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REDUCTION OF NITRITE TO NITROUS OXIDE BY A CYTOPLASMIC MEMBRANE FRACTION FROM THE MARINE DENITRIFIER *PSEUDOMONAS PERFECTOMARINUS*

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Summary

A cytoplasmic membrane fraction from the marine denitrifier *Pseudomonas perfectomarinus* reduced nitrite to nitrous oxide in a stoichiometric reaction without nitric oxide as free intermediate. The membrane system had a specific requirement for FMN with NAD(P)H as electron donors. Other electron donors were ascorbate-reduced cytochrome *c*-551 or phenazine methosulfate. The membrane fraction contained tightly bound cytochrome *cd* which represented only a small portion of the total cytochrome *cd* of the cell. As further terminal oxidase cytochrome *o* was identified. The membrane fraction produced also nitrous oxide from nitric oxide, however, at a substantially lower rate than from nitrite when using ascorbate-reduced phenazine methosulfate as electron donor.

Introduction

Nitrite reduction coupled to electron transport phosphorylation is a property of many facultative anaerobic and a few obligate anaerobic bacteria [1,2]. The process is assumed to include nitrous oxide (N_2O) and nitric oxide (NO) as gaseous intermediates. The role of N_2O is far better established [1,3—5], though still ocassionally questioned [6], than that of NO [5,7]. Although formation and reduction of NO by whole cells was shown, as well as by several in vitro systems [8—11], it remains uncertain whether NO is an obligate intermediate of nitrite respiration or whether the cell has the ancillary capacity of producing

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Abbreviation: TMPD. *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

and metabolizing NO under certain conditions. Formation of N_2O from nitrite via NO should require two separate enzymatic entities, of which only nitrite reductase has been studied to any depth.

Respiratory nitrite reductases are of an extremely diverse nature, comprising the cytochrome oxidase (EC 1.9.3.2) (cytochrome *cd*) of *Pseudomonas aeruginosa*, [12], *Pseudomonas perfectomarinus* [13], *Alcaligenes faecalis* [14], *Paracoccus denitrificans* [15], and *Thiobacillus denitrificans* [16], the copper-containing nitrite reductase (EC 1.7.99.3) of *Achromobacter cycloclastes* [17], *Alcaligenes spec.* [18], formerly described as *Pseudomonas denitrificans*, and *Rhodopseudomonas sphaeroides* f. sp. *denitrificans* [19], the cytochrome *c*-containing nitrite reductase of *Achromobacter fischeri* [20], and the nitrite reductase (EC 1.6.6.4) of *Escherichia coli*, which was tentatively described as a flavoprotein without a heme moiety [21]. The latter two enzymes are likely not to be of true respiratory nature. Most of these enzymes were soluble or were easily removed from the membrane on cell breakage.

Nitric-oxide reductase (EC 1.7.99.2) was also described as a soluble enzyme [8,10,22] and identified in *A. faecalis* as cytochrome *cd* [10], in *P. perfectomarinus* as an enzyme system containing cytochrome *c* [23]. *P. perfectomarinus* is a marine facultative anaerobic denitrifier, which shows high rates of gas evolution. We chose this organism since previous work indicated a different nature of the denitrifying enzymes in this species [8,23] than those of other Pseudomonads. Here we describe isolation and partial characterization of a cytoplasmic membrane fraction from *P. perfectomarinus* which reduces nitrite to N_2O without NO as free intermediate. The system does not require exogenous cofactors, with the exception of a suitable electron donor. With the focus on membranes rather than the soluble cell fraction studied previously, we see a fresh approach addressing the pathway of nitrite respiration, its intermediates, and participating enzymes.

Materials and Methods

Organism and growth conditions. P. perfectomarinus (ATTC 14405), a gift from Dr. W.J. Payne, was grown in artificial sea water [24], together with 5 g tryptone (Difco), 1.5 g yeast extract (Merck), 1.0 g KNO_3, and 25 mg $FeCl_3 \cdot 6\ H_2O$/l medium [8]. Stock cultures were maintained on 1.5% agar slants of this medium under aerobic conditions. For 20-l batch cultures an inocculum was grown for 24 h in a 2 l Erlenmeyer flask, containing 1 l of growth medium. The flask was kept at 29°C in a gyrotory shaker at 200 rev./min under air. During the growth cycle of the 20 l culture aerobic and anaerobic conditions were alternated in the following way: 7 h aeration, 6 h anaerobic conditions, 7 h aeration, and 6 h anaerobic conditions. The rate of aeration was approx. 400 ml/min; in addition the culture was stirred magnetically. After the second anaerobic period, gas evolution of the culture was boosted by addition of 17 g of $NaNO_2$. When gas evolution was maximal, usually less than 1 h after addition of nitrite, the culture was harvested in a continuous flow centrifuge (flow rate 60 l/h). The cell paste was resuspended in 2 vols. of cold 50 mM Tris-HCl, pH 7.0, containing 50 mM $MgCl_2$, centrifuged at 13 200 $\times g$ for 15 min and used immediately for experiments.

Membrane preparation. Cells were resuspended in a two-fold volume (w/v) of 50 mM phosphate buffer, pH 7.0. Per 10 g of cells 2 mg DNAase and 2 mg RNAase was added. The cell suspension was passed three times in the cold through a French pressure cell at 16 000 lb/inch^2. The resulting homogenate was centrifuged for 10 min at 10 000 × *g* and the supernatant was further fractionated by centrifugation. The sediment from a centrifugation for 60 min at 29 000 × *g* was discarded and the supernatant subjected to a second centrifugation for 100 min at 126 200 × *g*. The pellet obtained from this step was resuspended in 50 mM phosphate buffer, pH 7.0, and used as 'membrane fraction' for most experiments. The lower part of the supernatant with a strong red color was kept separately and could be cleared from smaller membrane fragments by a third centrifugation for 70 min at 371 000 × *g*.

