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SOME PROPERTIES OF A NITRATE REDUCTASE FROM *PSEUDOMONAS DENITRIFICANS*

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SUMMARY

A particulate nitrate reductase has been prepared from actively denitrifying cells of *Pseudomonas denitrificans*. The enzyme system which utilizes formate as an electron donor and contains flavin, sulphydryl groups and a metallic component(s) reduces NO_3^- stoichiometrically to NO_2^-. Actively growing cells and resting cell suspensions reduced NO_2^- mainly to N_2, but small amounts of NO and N_2O were also produced. ClO_3^- and BrO_3^- which were competitive inhibitors of NO_3^- reduction also served as electron acceptors for formate dehydrogenase.

The particulate nitrate reductase which was associated with the respiratory chain was solubilized by deoxycholate treatment and purified about 60-fold. The purified enzyme which has a cytochrome *b*-type absorption spectrum utilized reduced dyes such as benzylviologen as an electron donor, but was no longer active with the natural donors, formate and NADH. Horse heart cytochrome *c* reduced with ascorbate–tetramethylphenylenediamine did not serve as an electron donor. Metal chelating agents, especially those that bind molybdenum (dithiol and KCNS), and the sulfhydryl groups reagent *p*-chloromercuribenzoate were highly inhibitory.

INTRODUCTION

The reduction of NO_3^- to NO_2^- by bacteria can occur as the first step in one of two processes, NO_3^- assimilation or NO_3^- respiration (dissimilation). The former involves the reduction of NO_3^- to NH_3 which is incorporated into cellular materials, while in the latter case NO_3^-, which acts as a terminal electron acceptor in place of O_2, is reduced to N_2 or oxides of nitrogen[1].

The dissimilatory nitrate reductase in *Pseudomonas aeruginosa* has been shown to require iron in the form of cytochrome *c*, flavin, and molybdenum for activity. NADH was effective as the hydrogen donor[2].

In *Escherichia coli* formate or NADH can be utilized as an electron donor and cytochrome b_1 has been shown to be a hydrogen carrier[3].

COLE AND WIMPENNY[4] have established that in *E. coli* (K_{12}) formate is produced

Abbreviations: HQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide; PCMB, *p*-chloromercuribenzoate.

from pyruvate in cells grown anaerobically with NO_3^- thus establishing a physiological role for formate as a hydrogen donor.

HACKENTHAL AND ARBABZADEH[5] studied nitrate reductase systems from *P. aeruginosa* and *Bacillus cereus*. ClO_3^-, ClO_4^- and BrO_3^- were all competitive inhibitors of NO_3^- reduction and were found to be reduced by nitrate reductase.

In this paper a dissimilatory, particulate nitrate reductase from *P. denitrificans* is described which utilizes formate and is competitively inhibited by ClO_3^- and BrO_3^-. The NO_3^- reducing system appears to be similar to that in *E. coli*.

MATERIALS AND METHODS

P. denitrificans (A.T.C.C. 13867) was grown, harvested and cell extracts prepared as described previously[6].

Assay of nitrate reductase

(1) Nitrate reductase activity was assayed anaerobically by following the appearance of NO_2^- in the following reaction mixture: 1 μmole sodium formate or NADH, 0.5 μmole KNO_3, an appropriate amount of cell-free extract, and 0.1 M phosphate buffer (pH 6.5) to a final volume of 1 ml. The reaction was started by tipping the cell-free extract into the rest of the reaction mixture from the side arm of a Thunberg tube after rigorously evacuating the contents and equilibrating at 30° for 5 min. The reaction was stopped by exposing the tubes to air and precipitating protein with 0.1 ml 1 M zinc acetate and 1.9 ml redistilled 95 % (v/v) ethanol. NO_2^- was determined in portions of the supernatant solution left after centrifuging at 3000 × *g* for 5 min by the sulfanilamide and *N*-(1-naphthyl)ethylenediamine dihydrochloride reaction described previously[6].

(2) For the manometric assay of formate–nitrate reductase, the reaction was followed in Warburg manometer vessels with the following reactants: 1–6 μmoles sodium formate, 1–5 μmoles KNO_3, an appropriate amount of cell-free extract, 2.0 ml 0.1 M phosphate buffer (pH 6.5), and distilled water to a final volume of 3.0 ml. The evolution of CO_2 and the total amount of NO_2^- formed were determined.

(3) The activity of the solubilized nitrate reductase was determined using benzylviologen reduced by azotobacter particles and NADH as the electron donor. The azotobacter particles were prepared as described previously[6]. The reaction mixture contained: 0.5 μmole KNO_3, 1 μmole oxidized benzylviologen, 1 μmole NADH, 0.05 ml azotobacter particles, 0.01–0.05 ml solubilized nitrate reductase, and 0.1 M phosphate buffer (pH 7.5) to a total volume of 1 ml. The NADH was added to the reaction mixture last, immediately before evacuating the Thunberg tubes, to minimize loss due to the active NADH oxidase in the azotobacter particles. The reaction was initiated by tipping the NO_3^- from the side arm of the Thunberg tube into the rest of the reactants and terminated by exposing to air and precipitating with zinc acetate and ethanol as described above.

Assay of formate dehydrogenase

Formate dehydrogenase was assayed manometrically using the following reaction mixture: 5 μmoles sodium formate, 5 μmoles methylene blue, 2.0 ml 0.1 M phosphate buffer (pH 6.5), an appropriate amount of cell-free extract and distilled water to 3.0 ml.

