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NITRATE REDUCTASE FROM BACTEROIDS OF *RHIZOBIUM JAPONICUM*: ENZYME CHARACTERISTICS AND POSSIBLE INTERACTION WITH NITROGEN FIXATION

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Summary

The soluble nitrate reductase of *Rhizobium japonicum* bacteroids has been purified and its properties compared to those of aerobically grown cells. The enzymes from both sources are similar with molecular weights of about 70 000 suggesting no close relationship with the molybdo-protein component of nitrogenase.

Nitrite, the product of nitrate reductase, strongly inhibited the nitrogenase activity from bacteroids, at concentrations less than 100 μM. Thus, an interference in the rate of nitrogen fixation is possible as a result of nitrate reductase activity.

A study of the distribution of nitrate reductase in bacteroids indicates that a proportion of the total activity is membrane-bound but that this activity is similar to that in the soluble fraction. Purified nitrate reductase required reduced viologen dyes for activity. Neither NADPH or NADH or FAD could substitute as electron donors. Dithionite is a strong inhibitor and inactivated nitrate reductase from all sources examined. This inactivation is prevented by methyl viologen.

Purified nitrate reductase from bacteroids and bacteria *Rhizobium japonicum* is practically unaffected by exposure to oxygen.

Introduction

The presence of a constitutive nitrate reductase in *Rhizobium japonicum* has been known for many years [1—3]. Earlier studies emphasized the particulate nature of this activity [1,2] and showed its similarity to that in *Escherichia coli* exhibiting nitrate respiration [4]. Lowe and Evans [5] later succeeded

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in purifying a soluble form of nitrate reductase from laboratory-cultured cells of *R. japonicum*, utilizing reduced benzyl viologen as electron donor. This enzyme was claimed to be extremely sensitive to oxidation. This period preceded that when it has been possible to prepare nitrogenase from bacteroids of *R. japonicum* from soybean nodules [6—8], and a possible relationship of nitrate reductase activity to N_2 fixation was then examined, but found improbable [9,10].

Recently, interest in this nitrate reductase activity was revived when it was found that strain CC705 (syn. Wisconsin 505) was capable of anaerobic growth with nitrate as terminal electron acceptor in place of O_2 [11]. It was then shown that bacteroids of this strain could also use nitrate in place of O_2 to support N_2 or C_2H_2 reduction by nitrogenase [12], presumably utilizing ATP generated by nitrate respiration.

In this paper, we report further on the properties of particulate and purified soluble nitrate reductase from *R. japonicum*, with particular reference to bacteroids from soybeans. The characteristics of nitrogenase from strain CC705 are also briefly described and the possible effect of nitrate reductase activity on N_2 fixation by soybean nodules examined.

Methods

Nodules. Soybeans (*Glycine max* Merr. cv. Altona), inoculated with *R. japonicum* strain CC705 (syn. Wisconsin 505) were grown in a glasshouse and watered with a nitrogen-free mineral solution described previously [13]. Nodules were harvested 30—32 days after their appearance on the roots.

Bacteroid suspensions. Nodules (about 40—50 g fresh weight) were disrupted anaerobically in 0.1 M sodium phosphate buffer (pH 7.4) containing 0.2 M sodium ascorbate and polyvinylpyrrolidone (10 g/100 ml) using a modified Sorvall Omni-mixer equipped with a filtration system as described by Bergersen and Turner [8]. Bacteroids were washed twice after initial centrifugation with an anaerobic sodium phosphate buffer (25 mM, pH 7.4) containing 2 mM $MgSO_4$ and 0.3 M sucrose [14].

Laboratory-cultured bacteria. R. japonicum (CC705) were grown aerobically with continuous magnetic stirring in 1-l flasks containing 250 ml of liquid medium with the following composition (g/l) : NaCl, 0.2; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; $CaSO_4 \cdot 2H_2O$, 0.1; $CaCO_3$, 0.1; mannitol, 10.0; yeast extract concentrate, 0.5; sodium glutamate, 0.5. Cultures were harvested after 4 days growth yielding 2.6 g fresh weight of cells per l.