Inner and outer cell membrane were also prepared by lysozyme treatment and density gradient centrifugation combining two published methods [25,26]. Cells were resuspended in 50 mM Tris-HCl, pH 8.0, containing 0.6 M sucrose, supplemented with nucleases as specified above, and passed twice in the cold through the French press at 16 000 lb/inch^2. The homogenate was incubated for 30 min at room temperature with 10 mg lysozyme/10 g cells under continuous stirring and centrifuged afterwards for 10 min at 10 000 × *g*. The supernatant was diluted three fold and 50-ml portions were layered on top of a two-step sucrose gradient of 6 ml 55% (w/w) and 14 ml 15% (w/w) sucrose, containing 50 mM Tris-HCl, pH 7.0. The gradients were centrifuged in the cold for 60 min in a Beckman 45 Ti fixed-angle rotor at 142 800 × *g*, using a Kontron preparative ultracentrifuge equiped with a program for reorienting gradients. Red membranous material packed at the 55% sucrose interface was removed by syringe, diluted ten fold with buffer and collected by high-speed centrifugation. The crude membranes were treated with nucleases, washed as described by Scott et al. [25], and separated on a discontinuous sucrose gradient of 8 ml 55%, 10 ml 41.7%, 10 ml 24%, and 5 ml 15% (w/w) sucrose in 50 mM Tris buffer, pH 7.0, by centrifugation in a Beckman SW 27 rotor for 14 h at 58 000 × *g*. The gradients were fractionated into 1 ml portions by a fraction collector; membrane fractions were diluted ten fold with water or 50 mM phosphate buffer, pH 7.0, and the membranes were collected by high-speed centrifugation.

Enzymatic assays. The formation of N_2O from nitrite was assayed in 10-ml stoppered vials, containing 1 ml of liquid phase. The system comprised besides the membrane fraction, 150 μmol phosphate, pH 7.0; 25 μmol $NaNO_2$; 0.1 mg FMN, and 25 μmol NADH. Alternatively 40 μmol sodium ascorbate and 0.1 μmol phenazine methosulfate were used as the electron-donating system. The vials were filled with helium on a vacuum line, and incubated at 30°C in a shaking water bath; at regular intervals 50 μl of gas were withdrawn and analyzed by gas chromatography [27]. For the assay of nitric-oxide reductase nitrite was replaced by 10% NO in the vial, a concentration which was previously determined as optimal [9]. Nitrate reductase was assayed with NADH or reduced methyl viologen as electron donors [28]. NADH oxidase activity was followed with an oxygen electrode.

Preparation of cytochromes. Cytochrome *c*-551 was obtained from stationary-phase cells which showed a high tendency to leak this cytochrome into the

magnesium chloride solution used as wash liquid. Combined wash fractions from 500-g cells were diluted ten-fold with water. DEAE-cellulose (DE-52, Whatman) was stirred into this solution until all red material was adsorbed. The cellulose was then packed into a column, washed with 50 mM Tris-HCl, pH 8.0, and eluted with 0.5 M NaCl in this buffer. The cytochrome solution was dialyzed against Tris buffer and applied to a DEAE-cellulose column (2.5 × 30 cm), equilibrated with 50 mM Tris-HCl, pH 8.0. Two cytochrome fractions were recovered on elution of the column with a linear NaCl gradient, 0.05—0.5 M in Tris buffer, pH 8.0; the total gradient volume was 800 ml. The first eluting, principal cytochrome fraction was dialyzed, concentrated to 3 ml and chromatographed on a Sephadex G-100 column (2.5 × 80 cm) with Tris buffer. Three cytochrome-containing fractions were well resolved by this procedure. The slowest moving fraction was cytochrome *c*-551 which was identified by its pyridine-hemochrome spectrum.

Cytochrome *cd* (nitrite reductase) was purified from the soluble cell fraction according to the method of Zumft et al. [13], using chromatography on DEAE-cellulose and Sephadex, and ammonium sulfate precipitation.

Analytical methods. Nitrous oxide and nitric oxide were determined with known standards by gas chromatography using a thermal conductivity detector [27]. They were separated on a 1/8 inch steel column of 200 cm length, packed with Porapak Q, 80—100 mesh; helium was used as carrier gas with a flow rate of 30 ml · h^{-1}. Retention time for NO was 0.66 min, that for N_2O was 2.63 min. Nitrous oxide for gas standards was a product of Matheson, Belgium; NO was generated from acidified $NaNO_2$ and passed through KOH pellets [29]. Protein was determined by the Lowry method after digesting the membranes overnight in 1 N NaOH. Polyacrylamide gel electrophoresis of membranes followed a published method [25]. Nitrite was determined as azo dye [30]. Absorption spectra were recorded with a Perkin-Elmer model 557 dual-wavelength spectrophotometer.

Chemicals. Phenazine methosulfate, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, sodium ascorbate, lysozyme, grade I, from egg white, desoxyribonuclease I, DN-25, from bovine pancreas, ribonuclease A, type 1-A, from bovine pancreas, and buffer substances were from Sigma, München; FMN, FAD, NAD(P)H, methyl viologen, and *p*-chloromercuribenzoate were from Serva, Heidelberg. Sucrose, ultra-pure, was purchased from Becton and Dickinson, Heidelberg; other chemicals were from Merck, Darmstadt, and when available were of analytical grade.

Results

Growth mode and denitrification of the batch culture

The method of cell cultivation chosen here had as objectives high yield and actively denitrifying cells. Since the cell yield of aerobically growing cultures was considerably higher than that of anaerobic ones, each anaerobic growth phase, i.e. reduction of nitrate to nitrite and reduction of nitrite to dinitrogen, was preceded by 7 h continuous aeration (Fig. 1). By this culture method the yield could be nearly doubled from 1.31 ± 0.22 (S.D.) g/l of the anaerobic culture to 2.45 ± 0.59 (S.D.) g/l of the alternate aerated and anaerobic culture.

