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A SULPHITE-DEPENDENT NITRATE REDUCTASE FROM *THIOBACILLUS DENITRIFICANS*

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SUMMARY

1. A dissimilatory nitrate reductase from *Thiobacillus denitrificans* utilizes sulphite as an electron donor, with a pH optimum at 8.5. NADH which also serves as an electron donor has a pH optimum at 7.0.

2. Whole cells reduced NO_3^- to NO_2^-, NO and N_2O when Na_2S, Na_2SO_3, $Na_2S_2O_3$ or $Na_2S_2O_4$ was the electron donor. The cell-free extract however only reduced NO_3^- to NO_2^-.

3. Inhibitor effects suggest the involvement of an electron transport system containing cytochromes coupled to nitrate reductase.

4. Kinetic studies with the SO_3^{2-} linked nitrate reductase indicate a Ping–Pong mechanism and K_m values of $7.2 \cdot 10^{-5}$ M for NO_3^- and $2.5 \cdot 10^{-4}$ M for SO_3^{2-}.

INTRODUCTION

Thiobacillus denitrificans in common with other *thiobacilli*[1,2] is a chemoautotrophic bacterium oxidizing inorganic sulphur compounds to obtain energy for growth. It is a facultative anaerobe and utilizes NO_3^- instead of O_2 as a terminal electron acceptor under anaerobic conditions. Consequently, it resembles the heterotrophs *Escherichia coli* and the denitrifying bacteria in having a respiratory nitrate reductase system[3–5], which utilizes cytochromes as electron carriers[6].

AUBERT *et al.*[7] have purified a *c*-type cytochrome from *T. denitrificans* which appeared to be involved in transporting electrons from $S_2O_3^{2-}$ to NO_3^-. However little work has been done on the nitrate reductase from *T. denitrificans*. In this paper the properties and kinetics of a particulate nitrate reductase from *T. denitrificans* (EC 1.8.6.2) are described. This enzyme utilizes SO_3^{2-} for the reduction of NO_3^- to NO_2^-.

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

MATERIALS AND METHODS

Culture and harvest of organism

T. denitrificans "Oslo" strain was grown anaerobically for 2–3 days at 25–30° in 40-l carboys. The culture solution contained (g/l): $Na_2S_2O_3 \cdot 5H_2O$, 5.0; KOH, 0.2; $NaHCO_3$, 1.0; KNO_3, 2.0; NH_4Cl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; KH_2PO_4, 2.0; $FeSO_4$, 0.01. The pH which was adjusted to 6.9 with 20% (w/v) K_2CO_3 fell to 6.5 during growth. 10 l of inocolum were added to 30 l of medium to give a final volume of 40 l.

The cells collected at 0° in a Sorvall RC-2 refrigerated centrifuge fitted with a continuous flow head, were washed twice with either 0.05 M phosphate buffer (pH 6.7) or 0.05 M Tris–HCl buffer (pH 7.5).

Preparation of extracts

Cells suspended (25% w/v) in either 0.05 M Tris–HCl (pH 7.5) or 0.05 M phosphate buffer (pH 6.7), both containing 0.2 mM Na-EDTA, were crushed twice in a French Pressure Cell at 20 000 lb/inch^2 at 4°. The crude homogenate was centrifuged at 10 000 $\times$ *g* for 30 min. The supernatant fraction (S_{10}) was centrifuged at 144 000 $\times$ *g* for 1 h and the pellet (P_{144}) resuspended in the appropriate buffer. This suspension, treated with an MSE ultrasonic titanium probe (20 kcycles/sec) at 2° for three separate periods of 3 min was then centrifuged at 20 000 $\times$ *g* for 20 min. The supernatant fraction (Fraction IV) which contained the nitrate reductase enzyme was diluted with buffer and used for the studies reported herein. Portions of the particulate fraction (Fraction III, P144), were treated with lipase II (20 μg/mg protein) for 30 min at 30° and then with the ultrasonic probe as described previously to give Fraction V. 10 ml of this fraction were applied to a Sepharose-4B column (58 cm $\times$ 3 cm) equilibrated with 0.05 M phosphate buffer (pH 6.7) and the column eluted with the same buffer. The active fraction (VI) was eluted in the void volume.