Nitrogenase preparation. Bacteroids were suspended in 50 mM *N*-tris(hydroxymethyl) methyl-2-aminoethane sulphonic acid (TES) buffer (pH 7.4) normally containing 0.5 mM $Na_2S_2O_4$ and disrupted in an M.S.E. 16 Kc ultra sonicator for 3 min. Anaerobic conditions were maintained with a stream of O_2-free N_2 or of argon. Crude extract of nitrogenase, containing about 5—10 mg of protein per ml, was obtained by centrifugation at 35 000 × *g* for 30 min. Activity was measured by acetylene reduction [15] in rubber-capped vials (22 ml), containing in 2.0 ml (μmol): TES buffer, 90; ATP, 5; $MgCl_2$, 5; creatine phosphate, 20; and 100 μg of creatine kinase. The gas phase contained 10% acetylene in argon. Reaction was initiated by the addition of 20 μmol of

$Na_2S_2O_4$ or of nitrogenase extract. Gas samples (200 µl) were taken from the flasks at 5—10-min intervals during incubation at 30°C, for ethylene measurement by gas chromatography, using a Pye-Unicam instrument equipped with a column of Porapak R.

Preparation of nitrate reductase. All operations were carried out with N_2-saturated buffers or under a stream of N_2, using Beckman tubes for centrifugation to allow exclusion of air.

Soluble nitrate reductase was obtained by sonication of cells for 4 min at 0°C in the same system as for nitrogenase, using 0.1 M sodium phosphate buffer, (1 g fresh weight for 10 ml buffer), followed by centrifugation at 35 000 × *g* for 30 min. No $Na_2S_2O_4$ was added. Soluble nitrate reductase was purified as indicated in Table II, by $(NH_4)_2SO_4$ fractionation and Sephadex G-150 (Pharmacia, Uppsala) filtration. Gels beds (85—90 cm × 2.5 cm) were eluted with degassed 0.1 M phosphate buffer (pH 7.5). Fractions (4 ml) were collected by a Gilson collector placed in a refrigerated glove-box flushed with N_2.

The Sephadex G-150 columns were calibrated for molecular weight estimations with horse radish peroxidase, bovine serum albumin (Sigma Chemical Co, St Louis, Mo), γ-globulin Fraction II, (Calbiochem, San Diego Calif.), beef liver catalase (N.B.C. Cleveland Ohio) and urease (Fluka A.G. Buchs, Switzerland).

Particulate nitrate reductase was prepared by resuspension of the pellet after centrifugation of sonicated bacteria or bacteroids in 0.1 M sodium phosphate buffer (pH 7.5). Centrifugation at 5000 × *g* for 10 min removed the small proportion of unbroken cells and particles were then sedimented at 35 000 × *g* for 30 min. The supernatant was discarded and the pellet resuspended in 10 or 20 ml of the phosphate buffer used above.

Nitrate reductase assay. Assays were performed at 30°C in sealed flasks filled with N_2 after evacuation. The reaction mixture contained in 3.0 ml (µmol): sodium phosphate buffer (pH 7.5) 300; KNO_3, 15; methyl viologen, 2; $Na_2S_2O_4$, 10 or 20 and enzyme. Reactions were initiated by injection of either enzyme, or more commonly, dithionite; the order of addition made no difference to the activity recorded. For assays yielding up to 1.5 µmol of nitrite in 30 min, time courses were exactly linear and proportional to protein concentration. Reaction was stopped by agitation in a stream of O_2. Incomplete oxidation of dithionite interfered with nitrite estimations. Assays using benzyl viologen in place of methyl viologen were also successful, but only about half as active. The common practice of using $NaHCO_3$ to maintain anaerobiosis slightly depressed activity with both viologen dyes (Table III).