Mass spectrometry

Gases formed by actively growing cultures were identified by growing cultures for 24 h in standard medium with $K^{15}NO_3$ as the sole electron acceptor. The incubation was carried out in Rittenberg tubes which were rigorously evacuated to 10^{-5} mm Hg and placed in an anaerobic jar which was also evacuated to minimize risk of air contamination during the long incubation period. Resting cells or particulate fractions were incubated for 2 h before testing gas production in the mass spectrometer. About 2 ml of 20% (w/v) KOH were included in a side arm to absorb any CO_2 formed. The gases formed during the incubation period were transferred from the Rittenberg tubes under high vacuum into an A.E.I. MS-2 mass spectrometer for analysis.

Michaelis constants

Initial reaction rates were measured as a function of one substrate concentration while the other substrate was held at a constant, saturating level. Michaelis constants were calculated from double-reciprocal plots of reaction velocity *versus* substrate concentration[8].

Cofactors and other reagents

NADH, NADPH, FMN and bovine serum albumin were obtained from Sigma Chemical Corp., St. Louis, U.S.A.; $^{15}NO_3^-$ (33 atom%) was purchased from the Office National Industriel de l'Azote, France; methylene blue, benzylviologen and tetramethylphenylenediamine were obtained from British Drug Houses, Poole, Great Britain. All other reagents were of analytical grade.

Protein was determined by the Folin method[7] using bovine serum albumin as the standard.

RESULTS

Membrane fraction

Nitrate reductase activity was found to be associated with the cell-membrane fraction which was sedimented by centrifuging the crude cell-free extract at 144000 $\times$ *g* for 2 h (144P) as shown in Table I. No activity was detected in the 144000 $\times$ *g* supernatant solution in contrast to the nitrite reductase activity in this organism

TABLE I

DISTRIBUTION OF FORMATE–NITRATE REDUCTASE IN CELL-FREE EXTRACTS

Nitrate reductase activity was assayed anaerobically as described in MATERIALS AND METHODS.

Fraction		*Total enzyme units (μmoles NO_3^- reduced per 10 min)*	*Total protein (mg)*
(I)	Crude extract left after centrifuging at 10000 $\times$ *g* for 20 min	160	504
(II)	Pellet left after centrifuging Fraction I for 2 h at 144000 $\times$ *g* (144P)	160	296
(III)	144000 $\times$ *g* supernatant fraction from Fraction II	0	234

which is isolated entirely in the soluble fraction. Formate dehydrogenase activity was also localized in the membrane fraction (144P) and was present at higher levels in NO_3^--grown cells than in cells grown aerobically (Table II).

TABLE II

DISTRIBUTION OF FORMATE DEHYDROGENASE IN CELL-FREE EXTRACTS

Formate dehydrogenase activity in the various fractions from cell-free extracts of cells grown with NO_3^- or O_2 as terminal electron acceptor was assayed by the manometric method with methylene blue as acceptor as described in MATERIALS AND METHODS.

Fraction	*Cells grown on*	*CO_2 evolved ($\mu l/h$)*	*Protein (mg)*	*CO_2 evolved ($\mu l/mg$ protein per h)*
Crude extract	NO_3^-	111	2.2	50.4
Pellet (144P)	NO_3^-	81	1.1	73.8
Supernatant (144S)	NO_3^-	3	1.4	2.4
Crude extract	O_2	27	2.8	9.6
Pellet (144P)	O_2	15	1.6	11.4
Supernatant (144S)	O_2	0	1.8	0

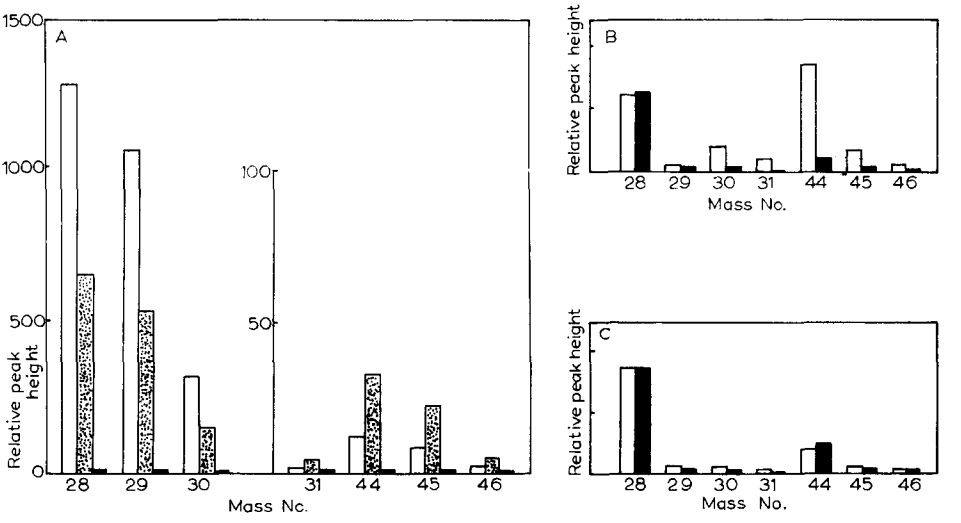

Fig. 1. Reaction products of the reduction of $^{15}NO_3^-$ by actively proliferating cells (A), resting cells (B) and cell membrane fraction (144P) (C). Conditions of incubation and assay are described in MATERIALS AND METHODS. Open bars, Culture 1; stippled bars, Culture 2; solid bars, controls.

