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A NITRATE REDUCTASE FROM *MICROCOCCUS DENITRIFICANS*

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SUMMARY

A nitrate reductase from *Micrococcus denitrificans* (N.C.I.B. 8944) was associated with cell membranes. This particulate system utilized NADH and succinate as electron donors for the reduction of nitrate to nitrite. Small amounts of nitric oxide, nitrous oxide and nitrogen gas were also produced. The K_m for NADH using nitrate and oxygen as acceptors were $1.8 \cdot 10^{-5}$ M and $2 \cdot 10^{-5}$ M, respectively.

The solubilized nitrate reductase purified 108-fold converts nitrate to nitrite stoichiometrically with reduced benzyl viologen as the electron donor. Reduced methyl viologen was also effective, but NADH, succinate and reduced cytochrome *c* were not utilized. The purified enzyme contained Mo as shown by isotope labelling. Its involvement in enzyme action is indicated by strong inhibition by KCNS and dithiol. Idoacetamide and *p*-chloromercuribenzoate also inhibited the enzyme, and the effect in the latter case was reversed by reduced glutathione. The purified enzyme did not contain any cytochrome or flavin. Maximum enzyme activity was observed at pH 6.3 and a K_m of $9.6 \cdot 10^{-4}$ M was recorded for nitrate when reduced benzyl viologen was the electron donor.

INTRODUCTION

Nitrate reductase systems in microorganisms are of two main types; the assimilatory enzyme and the system involved in nitrate respiration. The assimilatory enzyme is nonparticulate[1–4] and utilizes NADH or NADPH as an electron donor with flavin and Mo as cofactors[5,6]. The respiratory nitrate reductase system, however, is usually membrane bound and relies on cytochromes as electron carriers[9,10]. Exceptions do occur as in *Aerobacter aerogenes* where the nitrate reductase, apparently of the respiratory type, was reported not to require cytochrome as carrier[7].

The nitrate reductase from *Escherichia coli* utilizes reduced cytochrome *b* as donor but for the purified enzyme, reduced dyes only are effective[8]. The enzyme contains Mo and iron but no cytochromes or flavins.

Nitrate reductase systems from denitrifying bacteria were found to oxidize reduced cytochrome *c* under anaerobic conditions[9,10] and in the presence of nitrate.

Abbreviations: PCMB, *p*-chloromercuribenzoate; PMS, phenazine methosulphate.

Since many of these studies were made with either intact cells or with the membrane-bound enzyme, it is probable that other reductase systems which utilize nitrite and other intermediates might also bring about the oxidation of reduced cytochrome *c*. A purified enzyme from a halotolerant, *Micrococcus denitrificans* was shown to utilize reduced cytochrome *c*, although a brown protein from the same bacterium was a more effective electron donor[11]. Cytochromes were not detected in the purified enzyme.

M. denitrificans (N.C.I.B. 8944) uses nitrate for both assimilation[12] and respiration[13,14]. When the bacteria were grown under anaerobic conditions in cultures containing ammonium nitrate, a respiratory type nitrate reductase system was produced. This paper describes the purification and some properties of a terminal enzyme from the system.

MATERIALS AND METHODS

Organism

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

Experiments with ^{99}Mo

The bacteria were sub-cultured through 5 transfers of 1-l lots of liquid medium without added molybdenum and cultures which were 18 h old were transferred into 9 l of fresh medium containing 15 μC of ^{99}Mo per l (specific activity of ^{99}Mo equivalent to 89.5 mC/g). After 16 h growth at 37°, the cells were collected in a refrigerated centrifuge in the radioisotope laboratory.

Preparation of cell extracts

Cell extracts prepared by resuspending 1 part of cell paste in 2 parts of cold phosphate buffer (w/v) were crushed at 2° in a French Pressure Cell at 20 000 lb/inch^2. Intact cells and cell debris were removed by centrifuging at 10 000 × *g* for 15 min. The supernatant fraction (S-10) was further centrifuged at 144 000 × *g* for 2 h in a Spinco preparative ultracentrifuge. The pellet (P-144) was used in the studies with NADH–nitrate reductase and the enzyme was purified further from this preparation.