Colorimetric assays. Protein was determined by the method of Lowry et al. [16] using the Folin-Ciocalteu reagent with bovine serum albumin as standard.

For the estimation of nitrite, samples of reaction mixtures were further diluted with water to ensure complete elimination of dithionite and then assayed for nitrite by the method of Nicholas and Nason [17].

TABLE I

REACTIVATION OF NITROGENASE

Inactive extract (2.0 ml) was incubated as indicated at 30°C for 3 h under N_2 and 0.5-ml (1.6 mg protein) aliquots assayed as described in Methods. The enzyme extract was prepared without added dithionite and was kept at room temperature under N_2 until inactive.

Treatment	Specific activity (nmol ethylene/min per mg protein)
Control (fresh preparation)	12.7
Control (without treatment)	0
Dithiothreitol (5 mM)	0
$FeSO_4$ (5 mM)	0.05
Dithiothreitol (5 mM) + $FeSO_4$ (5 mM)	2.21
$MgCl_2$ (5 mM)	0
$Na_2S_2O_4$ (5 mM)	4.00
ATP generator (5 mM ATP)*	8.54

* Also containing $MgCl_2$ (5 mM), creatine phosphate (20 mM), creatine kinase (100 μg).

Results

General properties of nitrogenase

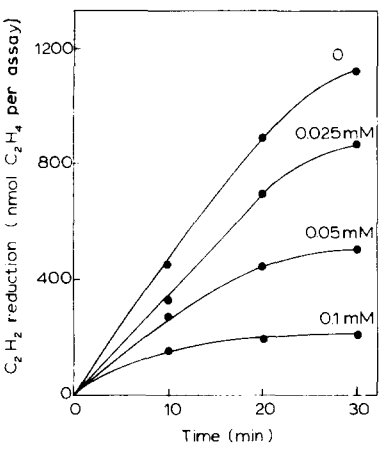
Nitrogenase isolated from soybean bacteroids (strain CC705) is similar to the enzyme reported from other strains capable of nodulating soybeans [6,7].

For example, specific activity of freshly prepared extracts ranged from 5 to 10 nmol/min per mg protein. Optimal concentrations of ATP and $Na_2S_2O_4$ are approx. 5 and 10 mM, respectively. Activity was immediately lost on exposure of extracts to O_2; ADP inhibited activity when ATP was used instead of the creatine kinase ATP-generating system.

However, we found the crude enzyme difficult to purify because of rapid inactivation of activity. Usually, activity was completely lost at room temperature within 3—5 h of preparation. Restoration of activity was possible to a certain degree (Table I). As shown, dithiothreitol was ineffective (it neither protects nor reactivates nitrogenase) however, together with $FeSO_4$, a degree of reactivation occurred. Incubation with dithionite or an ATP-generating system proved more effective. Since both ATP and ADP have been shown to interact with the smaller iron-sulphur component of nitrogenase [18], we conclude that both inactivation and restoration of activity was probably by interaction with this component.

Inhibition by nitrite

Time courses of nitrogenase activity of crude bacteroid extracts showed a strong inhibitory effect of KNO_2 (Fig. 1). The rates of acetylene reduction decreased rapidly with increasing KNO_2 concentrations. When the rates of reaction were plotted for the first 9 min, 50% inhibition was observed for concentrations of KNO_2 less than 0.1 mM (Fig. 2). Sensitivity to nitrite has also been shown using whole bacteroids of this strain [12] although a much higher concentration of KNO_2 (0.5 mM) was required to obtain 50% inhibition with bacteroids. This contrasts with nitrogenase from bacteroids of *Rhizobium lupini* which is inhibited by nitrite to the same degree (50% at 0.5 mM)