Nitrate reductase assay

This enzyme was assayed in open tubes with either Na_2SO_3 or NADH as electron donor. With Na_2SO_3 the reaction mixture contained: 1.7 ml 0.05 M Tris–HCl buffer (pH 8.5) containing 0.2 mM Na-EDTA; 2.0 μmoles $NaNO_3$; 5.0 μmoles Na_2SO_3 in 2 mM Na-EDTA and 0.1 ml enzyme in a final volume of 2.0 ml. The reaction, started by adding the electron donor, was incubated for 10 min at 30°. The reaction was terminated by adding 1.0 ml 1% (w/v) sulphanilamide in 1.5 M HCl and 1.0 ml 0.2% (w/v) *N*-(1-naphthyl)ethylenediamine hydrochloride. After 15 min the optical density was measured at 540 nm. When NADH was the electron donor the reaction mixture was as follows: 1.6 ml 0.05 M phosphate buffer (pH 7.0) containing 0.2 mM Na-EDTA; 2.0 μmoles $NaNO_3$; 2.0 μmoles NADH and 0.1 ml enzyme in a final volume of 2.0 ml. The reaction was initiated and incubated as in the previous assay. The reaction was terminated by adding 0.1 ml (N) acetaldehyde followed by 0.1 ml crystalline alcohol dehydrogenase (30 μg/0.1 ml phosphate buffer). This rapidly oxidised any residual NADH which would otherwise interfere with the diazotisation reaction for NO_2^-. The NO_2^- produced was then determined as described previously. Enzyme activity is expressed in nmoles NO_2^- produced after 10 min incubation at 30°.

Mass spectrometry

The reaction mixture in a RITTENBERG tube[17] contained 4.0 μmoles $NaNO_3$ and 10 μmoles of either Na_2S, Na_2SO_3, $Na_2S_2O_3$ or $Na_2S_2O_4$ in 0.4 ml in one of the arms. The other limb of the tube contained 3.4 ml 0.05 M Tris–HCl buffer (pH 8.5) containing 0.2 mM Na-EDTA, and 0.2 ml of either 25% (w/v) cell suspension in Tris buffer or of the undiluted supernatant fraction containing nitrate reductase. A roll of filter paper soaked in 10 M KOH was placed inside the cap of the tube to absorb any CO_2 produced. The tubes were rigorously evacuated to $1 \cdot 10^{-5}$ mm Hg and the contents were then mixed and incubated for an hour at 30°. The gases formed were then transferred under high vacuum from the Rittenberg tubes into an A.E.I. MS-2 spectrometer for analysis.

Stoichiometry

Two 0.1-ml aliquots of a standard 2 ml reaction mixture containing 0.5 mg protein were assayed at 15 min intervals for SO_3^{2-} and NO_2^-. Sulphite was determined by the basic fuchsin method of GRANT[8]. The 0.1-ml aliquot was diluted to 4 ml with water and mixed with 1 ml of the colour reagent. After 20 min the optical density at 600 nm was measured. The basic fuchsin and sulphite solutions were prepared daily.

Protein determination

The method of ITZHAKI AND GILL[9] was used.

Reagents

Yeast alcohol dehydrogenase, lipase II (hog pancreas), NADH, NADPH and 2-heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO) were obtained from Sigma Chemical Co., St. Louis, U.S.A. Mepacrine was purchased from Imperial Chemical Industries, England. Sepharose-4B was obtained from Pharmacia, Uppsala, Sweden. All other reagents were of analytical grade.

RESULTS

Preparation of sulphite-dependent nitrate reductase

The nitrate reductase enzyme was located in the particulate fractions. About 40% of the activity was recovered in Fraction IV and the specific activity of this fraction was doubled relative to Fraction I (Table I). Attempts to solubilize the system by lipase II treatment and passage through Sepharose-4B resulted in a loss of enzyme activity (Table I).

Electron donors for nitrate reduction

The most effective electron donors were Na_2SO_3, $Na_2S_2O_4$, NADH, NADPH and reduced methyl viologen (Table II). Mercaptoethanol, 2:3-dimercaptopropanol and Na_2SO_4 were not utilized. Experiments with Na_2S and $Na_2S_2O_3$ showed that these compounds inhibited NO_2^- determination. Consequently their effectiveness as electron donors could not be evaluated. The sodium salts of acetic, formic, succinic and malic acids were ineffective donors for nitrate reduction.

TABLE I

PREPARATION OF SULPHITE-DEPENDENT NITRATE REDUCTASE

Assay conditions as in MATERIALS AND METHODS. Specific activity: nmoles NO_2^- produced/10 min per mg protein.

