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A NITRITE REDUCTASE WITH CYTOCHROME OXIDASE ACTIVITY FROM *MICROCOCCUS DENITRIFICANS*

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SUMMARY

The formation of nitrite reductase and cytochrome *c* in *Micrococcus denitrificans* was repressed by O_2. The purified nitrite reductase utilized reduced forms of cytochrome *c*, phenazine methosulphate, benzyl viologen and methyl viologen, respectively, as electron donors. The enzyme was inhibited by KCN, NaN_3 and NH_2OH each at 1 mM, whereas CO and bathocuproin, diethyl dithiocarbamate, *o*-phenanthroline and α,α'-dipyridyl at 1 mM concentrations were relatively ineffective. The purified enzyme contains cytochromes, probably of the *c* and a_2 types, in one complex. A K_m of 46 μM for NO_2^- and a pH optimum of 6.7 were recorded for the enzyme. The molecular weight of the enzyme was estimated to be around 130000, and its anodic mobility was $6.8 \cdot 10^{-6}$ $cm^2 \cdot sec^{-1} \cdot V^{-1}$ at pH 4.55.

The most highly purified nitrite reductase still exhibited cytochrome *c* oxidase activity with a K_m of 27 μM for O_2. This activity was also inhibited by KCN, NaN_3 and NH_2OH and by NO_2^-.

A constitutive cytochrome oxidase associated with membranes was also isolated from cells grown anaerobically with NO_2^-. It was inhibited by smaller amounts of KCN, NaN_3 and NH_2OH than the cytochrome oxidase activity of the nitrite reductase enzyme and also differed in having a pH optimum of about 8 and a K_m for O_2 of less than 0.1 μM. Spectroscopically, cytochromes *b* and *c* were found to be associated with the constitutive oxidase in the particulate preparation. Its activity was also inhibited by NO_2^-.

The physiological role of the cytochrome oxidase activity associated with the purified nitrite reductase is likely to be of secondary importance for the following reasons: (a) it accounts for less than 10% of total cytochrome *c* oxidase activity of cell extracts; (b) the constitutive cytochrome *c* oxidase has a smaller K_m for O_2 and would therefore be expected to function more efficiently especially at low concentrations of O_2.

INTRODUCTION

Nitrite reductase is usually found in microorganisms which assimilate NO_3^- nitrogen or utilize NO_3^- as a terminal hydrogen acceptor. The assimilatory type

Abbreviation: PMS, phenazine methosulphate.

nitrite reductase found in *Azotobacter agile*[1], *Bacillus pumilis*[2], *Escherichia coli* strain B_n and *Neurospora crassa*[5,6] convert NO_2^- to NH_3 with NADH and NADPH as electron donors. The same enzyme may require flavin[1,6] and metal ions for maximum activity.

The respiratory type nitrite reductase has been found in *Thiobacillus denitrificans*[7], *B. subtilis*[8], *Pseudomonas aeruginosa*[9], *Ps. stutzeri*[10], *Ps. denitrificans*[11]. NADH and NADPH are the electron donors. In some bacteria, succinate and lactate were also effective. The product of NO_2^- reduction is NO, although further reduction products including N_2 have also been reported. The dissimilatory nitrite reductase has flavin as an electron carrier[11]. This enzyme from *Ps. aeruginosa* contains cytochrome as well as copper and iron[12]. A similar system from *Ps. denitrificans* was stimulated by flavin and inhibited by copper and iron chelates[11] but cytochromes were not required.

M. denitrificans dissimilates NO_3^- to N_2 (ref. 13) under anaerobic conditions. This bacterium also assimilates nitrate when it is the sole nitrogen source in the culture medium[14]. This paper describes some properties of the nitrite reductase enzyme obtained from cells grown under anaerobic conditions and also considers its role in the respiration of this bacterium.

MATERIALS AND METHODS

Organism

Micrococcus denitrificans (N.C.I.B. 8944) was grown in a modified Gröhmans medium at 37° for 18 h under N_2. The cells collected at 4° in a Sorvall RC-2 refrigerated centrifuge fitted with a continuous flow attachment were washed with cold 0.05 M phosphate buffer (pH 7.5) to remove NO_2^-. The cells were stored as a paste at −15°.

Effects of O_2 on the formation of NADH oxidase and nitrite reductase

The organism was subcultured through three transfers in a culture medium without NO_3^- but containing NH_4Cl as the sole nitrogen source. A portion (70 ml) from an 18-h culture, grown under aerobic conditions, was transferred into 630 ml of fresh sterile medium containing 4 g KNO_3. The cultures were kept at 37° and sparged with 1, 5, 10 or 20% O_2, or with O_2-free N_2 at 200 ml/min. The cells were collected as described previously after 18 h growth[15].