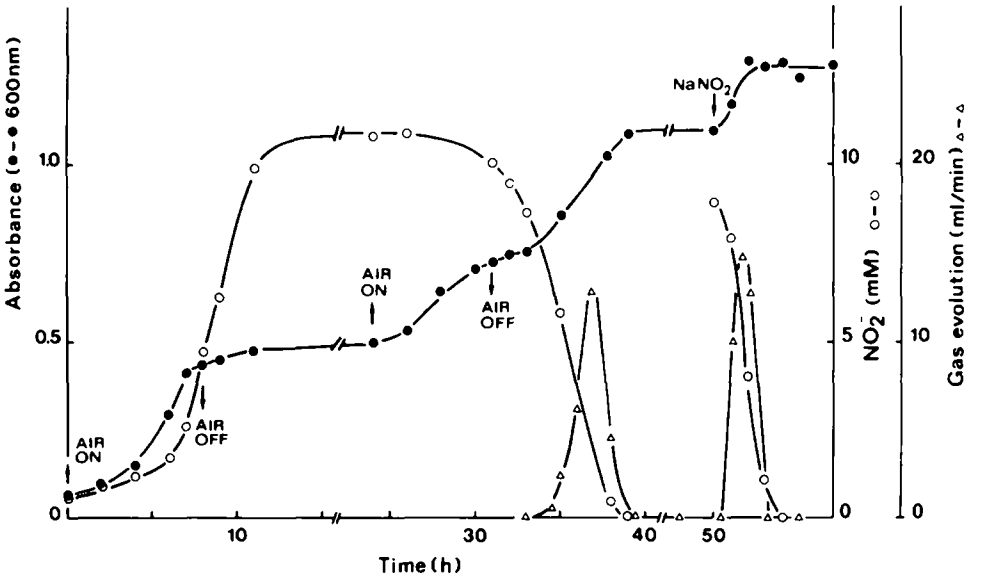

Fig. 1. Growth kinetics, nitrite excretion and denitrification of a 20-l batch culture of *P. perfectomarinus*. Where indicated by arrows, the culture was aerated at a rate of 400 ml/min. Gas evolution refers to the entire culture; after 50 h, 17 g $NaNO_2$ was added to the culture. Growth temperature was 29 ± 2°C.

Several features of the growth curve deserve comment. Nitrate was quantitatively converted to nitrite during the initial growth phase and excreted into the medium. Although the culture was aerated, conditions were certainly partially anaerobic since nitrate was reduced to nitrite and the growth kinetics changed abruptly to a lower rate even before aeration was cut off. With nitrate as initial oxidant the culture ceased growing when all nitrate was reduced to nitrite, and no denitrification took place even after 16 h of anaerobic incubation. Under the conditions employed here, nitrite was not used as electron acceptor unless oxygen was again provided (Fig. 1). Payne et al. [31] have made the interesting observation that a sudden switch from aerobic to anaerobic conditions prevented the synthesis of the denitrifying system, and a similar phenomenon may be operative here. Finally, a rather short period of gas evolution of only 2—3 h was observed. Nitrite was therefore added prior to cell collection to ensure harvest during active gas evolution and cells were used immediately for experiments without storage in the frozen state.

Isolation and characterization of the membrane fraction

Membranes of *P. perfectomarinus* with the highest specific activity of nitrous oxide formation from nitrite were obtained by a three-fold passage through the French press at 16 000 lb/inch^2. The membrane fraction sedimenting between 29 000 and 126 000 × *g* was two to four-fold enriched in this activity versus the crude extract. This membrane fraction was resolved by density gradient centrifugation in a component that banded at the density of the outer membrane (ρ = 1.258 g/ml), one corresponding to the position and density of the cytoplasmic membrane (ρ = 1.182 g/ml), and a broad band of very small cytochrome-void vesicles of lower density (Fig. 2). For comparative

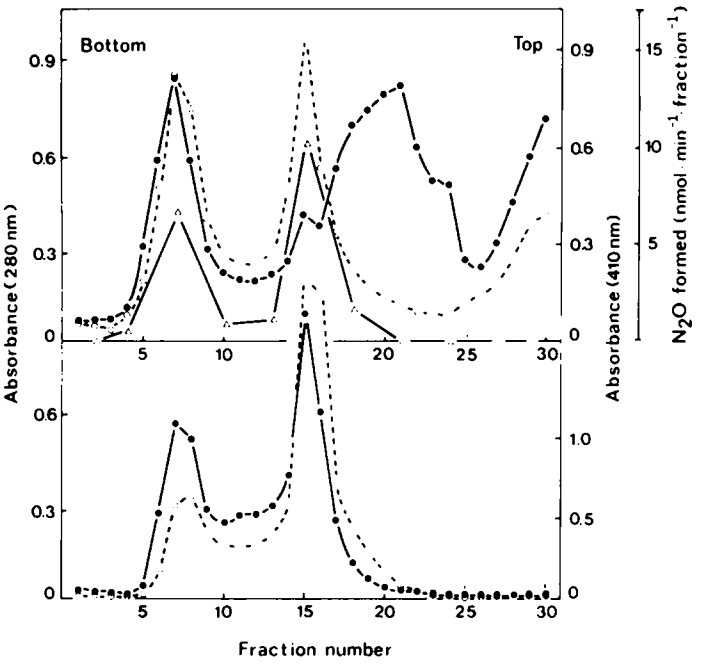

Fig. 2. Separation of cytoplasmic and outer membranes by sucrose density gradient centrifugation. Top part, separation of the membrane fraction of mechanically broken cells; lower part, separation of membranes from lysozyme-treated cells. The conditions of the gradient are given in Materials and Methods. For measurement of the 280 nm absorbance, samples were diluted 11-fold with sucrose; N_2O formation from nitrite was determined with phenazine methosulfate/ascorbate as electron donor system. ●———●, absorbance at 280 nm; ○ - - - - - - ○, absorbance at 410 nm; △———△, N_2O formation.

purposes outer and cytoplasmic membrane of this organism were also obtained by conventional lysozyme treatment (Fig. 2).

The protein profiles of the two types of membranes are shown in Fig. 3. The cytoplasmic membrane showed the more complex profile and indicated some contamination by outer membranes as evident from the major outer membrane protein. Cytoplasmic membranes from lysozyme-treated cells and from only mechanically homogenized cells showed a rather indistinguishable protein pattern. This is important since the nitrous oxide-forming activity was found to be associated with the cytoplasmic membrane. Examination by electron microscopy showed the presence of large and smaller vesicles in the membrane fraction (courtesy by Dr. H.J. Burkhardt).

The activity profile of nitrous oxide formation from nitrite matched the pattern of the 410 nm absorbance in the density gradient and activity was particularly high in the cytoplasmic membrane fraction (Fig. 2). Nitrous oxide-forming activity, and specific marker enzymes for the inner membrane such as NADH oxidase and nitrate reductase, a cytoplasmic transmembrane protein, were three- to four-fold enriched in the cytoplasmic membrane over the outer membrane (data not shown). The specific activity for nitrous oxide formation, however, could not be improved by this procedure.