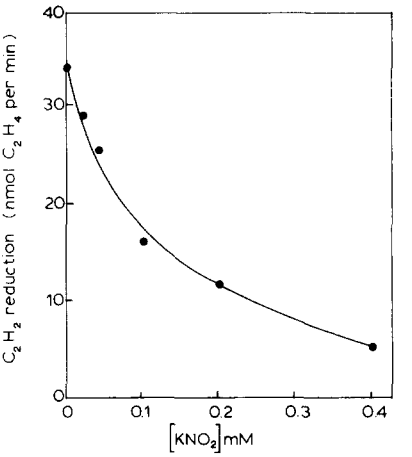


Fig. 1. Time courses of acetylene reduction by crude bacteroid extract in the presence of different concentrations of KNO_2. Assays were conducted as described in Methods with 2.7 mg of crude bacteroid protein.

Fig. 2. Inhibitory effects of nitrite upon acetylene reduction. Experimental conditions are identical as in Fig. 1, and rates are plotted for the first 9 min of reaction.

whether in whole bacteroids or as a partially purified enzyme (Kennedy, I.R., unpublished).

It was possible that nitrite inhibition was a result of it acting as an alternative substrate to acetylene. This was ruled out when assays catalyzing the formation of 250 nmol of ethylene in the controls did not cause significant nitrite disappearance in reactions of 2.0 ml containing 100 nmol KNO_2. The small disappearance of nitrite observed was also independent of the inclusion of ATP or $Na_2S_2O_4$. Because significant inhibition occurred at a nitrite concentration of less than 10^{-5} M, the possibility that some chemical impurity or decomposition product caused the inhibition is considered unlikely. For this to occur, such chemical species would need to act at extremely low concentrations, probably less than 10^{-7} M.

As shown in Fig. 1, inactivation of nitrogenase by nitrite was not apparent for KNO_2 concentrations lower than 0.1 mM. However, this is immediately evident for values of KNO_2 or $NaNO_2$ greater than 1 mM. Again, the nitrogenase of *R. lupini* is less sensitive in this respect, since inactivation does not occur, even at 2 mM KNO_2 (Kennedy, I.R., unpublished).

TABLE II

PURIFICATION OF SOLUBLE NITRATE REDUCTASE FROM BACTEROIDS

Extract	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Activity (nmol NO_2^-/min)	Specific activity (nmol NO_2^-/ min per mg)	Purification
Crude extract	38	5.9	224.2	2938.6	13.1	1
40—65% $(NH_4)_2SO_4$	2	39	78	3066.6	39.3	3
Sephadex G-150 eluate	16	0.27	4.32	746.6	172.8	13.2

General properties of nitrate reductase

As previously shown [3,5], free-living bacteria and bacteroids of *R. japonicum* contain both particulate and soluble activity. Specific activities of crude extracts from soluble enzyme were always higher for bacteroids than for bacteria (averaging 13 and 7.5 nmol of NO_2^-/min per mg, respectively). Nearly all the activity remained dissolved in the 40–65% $(NH_4)_2SO_4$ fraction (Table II). A final purification (10–15-fold) was obtained by Sephadex G-150 gel filtration. Considerable losses of activity occurred during purification, but the enzyme was stable once frozen. This nitrate reductase is apparently not strongly acidic, since it was not retained on beds of DEAE-cellulose. As indicated in

TABLE III

PROPERTIES OF SOLUBLE AND PARTICULATE NITRATE REDUCTASE

Assays were conducted anaerobically in 3.0 ml final volume as in Methods. The different fraction used in section 2 were prepared as described in Methods, by an initial centrifugation to remove any unbroken cells, followed by resuspension of sedimented fractions in the phosphate buffer mentioned and particles were immediately used. The concentration of $Na_2S_2O_4$ was 3.3 mM and viologen dyes 0.67 mM.