Preparation of cell extracts

Cell extracts were prepared by resuspending 1 part by weight of cell paste in 2 parts by volume of cold 0.05 M phosphate buffer (pH 7.5) and crushing at 2° in a French pressure cell at 20000 lb·inch^{-2}. Intact cells and cell debris were removed by centrifuging at 10000 × *g* for 15 min, and the supernatant fraction (S-10) was further centrifuged at 226000 × *g* for 3 h in a Spinco preparative ultracentrifuge. The supernatant fraction (S-226) contained the bulk of the nitrite reductase.

DEAE-cellulose and Biogel-200 column chromatography of the nitrite reductase enzyme

Washed DEAE-cellulose was equilibrated with 0.005 M phosphate buffer (pH 7.0) and packed into a column (1.2 cm × 20 cm). A cellulose–protein ratio of

1 ml to about 20 mg protein was used. After the sample was loaded onto the column, nitrite reductase was eluted between 0.30 and 0.45 M NaCl using a linear gradient. The Biogel-200 was equilibrated with 0.05 M phosphate (pH 7.5) containing 0.1 M NaCl and the enzyme was eluted with the same buffer. A column with void volume equivalent to about 30 times the sample volume was used.

For molecular weight determination, a Biogel-200 column (1.0 cm × 45 cm) was prepared and standardized by running through a number of known molecular weight markers separately, each in 0.4 ml buffer. The nitrite reductase was then loaded onto the column and developed with the buffer. The volume of buffer which passed through the column before the emergence of each marker protein was recorded and plotted against the log of the molecular weight of each protein. The approximate molecular weight of the most purified nitrite reductase was also estimated by determining its sedimentation coefficients in 0.005 M phosphate buffer (pH 7.5) in a Spinco analytical ultracentrifuge[24].

Assay of NADH oxidase and cytochrome c oxidase

NADH oxidase activity was determined at 340 mμ in a Unicam SP-700 recording spectrophotometer. The reaction mixture contained 0.01 ml enzyme in 0.05 M phosphate buffer (pH 7.5) in a final volume of 2.9 ml. The reaction was initiated by adding 0.4 μmole NADH.

Cytochrome *c* oxidase activity was determined at 550 mμ using 0.2 μmole of reduced cytochrome *c* as the electron donor.

Reduced cytochrome *c* was prepared by adding 4 times its molar amount of $Na_2S_2O_4$ and passing it through a Sephadex G-25 (coarse) column equilibrated with 0.05 M phosphate buffer (pH 7.5).

O_2 uptake

O_2 uptake was measured with the Beckman oxygen sensor (Model 39065) complete with an adaptor box (96260). The reaction mixture contained 0.05 ml enzyme in 0.05 M phosphate buffer (pH 7.5) in a final volume of 4.5 ml. One of the following electron donor systems was used, as indicated in RESULTS:

(*a*) *Lactate dehydrogenase–cytochrome c.* Reaction mixture contained 0.3 μmole cytochrome *c*, 40 μmoles sodium lactate and 0.02 ml of crystalline lactate dehydrogenase enzyme containing 40 μg protein. The reaction was started by adding 0.05 ml cytochrome oxidase through the substrate inlet.

(*b*) *Ascorbate–cytochrome c.* Reaction mixture contained 0.3 μmole cytochrome *c* and 40 μmoles of sodium ascorbate. The reaction was initiated by introduction of 0.05 ml of enzyme.

Preliminary experiments were performed to establish that reduced cytochrome *c* was in excess of that required for optimum oxidase activity.

The amount of O_2 present was determined by measuring the NADH required to use up all the O_2 in the reaction mixture by the NADH oxidase.

Assay of nitrite reductase

Nitrite reductase activity was measured in Thunberg tubes after evacuating and refilling them with O_2-free N_2. The residual NO_2^- at the end of the reaction was determined by the Griess and Ilosvay colorimetric method[15]. The following reaction mixture was used for the enzyme assay: 0.02 ml enzyme, 2 μmoles NADH

and 0.1 μmole phenazine methosulphate (PMS) in 0.05 M phosphate buffer (pH 7.5). The reaction mixture was preincubated for 5 min at 30° before adding the electron donor.

Mass spectrometry

The incubation was carried out in Rittenberg tubes using the same reaction mixture described for the nitrite reductase assay. After repeated evacuation to 10^{-5} mm Hg, the nitrite reductase *plus* 0.1 μmole PMS in the one limb was mixed into the other sidearm containing 2 μmoles NADH. The gases formed were monitored in the A.E.I. MS-2 mass spectrometer. The gaseous products were determined after the reaction mixture had been incubated for 1 h at 30°.