Step	*Fraction*	*Total activity (nmoles $NO_2^- \times 10^{-3}$)*	*Total protein (mg)*	*Specific activity*	*Yield (%)*
I	French pressure cell Extract in Tris buffer	513.6	2755	186.4	100
II	Supernatant fraction (S_{10})	381.8	1874	203.7	74
III	Pellet (P_{144})	329.1	691	476.3	64
IV	Ultrasonication of III	184.3	531	347.1	36
V	Lipase II treatment of III followed by ultrasonication	24.6	274	105.8	5
VI	Sepharose chromatography of V	20.4	263	77.6	4

TABLE II

ELECTRON DONORS FOR NITRATE REDUCTASE

The assay conditions for Na_2SO_3-dependent nitrate reductase are given in MATERIALS AND METHODS. The reaction mixture with $Na_2S_2O_4$ as donor was similar to that for the Na_2SO_3-dependent assay. The NADH- and NADPH-dependent activities were determined as described for the Na_2SO_3 system except that the reaction was terminated by adding 0.1 ml 1 M zinc acetate and 0.9 ml 95% (v/v) ethanol. Nitrite was then determined in the usual way. Reduced methyl viologen was generated by the method of PRABHAKARARAO AND NICHOLAS[10] and the enzyme assay conducted in Thunberg cuvettes.

Electron donor	*Final concentration (mM)*	*Nitrate reductase activity (nmoles NO_2^- formed/10 min per mg protein)*
Na_2SO_3	2.50	205
$Na_2S_2O_4$	2.50	157
NADH	1.00	92
NADPH	1.00	46
Reduced methyl viologen	0.85	1148

TABLE III

STOICHIOMETRY OF SO_3^{2-} UTILIZATION AND NO_2^- PRODUCTION

Assay conditions as in MATERIALS AND METHODS.

Time (min)	*SO_3^{2-} utilized (nmoles)*	*NO_2^- produced (nmoles)*	*$\frac{SO_3^{2-}}{NO_2^-}$*
1	0	0	0
15	6.9	6.3	1.1
30	11.9	11.5	1.0
45	14.5	13.9	1.0
60	21.0	16.1	1.3

TABLE IV

PRODUCTS OF NITRATE REDUCTION BY WHOLE CELLS

The assay conditions are given in MATERIALS AND METHODS. Gas production is expressed as relative peak heights (proportional to concentration) corrected for controls containing boiled cell suspensions.

Mass number	*Compound*	*Electron donor*			
		Na_2S	Na_2SO_3	$Na_2S_2O_3$	$Na_2S_2O_4$
30	NO	6.0	3.0	1.0	3.5
44	N_2O	26.0	18.5	9.0	19.0

Stoichiometry and products of nitrate reduction

The stoichiometry between SO_3^{2-} utilized and NO_2^- produced was 1:1 as shown in Table III. The cell-free enzyme preparation (Fraction IV) did not yield gaseous products from nitrate with either Na_2S, Na_2SO_3, $Na_2S_2O_3$ or $Na_2S_2O_4$ as an electron donor. A suspension of cells, however, produced NO and N_2O but N_2 was not detected in the mass spectrometer (Table IV). The most effective electron donor for gas production was Na_2S, whilst $Na_2S_2O_3$ was least effective.

Effect of pH

The enzyme was assayed with Na_2SO_3 and NADH as electron donors over a pH range of 5.4–9.5. Phosphate was used in the range 5.4–7.4, Tris–HCl for 7.0–9.5 and a borate–phosphate mixture[11] for 5.8–9.0. There was sharp pH optimum at 8.5 with Na_2SO_3 (Fig. 1) and at 7.0 with NADH.

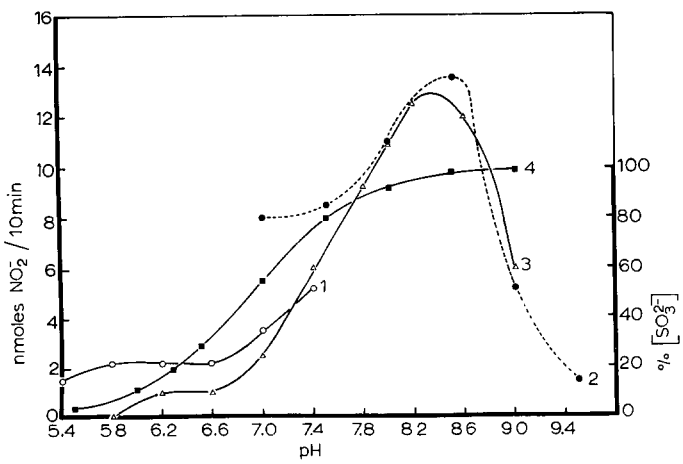


Fig. 1. Influence of pH. (a) Nitrate reductase activity with various buffers: Curve 1, phosphate (○—○); Curve 2 Tris–HCl (●.....●); Curve 3, borate–phosphate (△—△). (b) Curve 4, concentration of SO_3^{2-} (■—■). Protein, 0.9 mg/ml.