The distribution of nitrous oxide formation from nitrite with phenazine methosulfate/ascorbate as electron donor is shown in Table I. About 36% of

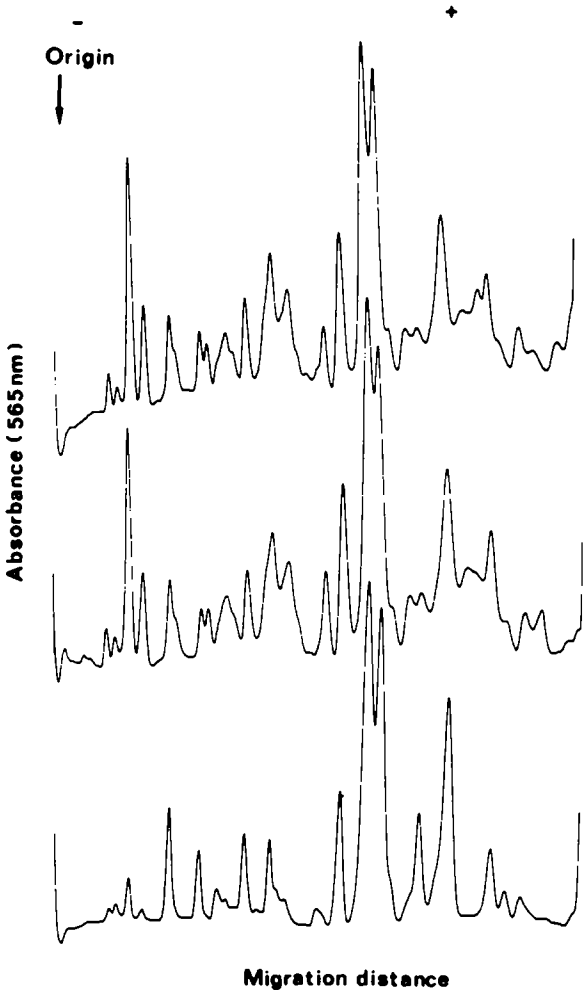

Fig. 3. Densitometer tracings of protein profiles of cytoplasmic and outer membranes after dodecyl sulfate polyacrylamide gel electrophoresis. Top, cytoplasmic membrane fraction from mechanically broken cells; middle, cytoplasmic membrane fraction from lysozyme-treated cells; bottom, outer membrane from lysozyme-treated cells. The membrane fractions correspond to the main bands of Fig. 2. Each trace was obtained from 0.12 mg protein.

TABLE I

DISTRIBUTION OF NITROUS OXIDE-FORMING ACTIVITY WITH PHENAZINE METHOSULFATE/ASCORBATE AS ELECTRON-DONATING SYSTEM

The activity unit is nmol N_2O formed · min^{-1}.

	Volume (ml)	Protein (mg)	Activity (units)	Specific activity (unit · mg^{-1})	Yield (%)
Crude extract	110	3168	16 790	5.3	100
Membranes (29 000 × *g*)	7.1	115	1 012	8.8	6
Supernatant (29 000 × *g*)	108	3110	15 861	5.1	94.5
Membranes (126 200 × *g*)	11	495	6 138	12.4	36.6
Supernatant (126 200 × *g*)	85	1777	4 264	2.4	25.4
Membranes (371 000 × *g*)	6.4	264	2 772	10.5	16.5
Supernatant (371 000 × *g*)	21	189	302	1.6	1.8

the total activity resided in the membrane fraction. An appreciable activity of 25% which remained in the 126 200 × *g* supernatant, was due to smaller membrane fragments which sedimented at a higher gravitational force (371 000 × *g* for 70 min). Together, these two membrane fractions represented about half of the total activity found in the crude extract. Measurement of nitrous oxide formation, however, did not account for the entire nitrite-reducing capacity of a cell fraction. The crude extract and all supernatants from centrifugations showed a high activity of nitric oxide formation from nitrite (not shown). This activity is most likely due to cytochrome *cd* which, as highly purified protein, was shown to release NO as end product of nitrite reduction [13]. The specific activity of nitric oxide formation increased slightly in the supernatants from high-speed centrifugations, strongly indicating that no nitric oxide-producing nitrite reductase was removed by the multiple centrifugation steps.

Spectral characterization of the membrane fraction

The reduced-minus-oxidized difference spectrum of the membrane fraction at 77 K showed absorbance characteristics due to cytochromes both of *b* and *c* type (Fig. 4). There were in all membrane fractions investigated, a weak band around 470 nm and accompanying weak bands beyond 600 nm, which were also present after passage of the membranes through a column of Sephadex G-200 (2.5 × 60 cm) or after further purification by sucrose density gradient centrifugation. These absorption bands were more prominent in the membrane-free supernatant and belonged to cytochrome *cd* (Fig. 4). The reduced-minus-oxidized spectrum of purified cytochrome *cd* is shown in Fig. 5. As mentioned above, this cytochrome is responsible for the reduction of nitrite to NO. More than 85% of this protein was found by spectral evidence in the soluble cell fraction. The membrane fraction, however, contained also another oxidase, cytochrome *o*, as indicated by the reduced plus CO minus reduced difference spectrum (Fig. 6).

Properties of the nitrous oxide-forming activity

Table II shows the efficiency of several electron donors in yielding N_2O from nitrite with the membrane fraction. Low activities were observed with reduced NAD(P), or dithionite-reduced FMN or FAD. Ascorbate alone gave higher activities, which could still be increased by addition of TMPD or phenazine methosulfate. Since ascorbate-reduced phenazine methosulfate gave consistently the highest activity it was used for the routine assay. Dithionite alone or in combination with methyl viologen was inactive.