Determination of NO

The assay of nitrite reductase was carried out in a double-armed Warburg flask. The reaction mixture was as follows; main compartment: 2.6 ml 0.05 M phosphate buffer (pH 7.5) containing 0.02 ml nitrite reductase, 0.1 μmole PMS; side-arm 1: 2 μmoles $NaNO_2$ *plus* 5 μmoles NADH; side-arm 2: 0.2 ml alkaline permanganate (0.125 M $KMnO_4$ in 1 M KOH); centre well: 0.2 ml 20% (w/v) KOH. The flask was rigorously evacuated and filled with O_2-free N_2. The reaction was started by mixing $NaNO_2$ *plus* NADH with the enzyme. After 1 h incubation at 30° the alkaline permanganate was removed and the NO absorbed by it determined by the method of ANDERSON[16].

Agar gel electrophoresis

Agar gel was prepared by dissolving 1 g of agar in 100 ml of 0.03 M citrate phosphate buffer (pH 6.75) at 100°. The melted agar was transferred into a semicircular trough (3 cm diameter × 20 cm length) and set at 2° for 2 h. The enzyme was inserted into the gel as described by SMITH[18]. The electrophoresis was carried out at 2° using 0.03 M citrate phosphate buffer (pH 6.75) and at a constant current of 20 mA.

Spectra

Absorption spectra were recorded with a Unicam SP-800 and a Cary-14 recording spectrophotometer.

Protein concentration was determined by the Folin method modified by LOWRY *et al.*[17].

NADH and cytochrome *c* (horse heart Type II) were purchased from Sigma Chemical Co., St. Louis. Lactate dehydrogenase (EC 1.1.2.3) was a gift from Dr. R. S. Symons, University of Adelaide. DEAE-cellulose (DE-11) and Biogel P-200 were supplied by Whatman, England and Bio-rad Laboratories, Richmond, respectively. Other chemical reagents were of analytical grade and were obtained either from British Drug Houses, Poole, or from May and Baker, Dagenham, England.

RESULTS

O_2 effect

Cell-free extracts were prepared from cells grown at different O_2 pressures (see MATERIALS AND METHODS) and the specific activities of NADH oxidase and nitrite

reductase as well as the concentration of cytochrome *c* determined (Fig. 1). The NADH oxidase activity of the bacteria remained essentially constant when O_2 pressures were varied from 0 to 20 %. The nitrite reductase activity was markedly reduced with increased O_2 supply, *e.g.*, 50 % activity was found in cells grown with 5 % O_2 compared with 100 % for cells grown with 1 % O_2. The concentration of cytochrome *c* increased proportionally to that of nitrite reductase activity.

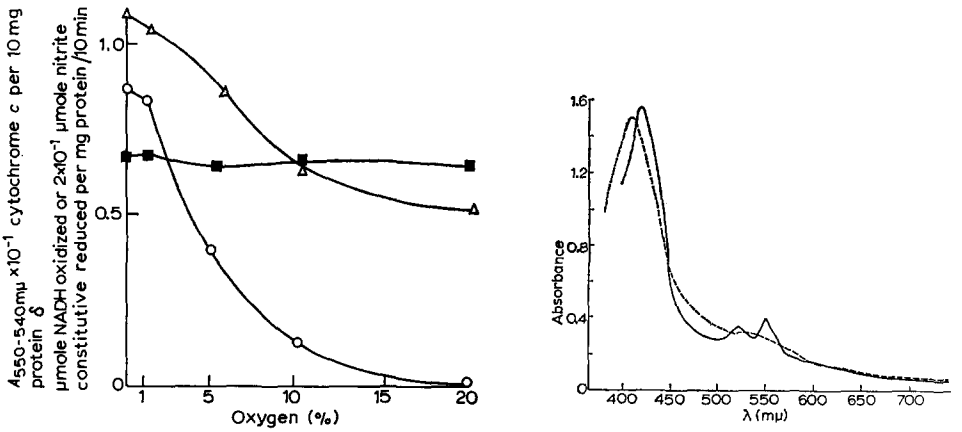


Fig. 1. Effects of O_2 on the formation of NADH oxidase, nitrite reductase and cytochrome *c*. NADH oxidase, nitrite reductase and cytochrome *c* content of the bacteria grown under different O_2 tensions were determined in crude cell extracts (S-10) as described in MATERIALS AND METHODS. △—△, cytochrome *c*; ○—○, nitrite reductase; ■—■ NADH oxidase.

Fig. 2. Absolute spectra of the particulate constitutive cytochrome *c* oxidase. The spectra were recorded in a Unicam SP-800 recording spectrophotometer. ———, absorption spectrum of the enzyme reduced with $Na_2S_2O_4$; – – – –, absorption spectrum of the enzyme in oxidized form. Concentration of enzyme was 3 mg/ml.