(1) Electron donors

	Specific activity (nmol NO_2^-/min per mg protein in bacteroids)	
	Purified soluble enzyme	Particles after 3 days at 3°C
$Na_2S_2O_4$ + methyl viologen (0.67 mM)	57.0	1.7
$Na_2S_2O_4$ + methyl viologen + $NaHCO_3$ (5 mM)	54.6	—
$Na_2S_2O_4$ + benzyl viologen (0.67 mM)	27.3	—
$Na_2S_2O_4$ + benzyl viologen + $NaHCO_3$ (5 mM)	23.1	—
Sodium succinate (16.7 mM)	0	6.7
Sodium malate (13.3 mM)	0	1.1
NADH (0.67 mM)	0	6.9
NADH (0.67 mM) + FAD (0.16 mM)	0	—
NADPH (0.17 mM)	0	—
NADPH (0.17 mM) + FAD (0.16 mM)	0	—
Nil	0	0

(2) Distribution of total activity

Substrate	Activity (nmol/min per g fresh cells) in each fraction				
	Bacteroids			Bacteria (free living)	
	35000 × *g* supernatant	7500 × *g* supernatant	3000 × *g* supernatant	35000 × *g* supernatant	Pellet
Nil	0	45	124	—	—
Sodium succinate	0	175	300	—	—
$Na_2S_2O_4$	0	4	5	0	18
$Na_2S_2O_4$ + methyl viologen	975.0	124	198	304	111

(3) Michaelis constant for nitrate

Soluble enzyme (bacteria)	$33 \cdot 10^{-5}$ M
Soluble enzyme (bacteroids)	$1.6 \cdot 10^{-5}$ M
Particulate enzyme (bacteroids)	$5.5 \cdot 10^{-5}$ M

Table III, the particulate form was able to utilize a wider range of electron donors, whilst the soluble enzyme functioned only with dithionite plus viologen dye, even in crude extracts. This confirms the earlier studies [3,5].

A significant proportion of the activity remained associated with broken cell membranes and smaller particles, even after prolonged sonication. Normally the particulate activity was well-supported by dithionite plus methyl viologen as electron donor but aged preparations (3–5 days at 3°C) were often inactive, despite persistently good activity with NADH as electron donor. We determined the Michaelis constant for nitrate with both particles and soluble enzyme (Table III). Differences are observable between the particulate preparations and the purified soluble enzyme, nevertheless the affinity for nitrate is invariably strong, indicating it to be a possible substrate in vivo. The ability of both NADH and succinate to act as electron donors, but only anaerobically in the complete absence of O_2, suggests that both utilize the naturally occurring respiratory electron carriers in this organism. These have been extensively described by Appleby [19].

The inability of the soluble enzyme to accept electrons from either NADH or NADPH, even in crude extracts supplemented with FAD suggests that it might also need electron carriers occurring in the particles. Normally, enzymes capable of accepting electrons from viologen dyes also accept electrons from iron-sulphur proteins such as reduced ferredoxin. Both *R. japonicum* bacteroids and cells grown anaerobically contain iron-sulphur electron-transferring proteins [20]. Presumably these could also act as electron donors to the soluble nitrate reductase.

Elution profile of soluble nitrate reductase activity on Sephadex G-150

Nitrate reductase activity of crude bacteroid extract elutes in a single symmetrical peak (Fig. 3b). Small traces of activity sometimes occur with the elution front. This was insignificant compared to the main peak shown. The elution profile for a preparation purified 4-fold by $(NH_4)_2SO_4$ precipitation (40–65% fraction) from bacteroids is shown in Fig. 3c. The elution pattern for crude enzyme from free-living bacteria is also shown (Fig. 3a). The enzyme activity had identical elution characteristics.

The molecular weight of the soluble enzyme from bacteria and bacteroids was estimated by gel filtration to be 70 000 and 66 000, respectively, using standard proteins indicated in Fig. 4 as markers. This difference is not significant for this technique.