The low activity of pyridine nucleotides could be attributed to a specific requirement for FMN (Table III). The activity of both nucleotides was considerably enhanced by the presence of FMN, a requirement which could only in part be satisfied by FAD. In the presence of both flavins, no additive effect was observed, presumably due to complete saturation of the flavin-susceptible site by FMN. The stimulatory effect was not observed with the artificial donor system phenazine methosulfate/ascorbate, indicating that the site for action of flavin lies prior to the enzyme, presumably in the electron transport chain. An equal stimulatory effect by FMN and FAD on nitrite reduction by a cell-free extract was previously shown, however, different from our system both soluble

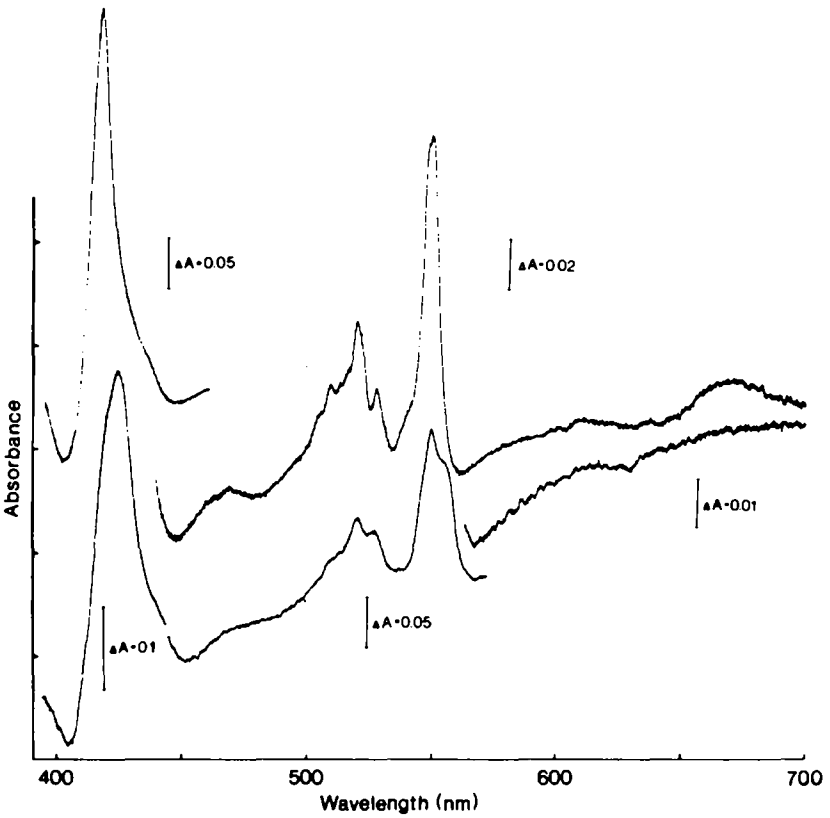

Fig. 4. Difference spectra of the membrane fraction and soluble cell compartment at 77 K. Top spectrum, dithionite-reduced minus ferricyanide-oxidized difference spectrum of a 235 800 × *g* supernatant; bottom, reduced-minus-oxidized difference spectrum of the membrane fraction. Protein concentration 8.1 mg/ml (top spectrum), 20 mg/ml (bottom spectrum); light path 1 mm; bandwidth 1 nm; solvent 0.05 M phosphate, pH 7.0.

and particulate cell fractions were required. FMN or FAD had no effect on the particulate fraction alone [4].

The stoichiometry of nitrite reduction to N_2O with NADH as electron donor in the presence of FMN is shown in Table IV. Per mol nitrite reduced, from 0.85 to 1.1 mol NADH were oxidized, and per mol nitrite reduced, from 0.47 to 0.50 mol N_2O were formed according to the equation:

$$2\,NO_2^- + 2(NADH + H^+) + 2\,H^+ \rightarrow N_2O + 3\,H_2O + 2\,NAD^+ \qquad (1)$$

The data of Table IV do not allow further reduction of N_2O to nitrogen, nor do they permit accumulation of an intermediate. By gas chromatographic analysis we have indeed not observed either N_2 or NO.

Cytochrome *c*-551 alone or together with azurin is the likely physiological electron donor of the cytochrome *cd* nitrite reductase [5]. Its effect on N_2O formation by the membrane fraction is shown in Table V. Ascorbate reduced cytochrome *c*-551 stimulated nitrous oxide formation about two fold but did not reach the activity of phenazine methosulfate. It was a much better mediator when used together with NADH, where, in its presence, the activity increased

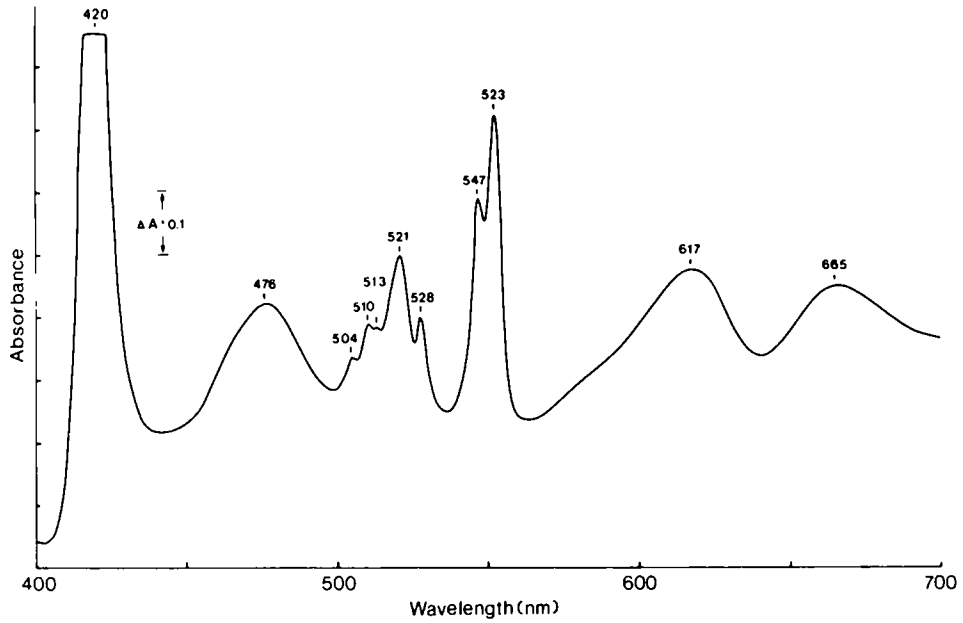

Fig. 5. Dithionite-reduced minus ferricyanide-oxidized difference spectrum at 77 K of purified cytochrome *cd* from *P. perfectomarinus*. Light path, 2 mm; bandwidth, 2 nm; solvent 0.05 M phosphate, pH 7.0.