TABLE I

DISTRIBUTION OF NITRITE REDUCTASE, NADH OXIDASE AND CYTOCHROME *c* OXIDASE

NADH oxidase and cytochrome *c* oxidase were assayed spectrophotometrically as described in MATERIALS AND METHODS. The nitrite reductase was assayed in Thunberg tubes; the reaction mixture contained the following: 2 μmoles NO_2^-, 0.02 ml enzyme in 0.05 M phosphate buffer (pH 7.5) using as an electron donor either, (a) 0.1 μmole benzyl viologen + 0.05 ml azotobacter particles + 2 μmoles NADH or (b) 0.10 μmole PMS + 2 μmoles NADH. Final volume of reaction mixture was 3.0 ml. Percentage of activity given relative to S-10 = 100%. Fractionation by centrifuging Fraction S-10 at 226000 × *g*.

Enzyme system	*Electron donor*	*Percentage of activity*	
		Pellet (P-226)	*Supernatant fraction (S-226)*
Nitrite reductase	Reduced benzyl viologen	30	70
	PMS + NADH	32	69
NADH oxidase	NADH	88	10
Cytochrome *c* oxidase	Reduced cytochrome *c*	90	10

Distribution of nitrite reductase, NADH oxidase and cytochrome c oxidase

Although it has been shown that nitrite reductase relies on the membrane-bound respiratory chain for its reducing equivalents, it is only loosely associated with this system. About 70% of the enzyme remained in the supernatant fraction after centrifuging the crude extract (S-10) at 226000 × *g* for 3 h, when NADH *plus* PMS or reduced benzyl viologen was used as the electron donor[20]. Under similar conditions about 90% of the NADH oxidase and cytochrome *c* oxidase activities were recovered in the particulate fraction (P-226) (Table I).

Purification of a particulate constitutive cytochrome oxidase

The particulate fraction (P-226), 25 mg protein per ml, was resuspended in 0.05 M EDTA (tetrasodium salt) (pH 10.5)[19]. After readjusting the pH to 10.5 with 0.2 M NaOH it was homogenized for 5 min in a glass tissue grinder and left for 30 min at 30°. The fraction was centrifuged at 20000 × *g* for 15 min and the colourless pellet discarded. The supernatant fraction was dialysed against 0.05 M phosphate buffer (pH 7.5) and the active cytochrome oxidase recovered by $(NH_4)_2SO_4$ precipitation between 0–25% saturation. A 10-fold increase in specific activity was obtained. The absorption spectra given in Fig. 2 indicate that the oxidase was still associated with cytochromes *b* (560 mμ) and *c* (550 mμ). This preparation was, however, free of nitrite reductase, using reduced cytochrome *c* as the donor, and was spectroscopically similar to the constitutive cytochrome oxidase from aerobic cells, grown without nitrate.

TABLE II

ELECTRON DONORS FOR THE CONSTITUTIVE CYTOCHROME OXIDASE

The cytochrome oxidase activity was assayed with a Beckman oxygen sensor (see MATERIALS AND METHODS). The reaction mixture contained the following: 0.05 ml enzyme in 0.05 M phosphate buffer (pH 7.5) in a final volume of 4.5 ml. The electron donor was introduced in 0.05 ml through the substrate inlet at the beginning of the experiment. 40 μmoles of ascorbate or hydroquinone were used as a reductant. When cytochrome *c* was used as an electron donor (300 mμmoles), it was reduced by adding sodium ascorbate (40 μmoles).

Electron donor	*μmoles O_2 uptake per 0.05 ml enzyme per 10 min*
Ascorbate	0.05
Hydroquinone	0
Mammalian cytochrome *c* + ascorbate	1.50
M. denitrificans cytochrome *c* + ascorbate	1.60

Properties of the constitutive cytochrome oxidase

The cytochrome oxidase utilized reduced cytochrome *c* prepared from either *M. denitrificans* or animal sources, as an electron donor. Hydroquinone and ascorbate were ineffective (Table II).

The effects of various inhibitors on the enzyme activity are shown in Table III. NaN_3 and KCN at 0.1 mM reduced enzyme activity by 80 and 90%, respectively. NH_2OH at 0.1 mM and $KClO_3$ at 1 mM resulted in a 30 and 35% inhibition, respectively. Diethyl dithiocarbamate, α,α′-dipyridyl and *o*-phenanthroline each at 0.1 mM showed less than 10% inhibition.

TABLE III

INHIBITORS OF THE CONSTITUTIVE CYTOCHROME OXIDASE

The enzyme assay was carried out as described in Table II (see MATERIALS AND METHODS). The inhibitor was introduced through the substrate inlet. Mammalian cytochrome *c* (300 mμmoles) reduced with sodium ascorbate (40 μmoles) was used as the electron donor. Enzyme activity in μmoles O_2 per mg protein as measured by a Beckman oxygen sensor, as described in MATERIALS AND METHODS.