Stability of nitrate reductase: inhibition by dithionite

Although both the particulate and soluble enzyme activities from both free-living bacteria and bacteroids are relatively stable; we have routinely employed anaerobic techniques similar to those employed in work with nitrogenase. Exposure to O_2 does not appreciably affect activity over periods of several hours, however, improved recoveries resulted when purification procedures requiring 24 h or more were conducted anaerobically. Frozen extracts were stable at −15°C for several weeks but steady losses of activity occurred at 0°C.

We have found that use of dithionite as electron donor is complicated by

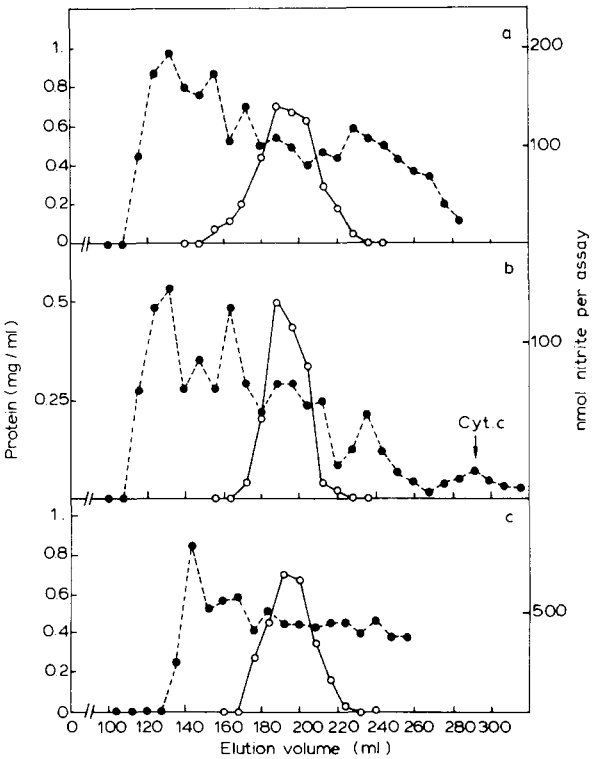


Fig. 3. Filtration of crude bacterial (a) and bacteroid (b) protein (35 000 × *g* supernatant) and fractionated (40—65% $(NH_4)_2SO_4$ saturation) bacteroid extract (c) on Sephadex G-150. Protein applied to the column was respectively, 111.6 mg (a) and 78 mg (b) after concentration in a Diaflo pressure membrane cell. For (c) 31.5 mg protein was employed. ●------●, protein estimated by the method of Folin; ○———○, nitrate reductase activity (in 0.5 ml eluate) measured as described in Methods. The position of bacteroid cytochrome *c* is indicated.

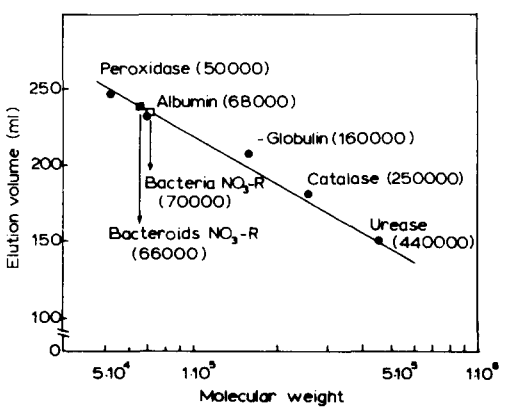
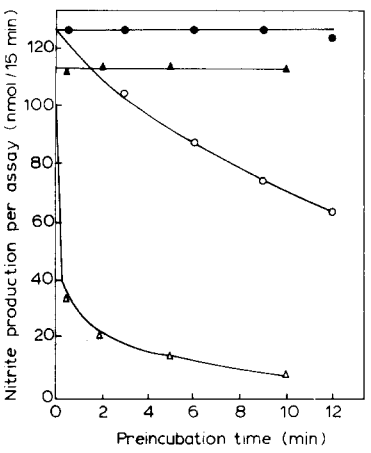


Fig. 4. Estimation of the molecular weights of nitrate reductase from free-living and bacteroid forms of *R. japonicum* (strain 705) on Sephadex G-150 by the method of Andrews [29]. The position of catalase (0.5 mg) and peroxidase (0.5 mg) was determined enzymically [30]. Other markers (5 mg of each) were located by the Lowry's technique [16].