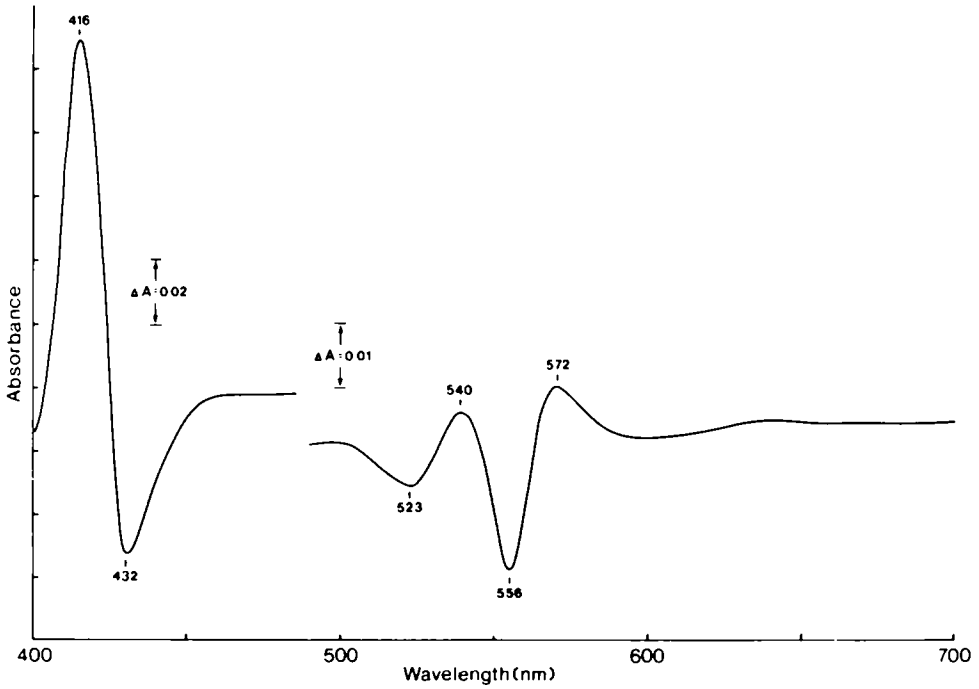

Fig. 6. Reduced plus CO minus reduced difference spectrum of the membrane fraction. Both cuvettes were reduced with a few crystals of dithionite; the sample cuvette was then flushed for 2 min with CO. Solvent 0.05 M Tris-HCl, pH 7.5; protein concentration, 1.9 mg/ml; light path 1 cm; bandwidth, 2 nm.

TABLE II

COMPARISON OF ELECTRON DONORS FOR N_2O FORMATION FROM NITRITE BY THE MEMBRANE FRACTION

PMS, phenazine methosulfate; MV, methyl viologen.

Electron donor	Concn. (mM)	N_2O formed (nmol · 10 min^{-1} · mg^{-1} protein)
NADH	16.6	2.9
NADPH	16.6	2.3
Ascorbate	26.6	9.4
+ PMS	0.01	114
+ TMPD	0.01	23.5
Dithionite		
+ FMN	2.2	3.3
+ FAD	1.3	2.1
+ FMN + FAD	2.2 and 1.3	3.3
+ MV	0.01	0

TABLE III

REQUIREMENT OF FMN FOR N_2O FORMATION FROM NITRITE BY MEMBRANES WITH NAD(P)H AS ELECTRON DONOR

Conditions were as in the standard assay; concentration of FMN was 2.2 mM; FAD was 1.3 mM. PMS, phenazine methosulfate.

System	N_2O formed (nmol · 10 min^{-1} · mg^{-1} protein)
NADH	2.8
+ FMN	35.2
+ FAD	17.5
+ FMN, + FAD	34.5
NADPH	2.3
+ FMN	32.8
Ascorbate	4.0
+ PMS	39.2
+ PMS, + FMN	37.3
+ PMS, + FAD	35.5
+ PMS, + FMN, + FAD	36.9

TABLE IV

STOICHIOMETRY OF NITRITE REDUCTION ACCORDING TO EQN. 1

Conditions as described for the standard assay with NADH and FMN.

Time (min)	NADH oxidized (μmol)	Nitrite reduced (μmol)	Nitrous oxide formed (μmol)	$\frac{[NO_2^-\text{ reduced}]}{[\text{NADH oxidized}]}$	$\frac{[N_2O\text{ formed}]}{[NO_2^-\text{ reduced}]}$	$\frac{[e^-\text{ consumed}]}{[N_2O\text{ formed}]}$
30	4.80	4.36	2.05	1.10	0.47	4.68
60	8.27	7.79	3.74	1.06	0.48	4.42
90	10.70	9.84	4.96	1.09	0.50	4.31
120	11.28	13.22	5.99	0.85	0.45	3.76

TABLE V

EFFECT OF CYTOCHROME c_{551} ON N_2O FORMATION FROM NITRITE BY THE CYTOPLASMIC MEMBRANE FRACTION

Conditions as described for the standard assay with phenazine methosulfate (PMS)/ascorbate or NADH/FMN. Where indicated, the assay system contained 0.45 μmol cytochrome c_{551} (ϵ^{red}_{mM}(551 nm) = 28.3) [41].

System	N_2O formed (nmol · 10 min^{-1} · mg^{-1} protein)
Ascorbate	7.5
Ascorbate + PMS	91.5
Ascorbate + c_{551}	14.3
Ascorbate + PMS + c_{551}	87.0
Ascorbate c_{551}, without membranes	0
NADH	1.5
NADH + FMN	26.9
NADH + c_{551}	20.7
NADH + FMN + c_{551}	28.4

14 fold to about 80% of the activity level of NADH with FMN. Highest activities were observed in a system containing both FMN and cytochrome *c*-551 besides NADH.