Inhibitor	*Concn.* (*mM*)	*Inhibition* (%)
KCN	0.1	90
NaN_3	0.1	80
NH_2OH	0.1	30
$KClO_3$	1.0	35
$NaNO_2$	0.1	50
KNO_3	0.1	0
Diethyl dithiocarbamate	0.1	<10
o-Phenanthroline	0.1	<10
α,α'-Dipyridyl	0.1	<10

The cytochrome oxidase activity was reduced by NO_2^- (about 50% at 0.1 mM) as measured by O_2 uptake using the lactate–cytochrome *c* system as the electron donor (*cf.* MATERIALS AND METHODS). The degree of inhibition at a fixed nitrite level was unaltered when the O_2 concentration was reduced from 100 to 50 μM. The rate of reoxidation of reduced cytochrome *c* by the enzyme also decreased in the presence of NO_2^-. These two observations indicate a noncompetitive-type inhibition. The K_m of the enzyme for O_2 was very low (<0.01 μM), and the optimum pH was around 8.0.

Purification of nitrite reductase

The specific activity of nitrite reductase was increased by about 140-fold after following the procedure outlined in Table IV.

Crude cell extracts (S-10), 150 ml, were prepared from bacteria grown anaerobically in a medium containing NO_3^-. This extract (Fraction 2) was centrifuged at 226000 × *g* (3 h), and the supernatant fraction (130 ml) was decanted (Fraction 3). Solid $(NH_4)_2SO_4$ was stirred slowly into this fraction at 4° with the aid of a magnetic stirrer. The pH was adjusted to 7.0 with 0.2 M NH_4OH. Protein fractions were precipitated at 25, 40, and 60% $(NH_4)_2SO_4$ saturation, respectively, and each fraction was centrifuged at 10000 × *g* (15 min). More $(NH_4)_2SO_4$ was then added to give an 80% saturation, and this precipitate which contained the nitrite reductase enzyme was centrifuged and resuspended in 15 ml 0.005 M phosphate buffer (pH 7.0). This fraction (Fraction 4) was dialysed against 2 l of the same buffer. The buffer was changed 3 times during a 12-h dialysis. The dialysate was loaded onto a DEAE-cellulose column (1.2 cm × 20 cm) previously equilibrated with the same buffer. The nitrite reductase was eluted with 35 ml phosphate buffer containing between 0.30 and 0.45 M NaCl, using a linear gradient (Fraction 5). A further fraction was collected between 80 and 90% saturation with $(NH_4)_2SO_4$, and the precipitate resuspended in 1 ml 0.05 M phosphate buffer (pH 7.5), Fraction 6, was passed through a Biogel P-200

TABLE IV

PURIFICATION OF NITRITE REDUCTASE

The bacteria were broken in a French pressure cell as described in MATERIALS AND METHODS. The enzyme was assayed using PMS + NADH as an electron donor as described in MATERIALS AND METHODS.

Fraction	*Total protein (mg)*	*Enzyme recovery (%)*	*Specific activity (μmoles NO_2^- per mg per 10 min)*	*Purification*
(1) Crude extract	—	—	—	—
(2) Supernatant fraction left after centrifuging crude extracts at 10000 × *g*·15 min	6150	100	0.09	1
(3) Supernatant fraction left after centrifuging fraction 2 at 226000 × *g*·3 h	1480	69	0.26	3
(4) Fraction 3 precipitated between 60–80% satn. of $(NH_4)_2SO_4$	358	45	0.71	8
(5) Fraction 4 dialysed against 0.005 M phosphate buffer (pH 7.0) and put onto a DEAE-cellulose column and eluted between 0.30–0.45 M NaCl by linear gradient	82	30	3.2	36
(6) Recovered from Fraction 5 by precipitating between 80–90% $(NH_4)_2SO_4$ satn.	25	28	6.2	69
(7) Fraction 6, chromatography through a Biogel P-200 column and collected between 1.2–1.4 times the void volume of the developing buffer, 0.05 M phosphate (pH 7.5) containing 0.10 M NaCl	12	28	12.7	140
(8) Agar gel electrophoresis (see MATERIALS AND METHODS)	—	—	—	—

column (1.2 cm × 60 cm) and developed with 0.05 M phosphate buffer containing 0.10 M NaCl. The fraction collected between 1.2 and 1.4 times the void volume (Fraction 7) was concentrated by negative pressure dialysis and further purified by agar-gel electrophoresis (Fraction 8; see MATERIALS AND METHODS).

The purified enzyme maintained full activity for 4 weeks at −15°.

Electron donors for nitrite reductase

The nitrite reductase in the S-10 (Fraction 2) utilized either NADH or succinate as an electron donor. In bacteria grown under H_2, the gas was also an effective electron donor. With the purified enzyme, reduced cytochrome *c* from either *M. denitrificans* or from animal sources, reduced PMS, reduced benzyl viologen or reduced methyl viologen and, to lesser extent, hydroquinone were also effective. Reduced forms of FMN or FAD were not utilized by the enzyme (Table V).