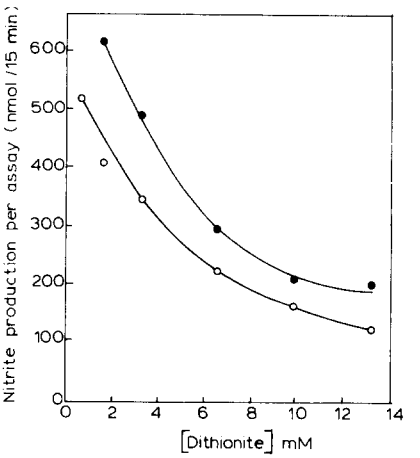


Fig. 5. Inactivation of nitrate reductase by $Na_2S_2O_4$. Purified enzyme was incubated in 0.1 M sodium phosphate buffer (pH 7.5) at 30° C for the times indicated, with added $Na_2S_2O_4$ (0.67 mM) plus methyl viologen (0.022 mM). Samples (0.5 ml) of the incubate were withdrawn and assayed (see Methods) in 3.0 ml; bacterial enzyme (0.27 mg/assay): ○——○, $Na_2S_2O_4$; ●——●, $Na_2S_2O_4$ plus methyl viologen; bacteroid enzyme (0.15 mg/assay); △——△, $Na_2S_2O_4$; ▲——▲, $Na_2S_2O_4$ plus methyl viologen.

Fig. 6. Effect of $Na_2S_2O_4$ concentration on purified nitrate reductase. Reactions were performed for 15 min as described in Methods: ○——○, bacterial enzyme (0.37 mg); ●——●, bacteroid enzyme (0.5 mg).

two aspects of its interaction with the enzyme extracts inactivation and inhibition. When purified preparations of soluble enzyme are incubated with $Na_2S_2O_4$, activity is rapidly lost (Fig. 5). This inactivation is particularly rapid with the purified enzyme from bacteroids, but the same effect occurs with enzyme from free-living rhizobia. It is not reversed after total oxidation of the $Na_2S_2O_4$ by agitation of the incubation mixture in a stream of O_2.

Fortunately, the inactivation is completely prevented by methyl viologen (see Fig. 5). Optimal concentration of dye was determined as shown in Fig. 7. To ensure complete protection against inactivation by dithionite, we routinely used 0.022 mM as final concentration for methyl viologen. Under this conditions the time course of enzyme activity is perfectly linear with time, showing an adequate protection of nitrate reductase. The rate of enzyme increased with decreasing $Na_2S_2O_4$ concentration (Fig. 6) to the point where it was not practicable to employ less reductant because of problems due to non-enzymic oxidation of $Na_2S_2O_4$ or limitation of electrons for nitrate reduction. Consequently, we have standardized the concentration of $Na_2S_2O_4$ at a non-optimal concentration but allowing good expression of activity. Clearly, it is necessary to limit the total enzyme activity used in assays, avoiding departures from linearity with time resulting from declining dithionite concentration.

The particulate activity is also sensitive to dithionite (see Table II, section 2); dithionite without added methyl viologen almost completely abolished activity compared to a control without added reductant relying on endogenous substrates. Here also, the addition of methyl viologen overcame this sensitivity.