Solubilization of nitrate reductase

The pellet (P-144) obtained by centrifuging the S-10 supernatant fraction at 144 000 × *g* for 2 h was resuspended in 0.05 M phosphate buffer (pH 8.0) containing 10^{-3} M KNO_3 and 15 mg/ml of sodium deoxycholate to give a final protein concentration of 30 mg/ml. The pH of the suspension was readjusted to 8.0 by adding 0.1 M NaOH solution. After homogenizing in a Teflon tissue grinder the suspension was incubated at 50° for 5 min and then put in ice for 12 h. It was then centrifuged at 144 000 × *g* for 2 h and the supernatant fraction (S-144) was dialysed against 2 l of phosphate buffer (pH 7.5) containing 10^{-3} M KNO_3 with 3 changes of buffer. The dialysis residue was used for purification of the nitrate reductase.

DEAE-cellulose and Sephadex G-100 column chromatography

DEAE-cellulose after precycling with NaOH and subsequently HCl was equilibrated with 0.005 M phosphate buffer (pH 7.5) before packing into a column of 1.2 cm × 24 cm. A cellulose protein ratio of 1 ml:15–20 mg protein was used. After the sample was loaded onto the column, the nitrate reductase was eluted between 0.20 and 0.36 M NaCl using a linear gradient. The Sephadex column was equilibrated with 0.05 M phosphate buffer (pH 7.0) containing 0.1 M NaCl and the enzyme was eluted with the same buffer. A column with void volume equivalent to around 30 times the sample volume was employed.

Preparation of Azotobacter particles

Particles from *Azotobacter vinelandii* containing an active NADH–benzyl viologen reductase was prepared as described by NAIK AND NICHOLAS[15]. Each preparation was checked to ensure that it was free from nitrate or nitrite reductase activity.

Nitrate reductase assay

The enzyme was assayed anaerobically in Thunberg tubes by following the reduction of NO_3^- or the formation of NO_2^-. The reaction mixture consisted of 2 μmole of KNO_3, 0.01 ml of enzyme in 0.05 M phosphate buffer (pH 7.5) in a final volume of 3.0 ml with either 2 μmoles of NADH or 2 μmoles of NADH *plus* 0.1 μmole benzyl viologen and 0.05 ml azotobacter particles to generate reduced benzyl viologen as an electron donor.

Assays for NADH oxidase and NADH–nitrate reductase

The two enzymes were assayed in a Unicam SP 700 recording spectrophotometer fitted with a constant temperature attachment set at 25°. The reaction mixture contained 0.4 μmole NADH, 0.01 ml P-144 particle in 0.05 M phosphate buffer (pH 7.5) 0.5 mg protein/ml in a final volume of 3.0 ml. The NADH oxidase activity was measured at 340 mμ in open cuvettes. The NADH–nitrate reductase activity was determined under anaerobic conditions in Thunberg cuvettes. For the NADH–nitrate reductase assay 2 μmoles of KNO_3 were also included in the reaction mixture, and the NO_2^- formed was determined at the end of the reaction.

Chemical determination of NO_3^- and NO_2^-

Nitrite was determined by the GRIESS AND ILOSVAY colorimetric method[15] and nitrate by the diphenylamine-*p*-diaminodiphenyl sulphone method of SZEKELY[17] as described by LAM AND NICHOLAS[18].

Measurement of ^{99}Mo

The ^{99}Mo activity was measured with a thallium-activated well scintillation counter linked to an EKCO scaler. The specific activity of ^{99}Mo in the enzyme fraction was related to a series of standards prepared from the same batch of the radioisotope.

Mass spectrometry

The reaction mixture in a Warburg flask contained 2 μmoles of NO_3^- and 2 μmoles of the appropriate electron donor in the side arm and 0.01 ml enzyme in the reaction flask in a final volume of 3.0 ml. The centre well contained 0.2 ml of

20% KOH to absorb CO_2. The reaction mixture was rigorously evacuated with a diffusion pump, and the gases present were measured before and after the enzyme reaction in an A.E.I. MS-2 mass spectrometer. The relative amounts of each gas present were related to argon which would come from residual air in the system.