The pH optimum for nitrous oxide formation from nitrite with phenazine methosulfate/ascorbate as electron donors was around pH 7.0 (phosphate or Tris-HCl buffer), with an almost equally high activity at pH 6.0 (phosphate buffer). Towards alkaline pH the activity dropped markedly, with 50% remaining at pH 8.0 (Tris-HCl) and only 25% at pH 9.0 (Tris-HCl), Morpholinopropane sulfonic acid, 2-[*N*-morpholino]ethanesulfonic acid, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, all at pH 7.0, came within 80—95% of the activity of phosphate buffer.

Table VI shows the effect of several inhibitors on nitrous oxide formation.

TABLE VI

EFFECT OF INHIBITORS ON N_2O FORMATION FROM NITRITE BY THE MEMBRANE FRACTION

Conditions as described for the standard assay with phenazine methosulfate/ascorbate; the membrane fraction was incubated for 15 min with each inhibitor and the reaction was started by the addition of ascorbate. Data are inhibition in percent of control. pCMB, *p*-chloromercuribenzoate.

Inhibitor	Concentration of inhibitor (M)		
	10^{-3}	$2 \cdot 10^{-3}$	$5 \cdot 10^{-3}$
Azide	0	7	13
Cyanide	5	4	0
pCMB	0	15	20
o-Phenanthroline	23	15	30
Bathophenanthroline disulfonic acid	3	8	17
2,2'-Bipyridine	12	11	11
Sodium diethyldithiocarbaminate	24	32	85
Salicylaldoxime	0	0	24
Bathocuproine disulfonic acid	0	10	35

TABLE VII

RATES OF NITROUS OXIDE FORMATION FROM NITRITE OR NITRIC OXIDE WITH NADH OR PHENAZINE METHOSULFATE/ASCORBATE AS ELECTRON DONORS

Conditions as described in Materials and Methods. PMS, phenazine methosulfate.

Electron donor system	Experiment	Rate of N_2O formation (nmol $N_2O \cdot 10\ min^{-1} \cdot mg^{-1}$ protein)		
		$NO_2^- \rightarrow N_2O$ (a)	$NO \rightarrow N_2O$ (b)	a/b
NADH/FMN	1	39	68	0.57
	2	46	96	0.48
	3	43	87	0.49
PMS/ascorbate	1	133	84	1.58
	2	177	121	1.46
	3	109	76	1.43

The activity was resistant towards cyanide, and azide inhibited only slightly at 5 mM. The -SH group reagent *p*-chloromercuribenzoate was ineffective at 1 mM, requiring higher concentration to exert any action. The most effective metal chelator was diethyldithiocarbaminate which at 5 mM inhibited the reaction to 85%. Other copper chelators did not show this strong inhibitory effect, neither did various iron chelators. A constant inhibition of approx. 60% was observed from 10% CO up to a complete atmosphere of CO in the reaction vial. On additional incubation with CO up to 10 min no stronger inhibition was achieved.

The membrane fraction was also capable of reducing NO to N_2O, both with phenazine methosulfate/ascorbate or NADH as electron donors (Table VII). The rate of nitrous oxide formation from NO with NADH as electron donor was about twice that of nitrous oxide formation from nitrite. Since this partial reaction was faster than the overall process it is clear that NO could not be observed as a free intermediate. Surprisingly nitrous oxide formation from NO was much slower with phenazine methosulfate/ascorbate as electron donors than nitrous oxide formation from nitrite; but also in this case no occurrence of NO during nitrite reduction was observed.

Discussion

The metabolic pathway of anaerobic nitrite respiration is not yet unequivocally established. Of particular concern is the role of NO as obligate free precursor of N_2O. The claim of a nitric oxide-binding protein [32] could not be upheld in a critical re-investigation [13], isotope studies are inconsistent with NO as intermediate [7], and the only characterized nitric-oxide reductase was shown in *A. faecalis* to be cytochrome *cd* [10]. Due to their role as terminal oxidases in a phosphorylating electron transfer chain, both nitrite and nitric-oxide reductase would be expected to be membrane bound. Other terminal oxidases of nitratre respiration are nitrate reductase [33] and the highly labile nitrous oxide reductase [34]. Nitrite as well as nitric-oxide reductase from *P. perfectomarinus* were soluble enzymes, although it was also men-

tioned that the enzymes of the entire pathway were contained in a membrane fraction sedimenting at 37 000 × *g* [8]. We have recently identified nitrite reductase of *P. perfectomarinus* as cytochrome *cd* which in highly purified form reduces nitrite to NO, but not further [13]. The major part of this enzyme was readily solubilized in accordance with a recent report about *P. aeruginosa* [35]. Using ferritin-conjugated antibodies, the authors showed association of cytochrome *cd* with the inner side of the cytoplasmic membrane [35]. However, for the same organism a periplasmic location for nitrite reductase was also reported [36].

Nitric oxide reduction was shown first in *Pseudomonas stutzeri* and required as cofactors NADH, FMN, and copper or iron [37]. The enzyme was purified 25-fold from a soluble fraction of *P. aeruginosa*, and described as a metalloflavoprotein [22]. Reduced pyridine nucleotides were ineffective for this enzyme. A particle-bound nitric-oxide reductase was partially characterized from a bacterium originally described as *P. denitrificans* [9], but later considered as an *Alcaligenes*-type organism (Ref. 34, and personal communication). The particulate fraction, however, also required the soluble cell fraction, contributing a copper-containing nitrite reductase, to reduce nitrite to N_2O. Reduced pyridine nucleotides alone or together with flavins were ineffective electron donors in this system. From *A. faecalis*, besides the soluble nitric-oxide reductase, identical with cytochrome *cd* mentioned above, a membrane-bound enzyme was described, whose nature remained obscure [38].

The enzymic system of nitrous oxide formation described in this paper is characterized by the following properties: (i) it reduces nitrite to N_2O stoichiometrically without detectable NO as intermediate; (ii) it also reduces NO to N_2O, however, the rate of N_2O formation from NO with phenazine methosulfate/ascorbate as electron donors is lower than that with nitrite; (iii) it is entirely membrane bound and with NADH as electron donor requires only FMN as external cofactor to achieve full activity, and (iv) it contains only the minor part of the total amount of nitrite reductase (cytochrome *cd*) present in the cell.