No inactivation of purified nitrate reductase from bacteroids occurred on incubation with NADH (1.6 mM) for 10 min. This was not unexpected, in view

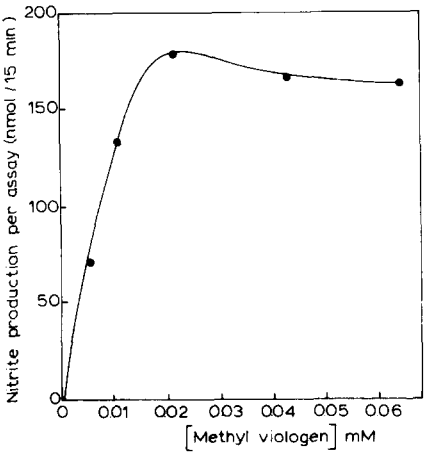


Fig. 7. Effect of methyl viologen concentration upon nitrate reduction by fractionated (40—65% $(NH_4)_2SO_4$ saturation) bacteroid extract. Assays were done as described in Methods with $Na_2S_2O_4$ (3.3 mM) and 2.06 mg of protein.

of the inability of NADH to provide electrons for nitrate reduction, however, such inactivation has been recorded with *Chlorella* [27].

Influence of nitrite

Neither preincubation of the purified nitrate reductase for 20 min in 0.5 mM KNO_2 nor carrying out assays with 0.5 mM KNO_2 present lowered activity. This contrasts with the strong inhibition of nitrogenase by nitrite.

Discussion

A possible role for nitrate reductase in leguminous nodules is not obvious from the results presented here. Indeed, its occurrence poses a specific problem for nitrogenase activity, because of the nitrite inhibition shown. Nitrite reductase is not present in bacteroids [11]. The enzyme in free-living organisms appears the same as that in bacteroids, and it is difficult to make claims for a functional role in soybean nodules, despite a higher specific enzyme activity. Nitrate respiration, shown earlier with isolated bacteroids [12], is nevertheless one such possible role [2], perhaps providing ATP under certain field conditions when O_2 is limited.

A possible similarity between a bacteroid constitutive subunit of nitrate reductase molecule and the molybdo-protein component of nitrogenase [21,22] is unlikely, because of the great difference observed in their respective weights. However, it remains possible that a low molecular weight molybdopeptide might be common to both enzymes. The small size of this nitrate reductase is in striking contrast to that of nitrate reductase occurring in higher plants (approx. 500 000 mol. wt) [24].

It appears highly probable that the particulate and soluble forms of nitrate reductase are the same enzyme, as suggested by Lowe and Evans [5]. The kinetic constants measured differ, but the common sensitivity to dithionite and the protective effect of methyl viologen shown here indicate this. Inability of

the soluble enzyme to utilize any of the common electron donors suggests that it would be associated with the cell membranes in vivo. This activity, always present in these cells whether nitrate is present or not, could thus be a respiratory form of reductase. Assimilatory forms of nitrate reductase such as those in plants [23,24] and bacteria [25] normally utilize either NADH or NADPH as electron donor. The close similarity between soluble nitrate reductases in free-living cells and bacteroids (molecular weight, electron donors, sensitivity to dithionite) are strongly indicative that these also are the same protein.

Inactivation of nitrate reductase by dithionite probably explains the difficulty experienced by Lowe and Evans [5] in purifying this enzyme without adding benzyl viologen. The conclusion that reduced benzyl viologen was necessary to maintain activity during purification was, paradoxically, a result of their assumption that the enzyme activity required protection from oxygen. Our results suggest that the role of viologen dye is to protect the activity against inactivation by dithionite, since the enzyme is virtually insensitive to O_2.

Inactivation of nitrate reductase by reducing conditions has been observed with a number of organisms including *Azotobacter* [26] and *Chlorella* [27]. In these organisms the inactivation was reversible but such a regulatory system would be more logical for nitrate assimilation under strongly oxidative conditions. Thus the inactivation of nitrate reductase observed here under reducing conditions would not be suited to a respiratory role for this enzyme. The nodule interior is normally regarded as strongly reducing [28] yet the activity of the enzyme carefully isolated anaerobically is greater than in aerobically grown cells. Whether this reductive inactivation could indicate an in vivo regulatory role in unclear.

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