Products of NO_3^- reduction. The gaseous products produced from $^{15}NO_3^-$ by either actively growing cells, resting cells or cell-free extracts was investigated by the mass spectrometric assay method. Fig. 1A shows that proliferating cells after 24 h growth produced large quantities of N_2 (mass 28 and 29) and smaller amounts of N_2O (mass 44, 45, 46). Culture 2 which showed less profuse growth at the time of sampling contained a higher proportion of N_2O. Resting cells (Fig. 1B) after 2 h incubation had produced NO and N_2O, while no gaseous products were detected with the particulate fraction (P144) as shown in Fig. 1C. The data indicate that the particu-

late fraction reduces NO_3^- only as far as NO_2^-, and that the final product of NO_3^- reduction in *P. denitrificans* is N_2. NO and N_2O appear to be minor products of the reduction process.

Electron donors. The relative efficiency of various electron donors with particulate nitrate reductase (144P) is shown in Table III. Activity with NADH was taken as 100. Very little or no activity was observed with either NADPH, sodium succinate, or sodium lactate as donors. Sodium formate was as effective with this preparation as was NADH.

TABLE III

THE RELATIVE EFFICIENCY OF VARIOUS ELECTRON DONORS FOR NITRATE REDUCTASE

Enzyme activity was assayed anaerobically in Thunberg tubes as described in MATERIALS AND METHODS using 144P fraction (1.9 mg protein).

Electron donor	*Final concn. (mM)*	*Relative efficiency (NADH = 100)*	*Redox potential at pH 7.0 (V)*
NADH	1	100*	−0.32
NADH + 0.1 mM FMN	1	89	
NADPH	1	13	−0.32
Sodium succinate	1	16	−0.03
Sodium formate	1	96	−0.42
Sodium lactate	1	0	−0.19
None	—	8	—

* 540 nmoles NO_3^- reduced per mg protein per 10 min.

pH effects and time of incubation. Optimal activity of formate–nitrate reductase occurred at pH 6.5. A sharp decline in activity was observed below pH 6.0 and a more gradual diminution occurred above pH 7.0. When NADH was the electron donor, maximal activity occurred at pH 7.5.

NO_2^- production by nitrate reductase was linear with time up to 20 min incubation when 60 % of the substrate had been consumed with either formate or NADH as electron donor. Thus enzyme activity was routinely determined over the first 10-min period of the reaction.

Stoichiometry of the reaction. For each mole of NO_3^- reduced, 1 mole of NO_2^- was formed; 1 mole formate or NADH was oxidized for every mole of NO_3^- reduced. These relationships are shown in Table IV.

The Michaelis constants for NO_3^- and formate in the system were determined. Fig. 2 represents double-reciprocal plots of initial reaction velocity when the concentration of one of the substrates was varied and the other substrate kept at a constant saturating level (1 mM formate or 0.5 mM NO_3^-). A slight substrate inhibition occurred at high concentrations of both formate and NO_3^-, *i.e.* above 1 mM, and a much more pronounced substrate inhibition occurred at NADH concentrations above 0.5 mM. The Michaelis constants determined by extrapolating the linear portion of each plot were: K_m (formate) = 0.38 mM; K_m (NO_3^-) = 0.69 mM; K_m (NADH) = 0.19 mM.

Inhibitor studies. In Table V the results of studies with a number of inhibitors are presented. When using the particulate fraction (144 P) with sodium formate as

the electron donor, NO_3^- reduction was strongly inhibited by KCN (92 % at 5 mM and 80 % at 0.5 mM). Nitrate reductase was also inhibited to a lesser extent by the metal chelating agents *o*-phenanthroline, KSCN, thiourea, and dithiol (43, 25, 20 and

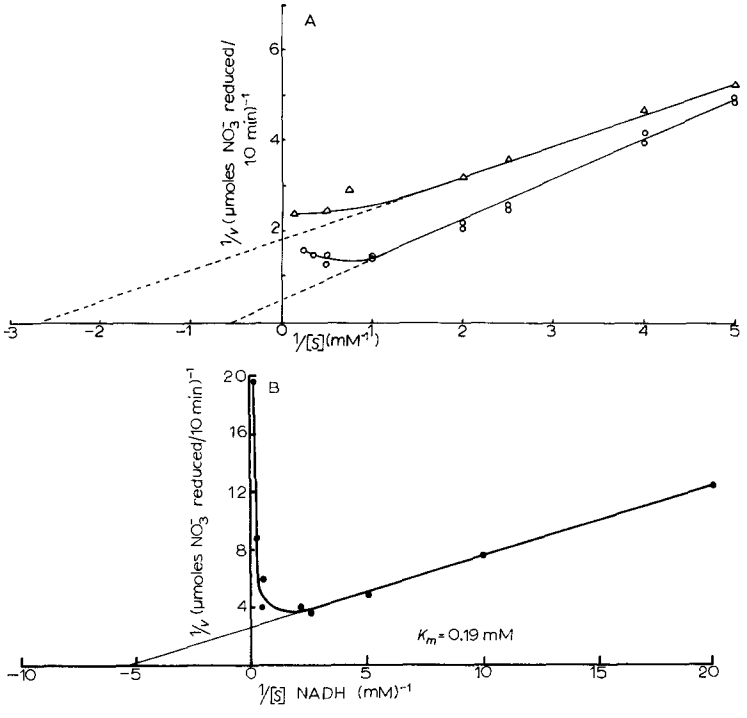

Fig. 2. Double-reciprocal plot of the rate of NO_3^- reduction in the 144P fraction with varying amounts of NO_3^- or formate. In each case the non-variable substrate was present at a saturating level: 1 mM formate or 0.5 mM NO_3^-. O—O, nitrate; △—△, formate; ●—●, NADH.