Due to the apparent diversity of enzymes involved in nitrous oxide formation from nitrite, no consistent pattern is emerging yet, although it appears that the initial event of nitrite reduction in several organisms, including the marine denitrifier *P. perfectomarinus*, is catalyzed by cytochrome *cd*, whereas another group of organisms utilizes a copper-containing enzyme (see Introduction). Only a very small percentage (less than 15%) of cytochrome *cd* in the system described here is bound to the cytoplasmic membrane, however, it might be the essential part exhibiting even different properties from those of the soluble enzyme. This part of cytochrome *cd* is rather tightly bound and cannot be removed from the membranes by gel chromatography or by sucrose density gradient centrifugation. Changes in activation energy and affinity for nitrite have been reported for *P. aeruginosa* nitrite reductase on release from the membrane [35]. Although our data do not discount two different enzymes for nitrous oxide formation in the cytoplasmic membrane, they are compatible with the assumption of a direct reduction of nitrite to N_2O by a single enzyme. We tentatively attribute this activity to the cytochrome *cd* nitrite reductase. According to its substructure and heme content [39], this enzyme can transfer

a total of four electrons of which in the reduction of nitrite to NO only one is being consumed, whereas in the reduction of oxygen its total capacity is required. Work is in progress to dissociate nitrite reductase from the membrane and study its catalytic properties. In this context it is of interest that preliminary data from other laboratories indicate formation of NO [14,40] as well as N_2O by cytochrome *cd* [10,16].

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References

1 Payne, W.J. (1973) Bacteriol. Rev. 37, 409—452
2 Hall, J.B. (1978) in Microbiology-1978 (Schlesinger, D., ed.), pp. 296—298, Am. Soc. Microbiol., Washington, DC
3 Payne, W.J. and Balderston, W.L. (1978) in Microbiology-1978 (Schlesinger, D., ed.), pp. 339—342, Am. Soc. Microbiol., Washington, DC
4 Miyata, M. and Mori, T. (1969) J. Biochem. 66, 463—471
5 Zumft, W.G. and Cárdenas, J. (1979) Naturwissenschaften 66, 81—88
6 Delwiche, C.C. and Bryan, B.A. (1976) Annu. Rev. Microbiol. 30, 241—262
7 St. John, R.T. and Hollocher, T.C. (1977) J. Biol. Chem. 252, 212—218
8 Cox, C.D., Jr. and Payne, W.J. (1973) Can. J. Microbiol. 19, 861—872
9 Miyata, M., Matsubara, T. and Mori, T. (1969) J. Biochem. 66, 759—765
10 Matsubara, T. and Iwasaki, H. (1972) J. Biochem. 72, 57—64
11 Pichinoty, F., Mandel, M., Greenway, B. and Garcia, J.-L. (1977) Ann. Microbiol. (Inst. Pasteur) 128A, 75—87
12 Horio, T., Higashi, T., Yamanaka, T., Matsubara, H. and Okunuki, K. (1961) J. Biol. Chem. 236, 944—951
13 Zumft, W.G., Sherr, B.F. and Payne, W.J. (1979) Biochem. Biophys. Res. Commun. 88, 1230—1236
14 Iwasaki, H. and Matsubara, T. (1971) J. Biochem. 69, 847—857
15 Lam, Y. and Nicholas, D.J.D. (1969) Biochim. Biophys. Acta 180, 459—472
16 Sawhney, V. and Nicholas, D.J.D. (1978) J. Gen. Microbiol. 106, 119—128
17 Iwasaki, H. and Matsubara, T. (1972) J. Biochem. 71, 645—652
18 Iwasaki, H., Shidara, S., Suzuki, H. and Mori, T. (1963) J. Biochem. 53, 299—303
19 Sawada, E., Satoh, T. and Kitamura, H. (1978) Plant Cell Physiol. 19, 1339—1351
20 Prakash, O. and Sadana, J.C. (1972) Arch. Biochem. Biophys. 148, 614—632
21 Coleman, K.J., Cornish-Bowden, A. and Cole, J.A. (1978) Biochem. J. 175, 483—493
22 Fewson, C.A. and Nicholas, D.J.D. (1960) Nature 188, 794—796
23 Cox, C.D., Jr., Payne, W.J. and DerVartanian, D.V. (1971) Biochim. Biophys. Acta 253, 290—294
24 Yoshinari, T. and Knowles, R. (1976) Biochem. Biophys. Res. Commun. 69, 705—710
25 Scott, C.C.L., Makula, R.A. and Finnerty, W.R. (1976) J. Bacteriol. 127, 469—480
26 Hancock, R.E.W. and Nikaido, H. (1978) J. Bacteriol. 136, 381—390
27 Jeffery, P.G. and Kipping, P.J. (1972) Gas Analysis by Gas Chromatography, Pergamon Press, Oxford
28 Showe, M.K. and DeMoss, J.A. (1968) J. Bacteriol. 95, 1305—1313
29 Jones, K. (1973) The Chemistry of Nitrogen, Pergamon Press, Oxford
30 Snell, F.D. and Snell, C.T. (1949) Colorimetric Methods of Analysis, Vol. 2, Van Nostrand, Toronto, New York and London
31 Payne, W.J., Riley, P.S. and Cox, C.D., Jr. (1971) J. Bacteriol. 106, 356—361
32 Rowe, J.J., Sherr, B.F., Payne, W.J. and Eagon, R.C. (1977) Biochem. Biophys. Res. Commun. 77, 253—258
33 Garland, P.B., Downie, J.A. and Haddock, B.A. (1975) Biochem. J. 152, 547—559
34 Matsubara, T. (1975) J. Biochem. 77, 627—632
35 Saraste, M. and Kuronen, T. (1978) Biochim. Biophys. Acta 513, 117—131

36 Wood, P. (1978) FEBS Lett. 92, 214—218
37 Chung, C.W. and Najjar, V.A. (1956) J. Biol. Chem. 218, 627—632
38 Matsubara, T. and Iwasaki, H. (1971) J. Biochem. 69, 859—868
39 Kuronen, T., Saraste, M. and Ellfolk, N. (1975) Biochim. Biophys. Acta 393, 48—54
40 LeGall, J., Payne, W.J., Morgan, V. and DerVartanian, D. (1979) Biochem. Biophys. Res. Commun. 87, 355—362
41 Horio, T., Higashi, T., Sasagawa, M., Kusai, K., Naki, M. and Okunuki, K. (1960) Biochem. J. 77, 194—201