Starch-gel electrophoresis

Starch gel was prepared as described by SMITH[19] using 0.08 M Tris–HCl buffer (pH 9.0) and 0.03 M sodium borate (pH 9.0) was used as a bridge buffer. The electrophoresis was carried out at 2° at a constant current of 20 mA. After the electrophoresis run the starch gel was cut horizontally into three slices. The top and bottom layers were stained with 0.1% nigrosin[19]. The middle slice was cut vertically into 1-cm portions starting from the origin. These were homogenized and tested individually for nitrate reductase activity in Thunberg tubes (see METHODS).

Co-factors and other reagents

NADH, NADPH, FMN, mammalian cytochrome *c* were obtained from Sigma Chemical Co., St. Louis, U.S.A. Cytochrome *c* from *M. denitrificans* was purified by $(NH_4)_2SO_4$ fractionation and DEAE-cellulose chromatography from the supernatant fraction left after centrifuging the S-10 at 144 000 × *g* for 2 h. Crystalline cytochrome b_2 from yeast was a gift from Dr. R. H. Symons of this University. Samples of DEAE-cellulose and Sephadex G-100 were purchased from Whatman Co., England, and Pharmacia, Uppsala, Sweden, respectively. All other reagents were of analytical grade.

RESULTS

Distribution of nitrate reductase

The nitrate reductase was associated with the membrane fraction which con-

TABLE I

DISTRIBUTION OF NITRATE REDUCTASE IN CELL-EXTRACTS

The enzyme activity was assayed at 30° in Thunberg tubes. The reaction mixture consisted of 2 μmoles of either NADH or succinate (electron donor), 2 μmoles KNO_3, 0.02 ml enzyme in 0.05 M phosphate buffer (pH 7.5) in a final volume of 3.0 ml. When reduced benzyl viologen was generated as an electron donor, 0.1 μmole benzyl viologen, 2 μmoles NADH and 0.05 ml azotobacter particles were used[15].

Fraction	*% of total nitrate reductase activity (crude extract = 100%)*		
	Electron donor: NADH	*Succinate*	*Reduced benzyl viologen*
1. Crude cell extract	100	100	100
2. S-10 (supernatant fraction left after centrifuging (1) at 10 000 × *g* for 15 min)	88	90	95
3. P-144 (particulate fraction left after centrifuging (2) at 144 000 × *g* for 2 h)	68	65	70
4. S-144 supernatant fraction from Fraction 3	18	20	32

TABLE II

PRODUCTS OF NITRATE REDUCTION

The assay conditions as in Table I except that the reactions were carried out in Warburg flasks. The gases present were assayed in the mass-spectrometer immediately before and after each reaction. The amounts of NO_3^- and NO_2^- left at the end of the assay were also determined (see METHODS). The results tabulated below were obtained from 3 separate experiments.

Electron donors	*Expt. No.*	*NO_3^- reduced (μmoles)*	*NO_2^- formed (μmoles)*
Succinate	1	0.95	0.91
	2	1.30	1.25
	3	0.71	0.69
NADH	1	1.93	1.88
	2	2.39	2.30
	3	1.54	1.50

tained the respiratory system. The bulk of the enzyme was located in the particulate fraction collected after centrifuging the crude extract for 2 h at 144 000 × *g* (Table I). Some enzyme activity, however, remained in the supernatant fraction, and this was probably associated with smaller membrane fragments since prolonged centrifugation of this fraction at 226 000 × *g* yielded a pellet which contained a nitrate reductase with similar properties to that in the P-144 fraction.

The electron donors and products of nitrate reduction

The most effective electron donors for NO_3^- reduction catalysed by either the crude extract or the P-144 fraction were NADH and succinate. Almost all the NO_3^- reduced by the P-144 fraction was converted into NO_2^- (Table II). A small amount of nitrite was, however, reduced further to NO, N_2O and N_2 (Fig. 1). The amount of nitrite reduced was usually less than 5% of that formed from nitrate. The K_m for NADH oxidation by the NADH–nitrate reductase was about $2.0 \cdot 10^{-5}$ M as compared with $1.8 \cdot 10^{-5}$ M for the corresponding NADH oxidase system.

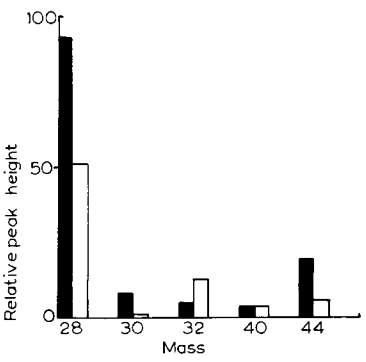


Fig. 1. Gaseous products formed during reduction of nitrate by P-144 using NADH as electron donor. Clear histogram represents measurement with mass spectrometer at zero time and black histogram represents those at the end of the incubation.

