine denitrifier *Pseudomonas perfectomarinus*. Biochim Biophys Acta 548: 484–499

Received April 18, 1983/Accepted October 4, 1983