TABLE IV

STOICHIOMETRY OF NO_3^- REDUCTION BY THE PARTICULATE PREPARATION (144P)

Formate- and NADH–nitrate reductase assays were performed as described in MATERIALS AND METHODS with one substrate present in limiting amounts and the other in excess as shown below. The reactions were allowed to go to completion and NO_2^- formed determined in the usual way.

Limiting substrate	*Amount of substrate (μmoles)*	*NO_2^- formed (μmoles)*	*NO_2^- formed per substrate*
NADH	0.225	0.295	1.31
	0.450	0.467	1.04
	0.675	0.630	0.93
Formate	2.0	2.2	1.10
	1.25	1.25	1.00
	0.50	0.48	0.96
NO_3^-	5.5	5.7	1.04
	2.5	2.45	0.98
	2.0	2.0	1.00

TABLE V

INFLUENCE OF INHIBITORS ON FORMATE–NITRATE REDUCTASE IN THE MEMBRANE FRACTION (144P)

The effect of inhibitors on particulate nitrate reductase activity assayed as described in MATERIALS AND METHODS. 1 mg protein per assay.

Inhibitor	*Final concn. (mM)*	*Inhibition (%)*	
	Donor:	*NADH*	*Formate*
KCN	5	90	92
	0.5	80	80
KSCN	5	30	25
Mepacrine	1	50	50
2,2′-Dipyridyl	10	0	0
o-Phenanthroline	5	45	43
	1	40	39
Thiourea	5	15	20
PCMB	2	71	70
	1	65	30
Dithiol	0.25	35	35
	0.50	50	52
Amytal	1	30	0
	2	75	20
HQNO	0.3	0	0
Antimycin A	50 μg/ml	0	0
Piericidin A*	0.2	70	35
	0.1	60	25
	0.4	45	—
Rotenone	1	50	0
	0.2	30	0
$KClO_3$	1	80	85
$NaBrO_3$	1	40	40
$KClO_4$	1	18	15
KIO_3	1	10	10
Na_2SO_4	1	0	0

* A 50 % reversal of inhibition occurred upon addition of a 10-fold excess of ubiquinone Q_{10}.

52 %, respectively, the first three at 5 mM and dithiol at 0.5 mM final concentration). Mepacrine inhibited NO_3^- reduction by 50 % at 1 mM suggesting the participation of a flavin component in the system. NO_3^- reduction was inhibited 70 % by *p*-chloromercuribenzoate (PCMB) at 2 mM thus implicating a requirement for sulfhydryl groups in the system. Piericidin A (0.2 mM) and amytal (5.5 mM) inhibited 35 and 20 % respectively. Rotenone had no effect.

With NADH as the electron donor, KCN and the other metal chelating agents and mepacrine inhibited to a similar extent as when the formate system was used. At low concentrations (1 mM) PCMB inhibited the NADH–nitrate reductase system more strongly than the formate system. Amytal inhibited 75 % at 5.5 mM. Piericidin A inhibited 75 % at 0.2 mM and this effect was reversed by about 50 % upon adding a 10-fold excess of ubiquinone Q_{10}. Rotenone inhibited 30 % at 1 mM.

Both systems were strongly inhibited by $KClO_3$, and to a lesser extent by BrO_3^-, ClO_4^- and IO_3^-. Antimycin A, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) and CO had no effect on either system.

Effect of ClO_3^- and BrO_3^-. The effect of ClO_3^- and BrO_3^- was found to be due to a competitive-type inhibition. Fig. 3 shows the effect of ClO_3^- and BrO_3^- on the

rate of NO_3^- reduction at different concentrations of NO_3^-. The K_i values calculated from these plots were: K_i (ClO_3^-) = 1.8 mM; K_i (BrO_3^-) = 12.5 mM.

The effect of these inhibitors on the rate of formate oxidation was studied. ClO_3^- and BrO_3^- were both suitable hydrogen acceptors for formate dehydrogenase with about 70 % of the efficiency of NO_3^- whereas ClO_4^- and IO_3^- were only 32 and 16 % as effective, respectively. Fig. 4A shows that CO_2 evolution from excess formate