Spectrophotometric assay in Thunberg cuvettes under anaerobic conditions showed that reduced mammalian cytochrome *c* was not oxidized by the P-144 fraction with NO_3^- as acceptor and the latter was not reduced. On exposing the reaction mixture to air, the reduced cytochrome *c* was reoxidized within 1 min, indicating the presence of an active cytochrome oxidase. The same system also oxidized NADH anaerobically with nitrate as the terminal acceptor.

The effects of pH and ionic strength

The activities of the NADH–nitrate reductase and NADH oxidase were measured over a range of pH values. Nitrate reductase had a similar activity at pH 6.0 and 7.3. At pH 8.0, however, a 30% reduction in nitrate reductase activity was observed but over a range of ionic strengths 0.02–0.20 M the enzyme activity was unchanged. The NADH oxidase activity was reduced by about 50% at pH 6.0 but was similar at pH 7.3 and pH 8.0 with the same buffer. Over a range of buffer concentrations (0.02–0.20 M) both enzyme reactions were unchanged.

TABLE III

PURIFICATION OF NITRATE REDUCTASE

The P-144 particles were solubilized as described in METHODS. Nitrate reductase activity using reduced benzyl viologen as the electron donor was determined by following nitrite formation.

Fraction	*Total protein (mg)*	*Enzyme recovery (%)*	*Specific activity (μmoles No_3^- reduced/ 10 min per mg protein)*	*Purification*
1. S-10 (supernatant fraction left after centrifuging crude extract at 10 000 × *g* for 15 min)	8960	100	0.25	1
2. P-144 (pellet, 144 000 × *g* for 2 h)	5060	80	0.36	1.4
3. S-144 (supernatant fraction left after centrifuging the solubilized P-144 pellet at 144 000 × *g* for 2 h)	1800	60	0.75	3.0
4. Fraction 3 dialysed against 0.05 M phosphate (pH 7.5) and precipitated with $(NH_4)_2SO_4$ between 0.35 and 0.45% satn.	380	44.5	2.65	10.5
5. Fraction 4 dialysed against 0.005 M phosphate (pH 7.5) and eluted from DEAE-cellulose column with the same buffer using a linear gradient between 0.20 and 0.36 M NaCl	31	14	10.2	41
6. Fraction 5 dialysed against 0.05 M phosphate (pH 7.5) and precipitated with $(NH_4)_2SO_4$ between 40 and 50% satn.	6	5.3	20.0	80
7. Fraction 6 resuspended in 0.05 M phosphate (pH 7.5) containing 0.1 M NaCl and developed through Sephadex G-100 (medium) column equilibrated in same buffer. The enzyme was eluted with buffer equivalent to 1.0–1.3 of the void volume of the column	4	4.8	26.9	108

Stabilization and purification

The nitrate reductase was solubilized from the P-144 particles by treating with deoxycholate (0.5 mg/10 mg protein) and then centrifuging at 144 000 × *g* (see METHODS). The solubilized nitrate reductase recovered in the supernatant fraction had very little overall NADH–NO_3^- reductase activity and was assayed by using reduced benzyl viologen as the artificial electron donor. The reduced benzyl viologen–nitrate reductase enzyme was purified 108-fold by procedures outlined in Table III. This enzyme remained active for 8 weeks at −15° in 0.05 M phosphate buffer (pH 7.5) containing 10^{-3} M nitrate.

Electron donors and products of nitrate reduction

A variety of compounds were tested as hydrogen donors for the reduced benzyl viologen–nitrate reductase. Only reduced benzyl viologen and methyl viologen were effective and reduced methylene blue and phenazine methosulfate (PMS) (reduced with NADH) were only 3 and 8% as efficient as reduced benzyl viologen. Reduced FMN, NADH, succinate, reduced cytochrome *c* from the bacterium and from horse heart were, however, ineffective.