TABLE V

EFFECTS OF INHIBITORS ON THE SULPHITE-DEPENDENT NITRATE REDUCTASE

Assay conditions were as described in MATERIALS AND METHODS. The inhibitor was preincubated with the enzyme and buffer, for 5 min at 25°, before adding the substrates. Protein 0.9 mg/ml.

Inhibitor	*Final concentration (mM)*	*% Inhibition*
Iodoacetamide	0.5	31
Arsenite	0.05	83
Piericidin A	1.0	100
Amytal	5.0	46
Antimycin A	0.5	73
HOQNO	0.005	87
2,2'-dipyridyl	5.0	21
o-Phenanthroline	5.0	33
NaN_3	0 05	96
KCN	0.5	99
CO	Bubbled through for 5 min	77
CO	Exposed to tungsten light for 5 min	28

Inhibitor studies

The effects of various inhibitors on the sulphite-dependent nitrate reductase activity are shown in Table V. Mepacrine, KCNS and *p*-chlormercuribenzoate (PCMB) were without effect but iodoacetamide reduced activity by about 30% at 0.5 mM. Azide, arsenite, piericidin A, antimycin A, KCN, HOQNO and CO were potent inhibitors. The CO inhibition was partly reversed by tungsten light. Amytal,

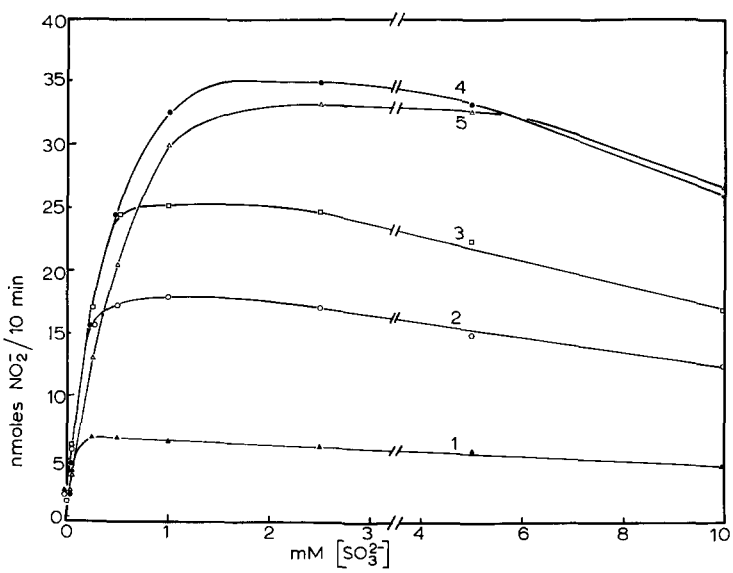


Fig. 2. Nitrate reductase activity with varying levels of SO_3^{2-} at constant levels of NO_3^-. Curve 1, 0.05 mM NO_3^- (▲—▲); Curve 2, 0.25 mM NO_3^- (○—○); Curve 3, 0.5 mM NO_3^- (□—□); Curve 4, 2.5 mM NO_3^- (●—●); Curve 5, 5.0 mM NO_3^- (△—△). Protein, 1.6 mg/ml.