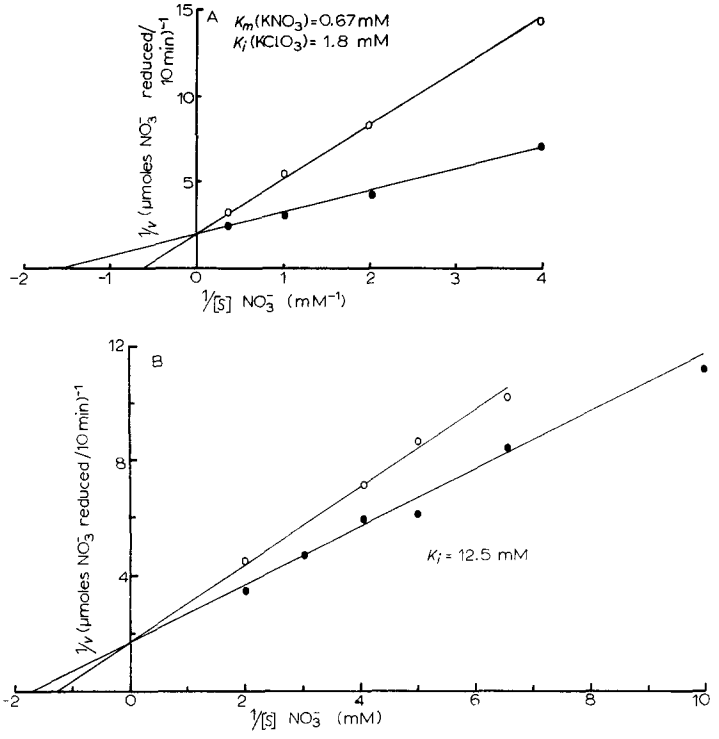

Fig. 3. Double-reciprocal plots of the rate of NO_3^- reduction in the 144P fraction with and without ClO_3^- or BrO_3^-. A. ●—●, no inhibitor; ○—○, 1 mM ClO_3^-. B. ●—●, no inhibitor; ○—○, 5 mM BrO_3^-.

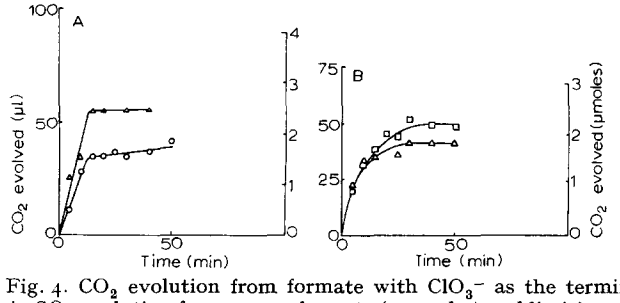

Fig. 4. CO_2 evolution from formate with ClO_3^- as the terminal acceptor in the 144P fraction. A. CO_2 evolution from excess formate (5 μmoles) and limiting amounts of ClO_3^-. △—△, 3 μmoles ClO_3^-; ○—○, 2 μmoles ClO_3^-. B. Effect on CO_2 evolution of adding ClO_3^- to NO_3^- as the terminal acceptor. △—△, 5 μmoles KNO_3; □—□, 5 μmoles KNO_3 + 5 μmoles $KClO_3$. Each assay mixture contained 2 μmoles formate. Manometric assay is described in MATERIALS AND METHODS.

is equivalent to the amount of ClO_3^- initially present in the reaction mixture. Fig. 4B shows the effect of added ClO_3^- on rate of CO_2 evolution with limiting amounts of formate.

The rate of CO_2 evolution is unchanged by the addition of ClO_3^- to the reaction mixture either alone or with NO_3^-, although the amount of NO_2^- formed is decreased by its presence. With NO_3^- alone as acceptor 2.1 μmoles of NO_2^- was formed; with ClO_3^- *plus* NO_3^- only 1.4 μmoles NO_2^- were formed although 2 μmoles of CO_2 were still evolved. It would appear that the inhibitory effect of these substances is due to their competition with NO_3^- for the hydrogen donor.

Table VI shows the effect of inhibitors on formate dehydrogenase activity when ClO_3^- was the acceptor. Dithiol (0.17 mM) inhibited the enzyme completely. KCN (5 mM) and *o*-phenanthroline (5 mM) inhibited 92 and 84 %, respectively. Mepacrine (1 mM), PCMB (2 mM), and KSCN (5 mM) inhibited 40, 70, and 30 %, respectively. Antimycin A, HQNO, and rotenone had no effect.

Purified nitrate reductase

Solubilization of nitrate reductase. In order to purify the nitrate reductase enzyme itself it was necessary to separate it from the rest of the cell-membrane bound respiratory chain. Incubation of the 144P membrane fraction with sodium deoxycholate (1 mg/mg protein) at pH 8.0 for 30 min at 30°, resulted in the solubilization of around 70 % of the protein originally present in the particles and a good recovery of the nitrate reductase activity with reduced benzylviologen as the donor. Formate dehydrogenase was completely inactivated, however, by this treatment. In Table VII details of the solubilization and purification scheme are set out. This procedure resulted in a 56-fold purification of nitrate reductase.

Spectrum of solubilized material. The solubilized material was a clear red solution. A difference spectrum (dithionite reduced *minus* oxidized) indicated that both *c*- and *b*-type cytochromes were present. The *c*-type cytochrome appeared to be lost upon further purification by $(NH_4)_2SO_4$ fractionation, but the *b*-type cytochrome persisted. Difference spectra are shown in Fig. 5. Absorption maxima and minima were as follows: 144P (maxima) 560 (shoulder), 534, 523 and 427 nm; (minima) 539 and

TABLE VI

INHIBITORS OF FORMATE DEHYDROGENASE WITH ClO_3^- AS ACCEPTOR

Effect of inhibitors on formate dehydrogenase activity in the particulate fraction (144P) (1 mg protein) with $KClO_3$ as electron acceptor was determined using the manometric assay described in MATERIALS AND METHODS.