No significant amounts of gaseous product were detected when 2 μmoles of NO_3^- was reduced to NO_2^- by the purified enzyme and a stoichiometric conversion of NO_3^- to NO_2^- was recorded. The K_m for the nitrate reductase with reduced benzyl viologen as a donor was found to be $9.6 \cdot 10^{-4}$ M using the HENRI equation[20].

Inhibitor studies

The effects of various metal-binding reagents on the activity of the purified nitrate reductase with reduced benzyl viologen as the donor are shown in Table IV (Fraction 7). Urethane, α,α′-dipyridyl, 8-hydroxyquinoline, bathocuproin, sodium

TABLE IV

EFFECT OF VARIOUS INHIBITORS

Enzyme assays were carried out in Thunberg tubes as described in METHODS. The inhibitor was incubated with the enzyme (Fraction 6) for 10 min prior to starting the reaction. Where inhibitors were dissolved in organic solvents, the same volume of solvent was included without the inhibitor in control experiments.

Inhibitor	*Final concn. (mM)*	*Inhibition (%)*
KCN	10^{-2}	30
	10^{-3}	0
KCNS	$3 \cdot 10^{-4}$	53
	$3 \cdot 10^{-5}$	20
Dithiol	$3 \cdot 10^{-4}$	100
	$3 \cdot 10^{-5}$	68
PCMB	10^{-4}	90
	10^{-5}	52
PCMB + GSH (10^{-4} M)	10^{-4}	30
Iodoacetamide	10^{-4}	67
	10^{-5}	45

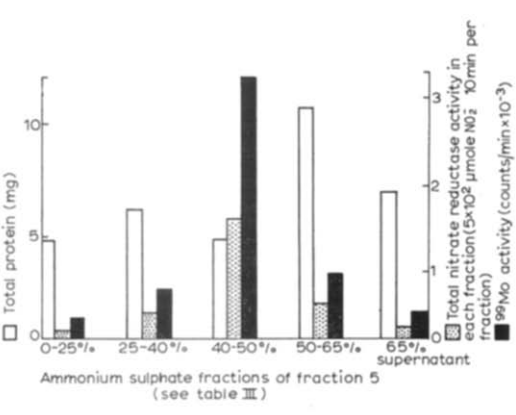
diethyldithiocarbamate each at 10^{-3} M had no effect. Although *o*-phenanthroline did not inhibit the reduced benzyl viologen-dependent purified enzyme, it reduced the activity of the NADH–nitrate reductase in the P-144 particles by about 50%. Toluene-3,4-dithiol and KCNS, which are known to chelate Mo, were strongly inhibitory (see Table IV), but KCN restricted enzyme activity (Fraction 7) at relatively high concentrations only.

The –SH inhibitors, *p*-chloromercuribenzoate (PCMB) and iodoacetamide reduced the activity of Fraction 7, and the inhibition by the former was partially reversed by reduced glutathione (see Table IV).

The respiratory inhibitors, amytal, mepacrine, rotenone and pericidin A which strongly affect the NADH–nitrate reductase system did not interfere with the activity of the purified enzyme. CO had no effect on nitrate reductase.

Studies with ^{99}Mo

To determine whether the purified reduced benzyl viologen–nitrate reductase contains Mo, the enzyme was purified from P-144 particles prepared from bacteria grown in culture medium containing ^{99}Mo (see METHODS) as shown in Table III. About 0.5 μC ^{99}Mo (6 μg Mo) was recovered in the protein of Fraction 3 (see Table III) even after extensive dialysis. Further purification of Fraction 3 as given in Table III resulted in the accumulation of Mo in the fractions with highest enzyme activity.



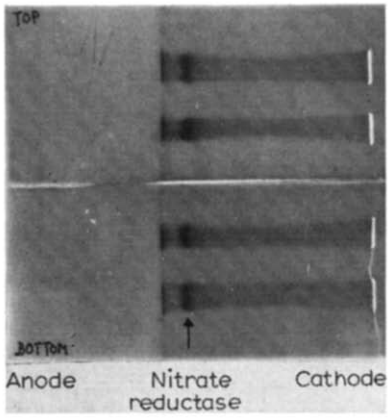


Fig. 2. Ammonium sulphate fractionation of the solubilized reduced benzyl viologen–nitrate reductase in Fraction 5.