TABLE V

ELECTRON DONORS FOR NITRITE REDUCTASE

The nitrite reductase was assayed under anaerobic conditions as described in MATERIALS AND METHODS. Benzyl viologen or methyl viologen were reduced with palladized asbestos and H_2. Cytochrome *c* from either *M. denitrificans* or from horse heart was reduced by coupling it with lactate dehydrogenase from yeast (cytochrome b_2). PMS or FMN was reduced chemically with NADH.

Electron donors	*Relative activity for nitrite reduction*	*Redox potential at pH 7.0 (V)*
NADH	0	−0.320
Succinate	0	−0.03
Lactate	0	
Reduced benzyl viologen	250	−0.359
Reduced methyl viologen	205	−0.466
PMS + NADH	90	
FMN + NADH	5	
FAD (reduced with H_2 + palladized asbestos)	10	
Hydroquinone	20	+0.277
Cytochrome *c* (horse heart)	90	+0.262
Cytochrome *c*-549 (*M. denitrificans*)	100	

The K_m for cytochrome *c* from *M. denitrificans*, coupled with the lactate dehydrogenase system from yeast, was approx. 46 μM, with a pH optimum between 6.5 and 7.0.

Products of NO_2^- reduction

The gaseous products of the purified nitrite reductase (Fraction 7), using reduced cytochrome *c* generated by lactate dehydrogenase as the donor, were measured in a mass spectrometer. A gas with a mass of 30 was recorded (Fig. 3) which was completely absorbed by alkaline permanganate and was identified as NO. More than 90 % of the NO_2^- reduced was recovered as NO.

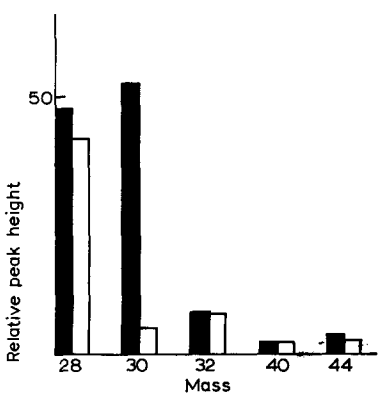


Fig. 3. End products of NO_2^- reduction. The gaseous products of NO_2^- reduction by purified nitrite reductase as recorded by mass spectrometer. White bars represent analysis of gaseous product at zero time and black bars represent analysis recorded after the reaction.

TABLE VI

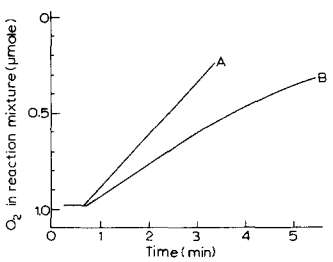
INHIBITORS OF NITRITE REDUCTASE ACTIVITY

The nitrite assay was carried out under anaerobic conditions in Thunberg tubes as described in MATERIALS AND METHODS. The reaction mixture contained 0.01 ml nitrite reductase (Fraction 7), 0.1 μmole PMS in 0.05 M phosphate buffer (pH 7.5) in the main tube with 2 μmoles NADH and NO_2^- in the side-arm. Total volume of reaction mixture was 3.0 ml. Enzyme units: mμmoles NO_2^- formed per mg protein.

Inhibitor	*Concn.* (*mM*)	*Inhibition* (%)
NaN_3	1.0	60
	0.1	45
KCN	1.0	72
	0.1	40
NH_2OH	1.0	50
CO (gassed for 2 min)		0

Inhibitors of nitrite reductase

The nitrite reductase activity was strongly inhibited by NaN_3, and KCN at 1 mM when PMS *plus* NADH was the donor. NH_2OH at 1 mM resulted in a 50% inhibition (Table VI). Metal-binding reagents such as sodium diethyl dithiocarbamate, bathocuproin, *o*-phenanthroline and α,α'-dipyridyl at 1 mM had no effect. Antimycin A, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide, amytal, mepacrine, rotenone and percidin A did not inhibit the purified enzyme when reduced benzyl viologen or reduced PMS was the electron donor, but when NADH was used with Fraction 2, the nitrite reductase activity was reduced by these inhibitors to a similar extent to the NADH oxidase system.