Inhibitor	*Final concn. (mM)*	*Inhibition (%)*
KCN	5	92
o-Phenanthroline	5	84
KSCN	5	30
Dithiol	0.17	100
PCMB	2	70
Mepacrine	1	40
HQNO	0.3	0
Antimycin A	50 μg/ml	0
Rotenone	1	0

TABLE VII

SOLUBILIZATION AND PURIFICATION OF NITRATE REDUCTASE FROM MEMBRANE FRACTION (144P)

Fraction	*Volume (ml)*	*Protein (mg)*	*Total activity (μmoles NO_3^- reduced per 10 min)*	*Specific activity (μmoles NO_3^- reduced per 10 min per mg protein)*	*Purification*
(1) Crude extract: supernatant left after centrifuging cell-free homogenates at 10000 × *g* for 20 min	20	1640	240	0.15	1
(2) 144P: pellet left after centrifuging at 144000 × *g* for 2 h resuspended in 0.1 M phosphate buffer (pH 7.0)	27	850	380	0.45	3
(3) Fraction 2 adjusted to pH 8.0, sodium deoxycholate (1 mg/mg protein) added and the mixture incubated at 30° for 30 min; the mixture then centrifuged at 225000 × *g* for 2 h. Activity in supernatant	50	510	1250	2.45	16
(4) $(NH_4)_2SO_4$ added to Fraction 3 to give 30% satn. Precipitate dissolved in 0.1 M phosphate buffer (pH 7.0) and dialysed for 12 h against the same buffer	25	88	750	8.5	56

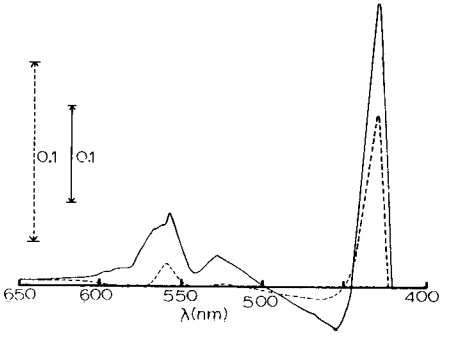

Fig. 5. Dithionite reduced *minus* oxidized difference spectra of solubilized nitrate reductase. A. Deoxycholate-solubilized preparation (Fraction 3, Table VII, 11 mg protein/ml). B. 0–30% satn. $(NH_4)_2SO_4$ precipitate (Fraction 4, Table VII, 3.5 mg protein/ml).

456 nm; solubilized nitrate reductase (maxima) 560, 521 and 431 nm; (minima) 540 and 461 nm.

Electron donors. The solubilized nitrate reductase was no longer active with the natural electron donors, formate and NADH. Reduced benzylviologen and methylviologen were, however, effective hydrogen donors. Ascorbate coupled to tetramethylphenylenediamine reduced cytochrome *c*, but not cytochrome *b* in the solubilized preparation. The ascorbate system was ineffective as an electron donor. NO_3^- reduction with reduced benzylviologen as donor was linear with time for the first 10 min when about 75 % of substrate had been utilized.

Optimal activity occurred at pH 8.0 with a sharp decline in activity at the more acid and alkaline pH values.

TABLE VIII

EFFECT OF INHIBITORS ON SOLUBILIZED AND PURIFIED NITRATE REDUCTASE

The effect of a variety of inhibitors on solubilized nitrate reductase was determined using reduced benzylviologen as electron donor as described in MATERIALS AND METHODS: 50 μg protein (Fraction 4) were used for each assay.

Inhibitor	*Concn.* (*mM*)	*Inhibition* (%)
KCN	1	81
KSCN	15	91
	1.5	75
Dithiol	0.1	95
o-Phenanthroline	3	25
PCMB	2	60
Sodium diethyldithiocarbamate	5	20
$KClO_3$	1	50
$KBrO_3$	1	50
$KClO_4$	1	33
KIO_3	1	40

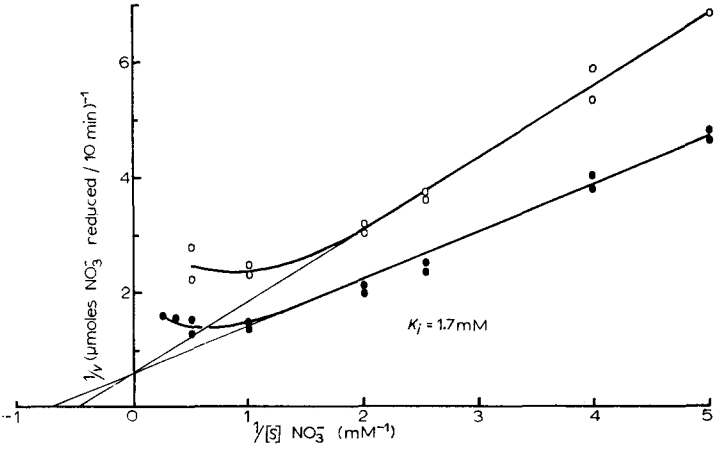

Fig. 6. Double-reciprocal plot of the rate of NO_3^- reduction by the purified enzyme (Fraction 4, Table VII), at different concentrations of NO_3^-. $KClO_3$ (5 mM) was added to one series of assays. Reduced benzylviologen was the electron donor, and purified nitrate reductase (Fraction 4, 0.14 mg protein) was used. ●—●, no inhibitor; O—O, 1 mM $KClO_3$.

Michaelis constant. NO_3^- was reduced quantitatively to NO_2^- by the purified preparation. The Michaelis constant for NO_3^- was 0.67 mM.

