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SOME PROPERTIES OF A NITRITE REDUCTASE FROM *PSEUDOMONAS DENITRIFICANS*

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SUMMARY

A nitrite reductase from extracts of actively denitrifying *Pseudomonas denitrificans* (A.T.C.C. 13867) was purified 160-fold. The enzyme utilized reduced benzyl or methyl viologen, leucomethylene blue, or reduced FMN as hydrogen donor. NADH and NADPH were active only in the presence of a flavin carrier (riboflavin, FMN, or FAD). The purified enzyme reduced nitrite stoichiometrically to nitric oxide, while suspensions of whole cells reduced nitrite to a mixture of nitric oxide and nitrous oxide. Inhibitor studies indicated the participation of a metal(s) and –SH groups. The inhibition of nitrite reduction by 2,4-dinitrophenol was due to a chemical reaction resulting in a competition for reducing power at the reduced flavin level and not to its uncoupling activity. The 2,4-dinitrophenol was reduced chemically by FMNH to 2-amino-4-nitrophenol.

INTRODUCTION

Nitrite reductase in denitrifying microorganisms was first reported by YAMAGATA[1] in cell-free extracts from *Bacillus pyocyaneus* (*Pseudomonas aeruginosa*). The product of the reaction was not determined. It was shown that nitric oxide occurs as a product of bacterial denitrification in whole cells and cell-free extracts from denitrifying organisms, *e.g. Thiobacillus denitrificans*[2], *Pseudomonas stutzeri*[3], a strain of *Bacillus subtilis*[4], and *Pseudomonas aeruginosa*[5].

Nitrite reductase from *P. stutzeri* required flavin, copper, and iron for its activity[3] as did the 600-fold purified enzyme from *P. aeruginosa*[5]. YAMANAKA and co-workers[6–8] prepared a crystalline nitrite reductase from *P. aeruginosa* which was found to be identical with its cytochrome oxidase.

Assimilatory-type nitrite reductase which reduces nitrite to ammonia has been found in extracts of *Bacillus pumilis*[9], *Azotobacter agile*[10], and in *Neurospora crassa*[11,12]. *Escherichia coli* strain Bn contains at least two systems which reduce nitrite to ammonia, one of them, however, acts as a sulphite reductase *in vivo*[13,14]. Nitrite reductase from both spinach leaves and *Anabaena cylindrica* requires ferredoxin as an electron carrier which can, however, be replaced by benzyl viologen or methyl viologen[15–17].

It is clear that there are at least two types of nitrite reductases in denitrifying

bacteria. In the dissimilatory system which is associated with cell particles, nitrite is a terminal acceptor and phosphorylation occurs during nitrite reduction. In the work reported here, however, another type of nitrite reductase from *Pseudomonas denitrificans* is described. This is a more soluble system requiring NADH and flavin for its activity and there is not an esterification of inorganic phosphate during enzyme action[24]. Some properties of this nitrite reductase are described in this paper.

MATERIALS AND METHODS

Organism

Pseudomonas denitrificans (A.T.C.C. 13867) was maintained on nutrient agar slopes containing 1 % glucose, 0.5 % yeast extract and 6 μg/l $FeCl_3$. The latter, sterilized separately, was added aseptically to each tube of medium. Fresh subcultures, grown for 18 h at 37°, were used to inoculate the culture solution. Cells were grown in a medium containing the following macronutrients (g/l): 10 KNO_3, 10 glucose, 1 peptone, 1.5 K_2HPO_4, 0.2 $MgSO_4 \cdot 7\ H_2O$, 0.2 NaCl; and micronutrients (mg/l) 2 Fe as $FeSO_4$, 1 Mo as Na_2MoO_4, 0.025 Mn as $MnCl_2$, 0.25 Cu as $CuSO_4$ and 0.25 Zn as $ZnSO_4$. The medium was adjusted to pH 7.0. The glucose was sterilized separately and added to the rest of medium aseptically. The organism was grown for 18 h at 37° in 10-l flasks sparged with nitrogen gas to ensure anaerobiosis.