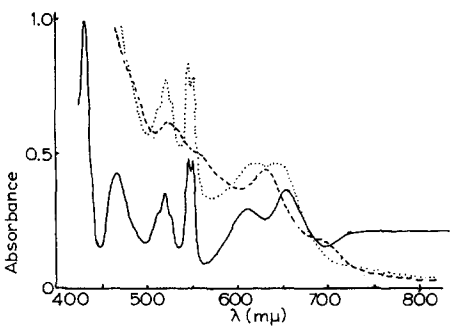


Fig. 4. Cytochrome *c* oxidase activity of the purified nitrite reductase (Fraction 7). The cytochrome *c* oxidase activity was determined as described in MATERIALS AND METHODS. The reaction mixture consisted of: 0.3 μmole cytochrome *c*, 0.02 ml (40 μg) lactate dehydrogenase, 40 μmoles of lactate in a final volume of 4.5 ml 0.05 M phosphate buffer (pH 7.5). The reaction was started by introducing 0.05 ml of nitrite reductase enzyme (Fraction 7) through the substrate inlet. A, O_2 uptake by the enzyme; B, O_2 uptake by the enzyme in a mixture containing 0.5 μmole NO_2^-. Less than 0.05 μmole NO_2^- was reduced during the experiment. NO_2^- had no effect on the lactate dehydrogenase.

Fig. 5. Spectral properties of nitrite reductase (Fraction 7). Reduced *minus* oxidized (———) difference spectrum, oxidized spectrum (— — —) and reduced spectrum (·····) of purified nitrite reductase from *M. denitrificans* in 0.1 M phosphate buffer (pH 7.0). $Na_2S_2O_4$ was used to reduce the enzyme. Oxidation was achieved by bubbling O_2 through the sample for 2 min. Spectra were determined with a Cary-14 recording spectrophotometer.

The cytochrome oxidase activity associated with the purified nitrite reductase

The oxidase activity of this enzyme with reduced mammalian cytochrome *c*, as a donor generated either by the lactate dehydrogenase system or by sodium ascorbate, was inhibited by NO_2^-. The degree of inhibition varied with the concentration of either O_2 or NO_2^- (Fig. 4) suggesting a competition of O_2 and NO_2^- for the enzyme. A small amount of NO_2^- was also reduced in the reaction. Both the K_m and v_{max} of this enzyme were altered when NO_2^- was present. The K_m for O_2 (Fraction 7) was about 27 μM and for NO_2^-, 46 μM, when reduced cytochrome *c* was the electron donor. The pH optimum was found to be 6.5–7.0 for the cytochrome oxidase activity.

Spectral properties of nitrite reductase

The spectra of the purified nitrite reductase prepared by agar gel electrophoresis indicate that it is a haemoprotein complex closely resembling those reported for a cytochrome oxidase from *Ps. aeruginosa, viz.*, cytochromes *c* and a_2 types (Fig. 5 and Table VII). The cytochromes were strongly autooxidizable and normally exist in oxidized form in purified preparations. Reduction of both cytochromes *c* and a_2 was achieved by titrating with either reduced PMS, ascorbate or hydroquinone. The cytochrome components of the enzyme may also be reduced by cytochrome *c* (549 mμ) from *M. denitrificans* coupled with a lactate dehydrogenase (cytochrome b_2) from yeast. The reduced enzyme was reoxidized on the introduction of air or by the addition of NO_2^- in the following sequence: first the band at 465 mμ and those between 615 and 655 mμ, followed by cytochrome *c* at 549 mμ. When NO_2^- was present, the absorption band at 465 mμ and those between 615 and 655 mμ remained oxidized even when the reducing substrate or $Na_2S_2O_4$ was in excess.

Although CO formed a complex with the cytochrome components of the purified enzyme, treating with this gas for 2 min did not affect the enzyme activity.

Chemical composition of nitrite reductase

The partially purified enzyme (Fraction 6) contained 10–20 % lipid materials. About 5 % of the total lipid consisted of saturated fatty acids of the 14, 16 and 18

TABLE VII

SPECTRAL PROPERTIES OF PURIFIED NITRITE REDUCTASE (FRACTION 8)

The maxima and minima of the purified nitrite reductase recorded under different conditions as described in MATERIALS AND METHODS.

Spectra (*conditions of recording*)	*Maxima* (mμ)	*Minima* (mμ)
Oxidized enzyme (pH 7.5)	408, 524, 628, 700	—
Reduced with $Na_2S_2O_4$ (pH 7.5)	417, (460), 521, 548–553, 615, 654	—
Reduced with $Na_2S_2O_4$ (pH 6.0)	417, (460), 521, 548–553, 615, 650	—
$Na_2S_2O_4$ reduced *vs.* oxidized	421, 468, 520, 549, 553, 615, 658	403, 440, 635
CO–$Na_2S_2O_4$ reduced *vs.* $Na_2S_2O_4$ reduced	415, (429), 540–570, 680	429, 460, 600–665
Alkaline pyridine haemochromagen	414, (462), 521, 549, 617	—
Acid acetone treatment—residue	417, 521, 547, 553	
Acid acetone treatment—supernatant fraction	432, (534), (560), (606)	

carbon units. Studies with ^{99}Mo showed that the nitrite reductase did not contain this metal. Copper was detected by neutron-activation analysis and by ESR measurements, but its concentration was very low in the purified enzyme: 5.5 ng/mg of protein (Fraction 6).