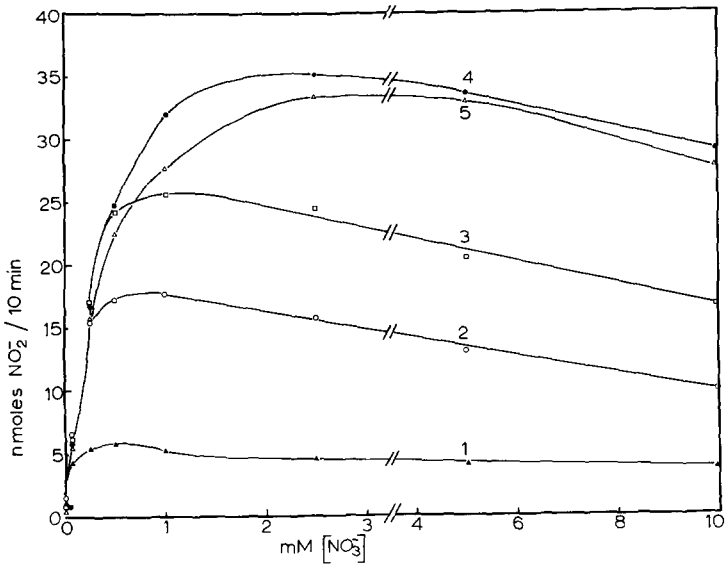


Fig. 3. Nitrate reductase activity with varying levels of NO_3^- at constant levels of SO_3^{2-}; Curve 1, 0.05 mM SO_3^{2-} (▲—▲); Curve 2, 0.25 mM SO_3^{2-} (○—○); Curve 3, 0.5 mM SO_3^{2-} (□—□); Curve 4, 2.5 mM SO_3^{2-} (●—●); Curve 5, 5.0 mM SO_3^{2-} (△—△). Protein, 1.6 mg/ml.

2,2'-dipyridyl and *o*-phenanthroline reduced activity by less than 50% even at 5 mM. The inhibitors had no effect on the diazotisation reaction for NO_2^- determination at the concentrations shown in Table V.

Kinetic studies

There was a linear relation between NO_2^- production and time over 1 h and with enzyme concentration over the range of 0.5 mg – 500 mg protein.

The effects of varying amounts of Na_2SO_3 and $NaNO_3$ on enzyme activity are presented in Figs. 2 and 3. A detailed study of the reaction kinetics was made over a smaller substrate range. Thus, the enzyme was diluted 5-fold and the incubation temperature was set at 20°.

Double reciprocal plots of the data are shown in Figs. 4 and 5. Each point is an average of three determinations. The lines were fitted by the method of least squares and a *t*-test of the slopes for each set of data indicates that they are not significantly different. The intercept values abstracted from Figs. 4 and 5 were then plotted against reciprocal substrate concentrations and the K_m value for each substrate calculated. The K_m value for NO_3^- was $7.2 \cdot 10^{-5}$ M and for SO_3^{2-} $2.5 \cdot 10^{-4}$ M.

DISCUSSION

A preparation of the nitrate-reductase activity was always associated with the cell debris which sedimented at 10 000 × *g*. Attempts to purify the enzyme were unsuccessful and it appears that a particulate fraction is required for sulphite-dependent nitrate reductase activity. Lipase II treatment, ultrasonication and

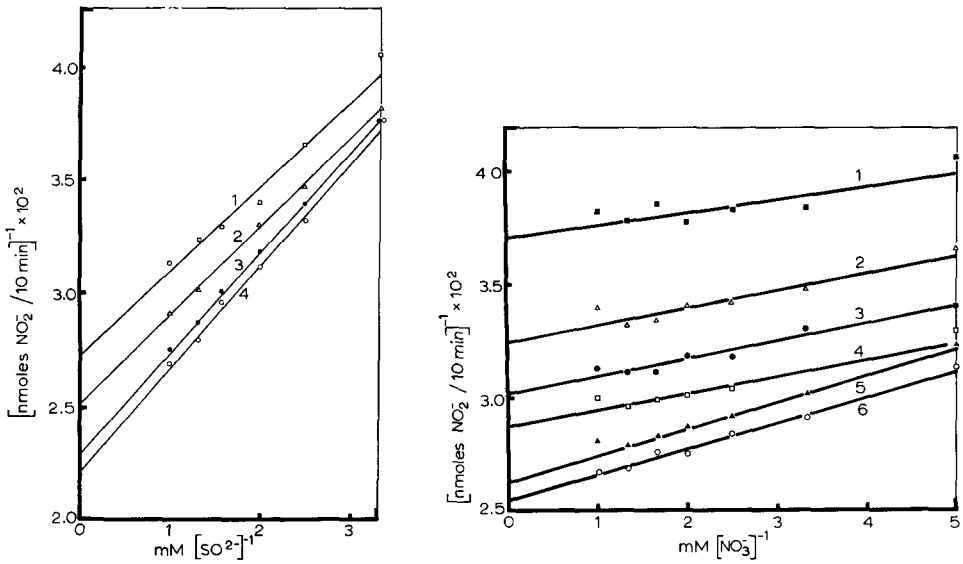


Fig. 4 Double reciprocal plots of nitrate reductase activity *versus* SO_3^{2-} concentration at different NO_3^- levels. Curve 1, 0.2 mM NO_3^- (□—□); Curve 2, 0.3 mM NO_3^- (△—△); Curve 3, 0.5 mM NO_3^- (●—●); Curve 4, 0.75 mM NO_3^- (○—○). Protein 3.2 mg/ml.