Inhibitors. The effect of a variety of inhibitors on the purified benzylviologen (reduced) enzyme is shown in Table VIII. Dithiol (0.1 mM) and KSCN (1.5 mM), agents that bind molybdenum, inhibited NO_3^- reduction by 95 and 75 %, respectively. The sulfhydryl inhibitor PCMB inhibited 60 % at 2 mM. The metal-chelating agents KCN (1 mM) and sodium diethyldithiocarbamate (5 mM) inhibited 81 and 20 %, respectively. Amytal, piericidin A, rotenone, CO, mepacrine, 8-hydroxyquinoline, and 2,2'-dipyridyl were all without effect. ClO_3^-, ClO_4^-, BrO_3^- and IO_3^- all inhibited as they did in the case of the particulate NO_3^--reducing system.

Effect of ClO_3^-. Fig. 6 shows the effect on the rate of NO_3^- reduction by the purified enzyme of adding 1 mM $KClO_3$ to the reaction mixture. This graph indicates that ClO_3^- is a competitive inhibitor of the purified nitrate reductase. The K_i for ClO_3^- was 1.7 mM.

DISCUSSION

The NO_3^--reducing system that utilizes NADH or formate as electron donor is localized in the cell-membrane fraction from *P. denitrificans* that also contains the respiratory electron transfer chain. The association of the NO_3^--reducing system with the electron transfer apparatus of the cell is a characteristic feature of nitrate reductase of the respiratory type as observed in *E. coli*[9], *P. aeruginosa*[10] and *Micrococcus denitrificans*[11].

Suspensions of whole cells reduced NO_3^- to NO, N_2O and N_2. When the particulate fraction alone was used with NADH as the donor no gaseous products were detected. 1 mole of NO_2^- was recovered per mole of NO_3^- reduced or per mole of NADH or formate oxidized. The particulate NO_3^--reducing system in *P. denitrificans* differs from that of *M. denitrificans* which produces traces of gaseous products from NO_3^-. In the Micrococcus system stoichiometric recovery of NO_2^- was only observed in the purified, soluble preparations using reduced viologen dyes as the electron donors[11].

NADH and sodium formate were the most effective electron donors for NO_3^- reduction by the particulate preparation whereas succinate, lactate or NADPH were relatively ineffective. Succinoxidase activity was greatly depressed in cells grown anaerobically with NO_3^-, and this effect probably accounts for the lack of activity of succinate as an electron donor for NO_3^- reduction.

Formate is an efficient electron donor for NO_3^- reduction in *E. coli*[12]. The physiological significance of formate as an electron donor for NO_3^- reduction in *E. coli* has been established by COLE AND WIMPENNY[4] who have shown that cell-free extracts can, in their system, produce formate from pyruvate by way of the phosphoroclastic reaction.

In *P. denitrificans*, formate dehydrogenase activity was increased 9-fold by growing the cells anaerobically in NO_3^- medium while the succinate dehydrogenase activity was depressed to an undetectable level. Although it was not possible to demonstrate a metabolic pathway from pyruvate to formate in *P. denitrificans*, it is known to be widely distributed in anaerobically grown microorganisms[4,13,14]. It has been observed that in some microorganisms such as *Streptococcus faecalis* this system

is extremely labile and sensitive to O_2 (ref. 15). It is possible that despite all precautions taken to exclude O_2 the formate–pyruvate exchange system was denatured during the preparation of the extracts. The observation that O_2 inhibits formate–nitrate reductase while having little effect on NADH–nitrate reductase suggests that O_2 may be detrimental to the functioning of the entire formate system.

The presence of an active formate dehydrogenase system in the cells grown anaerobically on NO_3^- and the much lower level of activity in cells grown on O_2 suggest that this system is an integral part of the nitrate respiration system and that formate is of physiological importance as a hydrogen donor for NO_3^- reduction *in vivo* in *P. denitrificans* as well as in *E. coli*.

NO_3^- acts as an alternative acceptor to O_2 for electron transfer in the membrane fraction (144P) *via* the cytochrome chain. Antimycin A and HQNO, inhibitors that block electron transport between cytochrome *b* and cytochrome *c* in animal mitochondria[16] had no effect on NO_3^- reduction with either formate or NADH as electron donor. However, these compounds inhibited NADH oxidation with O_2 as the terminal acceptor. These observations suggest that nitrate reductase derives its electrons from the cytochrome chain at a site prior to cytochrome *c*.

NO_3^- reduction with NADH as the donor was not inhibited by O_2 in the membrane fraction (144P) although adding NO_3^- to particles aerobically oxidizing NADH decreased the rate of O_2 uptake. When NO_3^- was added it was reduced to NO_2^- in amounts stoichiometric with the degree of inhibition of O_2 uptake. This observation also suggests that nitrate reductase derives its electrons at an earlier point in the electron transfer chain than does cytochrome oxidase and is therefore able to deplete the reducing equivalents available to O_2.

Nitrate reduction in the intact membrane system (144P) with NADH as the electron donor was inhibited by mepacrine, amytal, piericidin A, and rotenone. When formate was the electron donor, mepacrine inhibited to the same extent as when NADH was used. Piericidin A and amytal restricted NO_3^- reduction to a lesser extent with formate than with NADH as the donor, and rotenone was without effect on the formate system. Amytal and rotenone usually inhibit at the NADH dehydrogenase level of the electron transfer chain in animal mitochondria, and mepacrine acts as a flavin antagonist[15].

Metal chelating agents, especially dithiol which binds molybdenum[17] inhibited NO_3^- reduction in the membrane (144P) fractions with either formate or NADH as an electron donor. The participation of iron in the overall NO_3^- reduction was implicated by a depression of nitrate reductase activity of the membrane fraction prepared from cells grown in iron-deficient medium.