Physical properties of nitrite reductase

The molecular weight of the partially purified enzyme (Fraction 6) was determined by ultracentrifugation and by filtration through Sephadex G-200 column using markers of known molecular weights. The weight-average molecular weight calculated from ultracentrifugal data was about 150000. The molecular weight by gel filtration was estimated to be around 130000.

The anodic mobility of the nitrite reductase in starch gel using 0.03 M acetate buffer (pH 4.55) was $6.8 \cdot 10^{-6}$ $cm^2 \cdot sec^{-1} \cdot V^{-1}$ as compared with horse heart cytochrome *c* Type II which has a cathodic mobility of $2.0 \cdot 10^{-5}$ $cm^2 \cdot sec^{-1} \cdot V^{-1}$ under similar conditions.

DISCUSSION

In our earlier work we have shown that nitrite reductase was produced in response to its substrate[20]. The current experiments show that this induction was repressed by O_2 as was the production of cytochrome *c*, but the NADH oxidase system was unaffected. This is in contrast with the nitrite reductase in *Ps. aeruginosa* which was unaffected by the O_2 concentration in the culture medium[21].

The nitrite reductase was found to be associated with particles when NADH was the electron donor[22]. The results obtained here with reduced forms of either benzyl viologen or PMS as electron donor clearly show that the bulk of the nitrite reductase activity remained in the supernatant fraction after centrifuging at $226000 \times g$. On the other hand, about 88% of the NADH oxidase activity was found in the sedimented membrane fraction. Thus, the earlier observation that the enzyme is associated with particulates reflects the fact that the NADH donor system is associated with the membrane fraction rather than the nitrite reductase enzyme itself.

Both nitrite reductase and cytochrome *c* oxidase activities were found together even in the most highly purified enzyme. It is spectrophotometrically similar to the cytochrome oxidase prepared from *Ps. aeruginosa* and probably consists of a complex of cytochromes *c* and a_2 (ref. 23). Spectroscopic observations on the oxidation of the purified enzyme by O_2 or NO_2^- suggest that cytochromes *c* and a_2 are involved. Nevertheless the physiological role of this nitrite reductase as a cytochrome *c* oxidase is likely to be a minor one. This is suggested by the fact that although 70% of the nitrite reductase remained in the supernatant fraction after centrifuging at $226000 \times g$, only about 10% of the total cytochrome *c* oxidase activity was present in this fraction.

The rest of this activity was associated with a constitutive cytochrome oxidase which is spectroscopically distinct from the nitrite reductase as well as having a different pH optimum, *i.e.*, at pH 8.0 compared with 6.7 for nitrite reductase. The constitutive oxidase is also affected by lower amounts of KCN and NaN_3 and it did not utilize hydroquinone. Further, it had a very low K_m for O_2, estimated to be <0.1 μM. This latter observation also suggests that the nitrite reductase does not

function as a cytochrome *c* oxidase under low O_2 pressures, since it has a higher K_m for O_2 (27 μM).

The inhibitory effect of NO_2^- on O_2 uptake by the nitrite reductase varied with the concentration of both O_2 and NO_2^-. It is likely that this inhibition resulted from a competition of NO_2^- and O_2 for the enzyme. Kinetic data showed that both K_m and v_{max} for O_2 uptake were changed when NO_2^- was present, indicating that the effect is more complex than can be satisfactorily accounted for by a competitive-type inhibition. It is possible that both O_2 and NO_2^- affect the activity of the nitrite reductase by producing conformational changes in the enzyme.

In the intact system, nitrite reductase derives its reducing power from the electron transfer chain. It is interesting to note that NO_2^- inhibited the NADH-oxidase system isolated from anaerobically grown cells as well as the constitutive cytochrome *c* oxidase. This effect was not observed with the constitutive oxidase prepared from aerobically grown cells when nitrite reductase was absent. Spectroscopic studies show that the constitutive oxidases from either aerobic or anaerobically grown cells are similar if not identical. The kinetic data indicate that the inhibition of the constitutive cytochrome oxidase by NO_2^- was not affected by altering the concentration of either reduced cytochrome *c* or O_2 (0.5 to 1.1 μmoles). This suggests a noncompetitive-type inhibition and it may also indicate a modification of the electron transfer chain between reduced cytochrome *c* and the terminal oxidase.

The purified nitrite reductase still contained a small amount of copper as detected by neutron-activation analysis. However, the low concentration of the metal (5.5·ng per mg enzyme protein) and the lack of inhibition of the enzyme by copper chelating agents seem to preclude its actual involvement in this enzyme.

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