Fig. 3. Starch-gel electrophoresis of nitrate reductase (Fraction 5a). The purified nitrate reductase from the isotope-labelling experiment was run on starch gel prepared as described in METHODS. The conditions of separation were as follows: gel buffer: Tris–HCl (pH 9.0, 0.08 M); bridge buffer: borate (pH 9.0, 0.03 M); conditions: 150 min, 20 mA. Arrow shows the location of protein and nitrate reductase activity at the end of electrophoresis.

When Fraction 5 was fractionated with ammonium sulphate, both the reduced benzyl viologen–nitrate reductase and ^{99}Mo were precipitated between 40 and 50% $(NH_4)_2SO_4$ saturation (Fraction 5a). The other ammonium sulphate fractions of Fraction 5 contained very little reduced benzyl viologen–nitrate reductase and ^{99}Mo activity as shown in Fig. 2. Reduced benzyl viologen–nitrate reductase (Fraction 5a)

after dialysis ran as a single band on starch gel electrophoresis (Fig. 3). However, it was not possible to determine quantitatively the amount of Mo in this band because of the difficulty of extracting the protein from the starch gel.

pH optimum and temperature stability and spectra

The pH optimum of the reduced benzyl viologen–nitrate reductase was measured in Tris–maleate and phosphate buffers (0.05 M) both prepared from potentiometric titration. Maximum activity was observed at pH 6.3. The activity fell rapidly on the acid side and at pH 5.8 it was 50% of the optimum and at pH 7.3 it was 80%. The enzyme was stable at 50° for 10 min but inactivation occurred after further incubation.

Spectra of the purified enzyme measured between 260–750 mμ showed only an absorption peak at 280 mμ. No cytochrome or flavin was detected.

DISCUSSION

In earlier reports, we have pointed out the similarities which exist between the NADH oxidase and the NADH–nitrate reductase systems[18]. The present investigation shows that both NADH oxidase and NADH–nitrate reductase of *M. denitrificans* are bound to membranes. This is in agreement with observations on other bacteria which use nitrate for respiratory purposes. The similarity of the K_m for NADH with either NO_3^- or O_2 as the electron acceptor, supports our earlier suggestion that the two systems probably share the same primary dehydrogenase[18]. Although the optimum pH for the two systems are dissimilar, this probably reflects a difference in the terminal enzymes, *viz.* cytochrome oxidase and nitrate reductase rather than on other components of the respiratory chain. It is noteworthy that the pH optimum for the NADH–nitrate reductase in crude extracts (S-10) is similar to that of the purified enzyme (Fraction 7).

The products of nitrate reduction in the particulate fraction (S-10) were nitrite, nitrous oxide, nitric oxide and nitrogen gas but the purified reduced viologen-dependent enzyme (Fraction 7) reduced NO_3^- to NO_2^- only.

HORI[11] has reported that in a halotolerant micrococcus, reduced bacterial cytochrome *c* was reoxidized by a nitrate reductase when NO_3^- was the electron acceptor. Nitrate reductase from *Pseudomonas aeruginosa*[21] also utilized reduced mammalian cytochrome *c* as an electron donor. The particulate preparations from *M. denitrificans* did not, however, oxidize exogenous reduced cytochrome *c* under anaerobic conditions with NO_3^- but rapid reoxidation occurred readily when O_2 was introduced into the system. The reduced benzylviologen–nitrate reductase did not utilize reduced cytochrome *c*-549 from the same bacterium. It is interesting to note that whereas the NADH–nitrate reductase in the halotolerant micrococcus was inhibited by antimycin A, the same system in *M. denitrificans* was unaffected. In this respect, the NADH–nitrate reductase from *M. denitrificans* is probably similar to that in *E. coli*[8] where cytochrome *b* rather than cytochrome *c* is the electron carrier. Both the halotolerant micrococcus and *E. coli* are known to contain active formate-dependent nitrate reductases, this hydrogen donor was relatively ineffective for the enzyme in *M. denitrificans*.

The purified nitrate reductase from *M. denitrificans* shares some common

characteristics with the purified enzyme from both *E. coli* and the halotolerant micrococcus. It did not contain cytochromes or flavin and required –SH groups for its activity. A Mo requirement for enzyme activity is implicated by ^{99}Mo labelling of the enzyme and its inhibition by KCNS and dithiol.

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