The participation of a ubiquinone-type carrier in the electron transfer chain is suggested by the inhibition of NADH–nitrate reductase by piericidin A at 0.2 mM. This inhibition was reversed by about 50 % on adding ubiquinone Q_{10} at a concentration of 2 mM. Piericidin A has been shown to inhibit electron transfer in animal mitochondria at or near the ubiquinone site[18]. *P. denitrificans* contained appreciable amounts of ubiquinone (1.2 μg/mg protein) when grown with either O_2 or NO_3^- as the terminal acceptor. DOWNEY[19] has shown that irradiation of particulate fractions of bacteria with 360-nm light destroys vitamin K-type electron carriers. This treatment had no effect on either the nitrate reductase activity or the level of ubiquinone in particles from *P. denitrificans*.

Reduced cytochrome *b* appears to be a physiological electron donor for nitrate reduction as indicated by the following observations: (a) HQNO and antimycin A, compounds that block electron transfer between cytochrome *b* and cytochrome *c* in animal mitochondria and in some bacteria, inhibited NADH oxidation by O_2 but not by NO_3^-. (b) Ascorbate *plus* tetramethylphenylenediamine reduced cytochrome *c*, but failed to act as an electron donor for NO_3^- reduction in either the particulate or solubilized preparations. (c) Cytochrome *b* level was increased 70% by growth on NO_3^-, but cytochrome *c* content was little changed. (d) Cytochrome *c* was lost upon purification of the soluble benzylviologen (reduced)–nitrate reductase which retained a cytochrome *b*-type absorption spectrum.

Cytochrome *b* has also been established as the penultimate electron carrier in respiratory NO_3^- reduction in *M. denitrificans*[11]. In this organism NADH oxidation by O_2 was inhibited by antimycin A and HQNO, while electron transfer to NO_3^- was unaffected. The rate of NO_3^- reduction by particles from this organism was also unaffected by NO_3^-, while the rate of O_2 uptake was decreased by adding NO_3^- to the reaction mixture.

In some other microorganisms, nitrate reductase was linked with cytochrome *c*. FEWSON AND NICHOLAS[10] found that the NO_3^--reducing system from *P. aeruginosa* required cytochrome *c* for full activity and also suggested that cytochrome *c* was involved in NO_3^- reduction by *M. denitrificans*[11]. A purified nitrate reductase from *Achromobacter fischeri* (*Photobacterium sepia*) exhibited a cytochrome *c*-type absorption spectrum[20].

It was observed that ClO_3^-, $HClO_4^-$, BrO_3^-, and IO_3^- inhibited NO_3^- reduction by both the particulate and the solubilized nitrate reductase preparations. ClO_3^- and BrO_3^- proved to be competitive inhibitors of NADH–nitrate reductase in the particles with K_i values of 1.7 mM (ClO_3^-) and 1.25 mM (BrO_3^-). ClO_3^- and BrO_3^- served as acceptors for the formate dehydrogenase system in the particles. The Michaelis constant for ClO_3^- in this system was calculated to be 2.8 mM which is close to the value of the K_i of ClO_3^- as an inhibitor of NO_3^- reduction. When ClO_3^- was the acceptor for the formate dehydrogenase system, activity was completely inhibited by 0.17 mM dithiol. KCN and *o*-phenanthroline, each at 5 mM, inhibited 92 and 84%, respectively, while mepacrine (1 mM), PCMB (2 mM), and KSCN (5 mM) inhibited 40, 70 and 30%, respectively. Antimycin A, HQNO, or rotenone had no effect. These inhibitions are similar in pattern to those observed for NO_3^- reduction by the particulate preparation with formate as the electron donor. ClO_3^- also acted as a competitive inhibitor for NO_3^- reduction by the solubilized enzyme. A K_i value of 1.8 mM was found.

The competitive nature of the inhibition by ClO_3^- of NO_3^- reduction by either the particulate or the purified nitrate reductase suggest that ClO_3^- may act as an alternative electron acceptor to NO_3^-. Other evidence supporting this hypothesis includes the similarity of K_m and K_i values for ClO_3^- as an acceptor in the formate dehydrogenase system and as an inhibitor of the nitrate reductase system respectively, and also the similar pattern of inhibition for the formate to NO_2^- system and the formate to ClO_3^- system.

ClO_3^- inhibition has been observed in a number of other bacterial nitrate reductases. HACKENTHAL AND HACKENTHAL[21] reported that nitrate reductase from *B. cereus* was competitively inhibited by ClO_3^-. ClO_3^- and ClO_4^- were shown to be alternative substrates for nitrate reductase[22].

STOUTHAMER[23] found that mutants of *A. aerogenes* lacking nitrate reductase were also unable to reduce ClO_3^-. The nitrate reductase from *P. aeruginosa* was competitively inhibited by ClO_3^- and it was suggested that ClO_3^- may act as an alternative substrate for the enzyme[10].

The nitrate reductase from *E. coli* was competitively inhibited by ClO_3^-, BrO_3^- and IO_3^-. ClO_3^- and BrO_3^- acted as substrate for the enzyme[1]. PICHINOTY[24] reported that the nitrate reductase from *M. denitrificans* also reduced ClO_4^-. FAULL *et al.*[25] found that ClO_4^- acted as a competitive inhibitor of the nitrate reductase from *Nitrobacter agilis*.

Nitrate reductase from *P. denitrificans* seems to conform to a general pattern of being inhibited by ClO_3^- and BrO_3^- which compete with NO_3^- for the active site of the enzyme.

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