Fig. 5. Double reciprocal plots of nitrate reductase activity *versus* NO_3^- concentration at different SO_3^{2-} levels. Curve 1, 0.3 mM SO_3^{2-} (■—■); Curve 2, 0.4 mM SO_3^{2-} (△—△); Curve 3, 0.5 mM SO_3^{2-} (●—●); Curve 4, 0.6 mM SO_3^{2-} (□—□); Curve 5, 0.75 mM SO_3^{2-} (▲—▲); Curve 6, 1.0 mM SO_3^{2-} (○—○). Protein, 3.2 mg/ml.

Sepharose chromatography resulted in decreased activity (Table I). A range of detergents and organic solvents also inactivated the enzyme.

At pH 8.5 reduced methyl viologen was the most effective electron donor, followed by Na_2SO_3 and $Na_2S_2O_4$. Whole cells of *T. denitrificans* catalyzed the oxidation of $Na_2S_2O_3$, Na_2S and Na_2SO_3 with either NO_3^- or O_2 as the terminal acceptor[12]. Cell-free extracts of *Thiobacillus X* are known to oxidise mercaptoethanol aerobically[13]. We have been unable to test $Na_2S_2O_3$ and Na_2S as donors in our system because they interfere with NO_2^- determination but mercaptoethanol was ineffective. In contrast to the heterotrophic denitrifying bacteria[4,5] organic acids were not utilized for nitrate reduction by *T. denitrificans*.

The production of NO and N_2O by intact cells indicates that the bacterium utilizes Na_2S, Na_2SO_3, $Na_2S_2O_4$ and $Na_2S_2O_3$ for denitrification. The cell-free preparation reduced NO_3^- to NO_2^- only. These results are comparable to those obtained with extracts of *Pseudomonas denitrificans*[4].

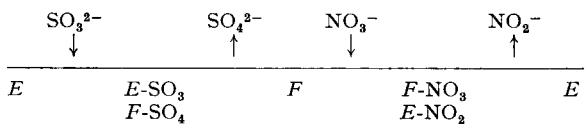
Over the pH range of 5.4–8.5 sulphite-dependent nitrate reductase activity closely correlates with SO_3^{2-} concentration calculated from the pK_2 of sulphurous acid, 6.91[14], and the Henderson–Hasselbach equation (Fig. 1). This suggests that SO_3^{2-} and not HSO_3^- is the ionic species utilized by the nitrate reductase. Moreover, since the enzyme was active with NADH down to pH 5.4, the relatively low sulphite-dependent activity below pH 7.5 is unlikely to be due to enzyme denaturation.

The results of inhibitor studies agree quite closely with those of PEETERS AND

ALEEM[12] with intact cells. This suggests that the nitrate reductase may be dependent on an electron transport system containing cytochromes of the *b* and *c* types and possibly a cytochrome oxidase coupled to nitrate reductase. Spectral evidence for a *c*-type cytochrome and of cytochrome oxidase in *T. denitrificans* has been reported previously[7]. Sulphydryl groups do not appear to be important since PCMB was without effect and iodoacetamide was only slightly inhibitory.

The enzyme rapidly responds to very low substrate concentrations (Figs. 2 and 3) and over 90% of the maximum rate was obtained with only 10% of the maximum substrate concentration. This suggests a co-operative mechanism.

The kinetic data illustrated in Figs. 4 and 5 were analyzed according to the system of CLELAND[15,16]. Double reciprocal plots of rate data for a two substrate enzyme yielding families of parallel lines suggest a Ping–Pong Bi–Bi mechanism[15]. This model requires the enzyme to oscillate between two stable forms, in this case, presumably oxidized and reduced. The reaction mechanism may therefore be depicted as follows:



E, represents oxidized enzyme; *F*, represents reduced enzyme.

CLELAND[16] further predicts that if each substrate combines with only one enzyme form then intercept *versus* reciprocal substrate plots should be linear and this was found to be the case.

The K_m for NO_3^- at $7.2 \cdot 10^{-5}$ M is an order of magnitude lower than that for nitrate reductase from *Pseudomonas denitrificans*[4]. The K_m for SO_3^{2-} at $2.5 \cdot 10^{-4}$ is higher than for NO_3^-.

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