Harvesting and preparation of extracts

The cells, collected at 0° in a Sorvall RC-2 refrigerated centrifuge fitted with a continuous-flow head, were washed with cold 0.85 % (w/v) saline until all nitrite was removed, before storage at −17°. The cells were suspended in 2 vol. 0.05 M Tris–HCl buffer (pH 7.4) and crushed at 2° in a French pressure cell (7000 lb·inch^{-2}). Whole cells and cell debris were removed by centrifuging at 20000 × *g* for 30 min.

Preparation of Azotobacter particles

Azotobacter particles (P6) with an active NADH–benzyl viologen reductase were prepared from *Azotobacter vinelandii* (O) grown in Burk's nitrogen-free medium, as described by NAIK AND NICHOLAS[18]. These particles were devoid of nitrite-reducing activity.

Assay of nitrite reductase

Nitrite reductase was assayed anaerobically by following the reduction of nitrite in the following reaction mixtures.

(1) NADH–FMN system: 1 μmole $NaNO_2$, 0.1 μmole FMN, 0.05–0.1 ml enzyme, 1 μmole NADH, 0.1 M phosphate buffer (pH 6.0) to a final volume of 1 ml. The reaction was started by tipping in the nitrite from the side-arm of the Thunberg tube after rigorous evacuation of the contents and temperature equilibration at 30° for 5 min. The reaction was stopped by opening the tubes to air and adding 0.1 ml 1 M zinc acetate and 1.9 ml redistilled 95 % (v/v) ethanol to precipitate any residual NADH that might interfere with the chemical determination of nitrite[19]. Nitrite was determined in 0.1-ml aliquots of the supernatant solution, left after centrifuging at 3000 × *g* for 5 min, by adding 0.5 ml 1 % (w/v) sulphanilamide in 1 M HCl and 0.5 ml 0.02 % (w/v) *N*-(1-naphthyl)ethylenediamine dihydrochloride.

(2) Reduced benzyl viologen system: 2 μmoles NADH, 0.01–0.05 ml enzyme, 1 μmole $NaNO_2$, 0.05 ml Azotobacter (P6) particles, 1 μmole oxidized benzyl viologen, 0.1 M phosphate buffer (pH 7.0) to a final volume of 1 ml. NADH was added last, immediately before evacuating the Thunberg tube, to minimize loss due to NADH-oxidase activity in the Azotobacter particles. The incubation procedure and the determination of nitrite were done as described in (1).

(3) Warburg manometer experiments: The reaction was carried out in Warburg flasks fitted with double side-arms with the following reactants: *Main compartment*: 2.4 ml of 0.1 M phosphate buffer (pH 6.0) containing 5 μmoles NADH, 0.2 μmole $NaNO_2$, 0.2 μmole FMN, and 0.2 ml enzyme; *side-arm 1*: 5 μmoles $NaNO_2$; *side-arm 2*: 0.2 ml alkaline sulphite (5 % (w/v) Na_2SO_3, 2 % (w/v) KOH) or 0.2 ml alkaline permanganate (0.125 M $KMnO_4$ in 1 M KOH) or 0.2 ml water; *centre well:* 0.2 ml 20 % (w/v) KOH. The flasks were flushed for 40 min with oxygen-free nitrogen gas. The reaction, started by adding $NaNO_2$ from the side-arm 1 was continued for 2 h or until gas production had ceased.

Reaction of 2,4-dinitrophenol with reduced FMN

The reaction of FMNH and 2,4-dinitrophenol was carried out anaerobically by injecting 80 μmoles of FMN reduced by hydrogen gas with palladised asbestos through a serum cap into a deoxygenated solution containing 40 μmoles of 2,4-dinitrophenol. The mixture was allowed to react for 16 h at 37°. The products of reactions were separated by thin-layer chromatography on silica gel plates using a 10 % ethanol–90 % benzene (v/v) mixture.

Assay of NAD^+

The fluorimetric method of LOWRY, ROBERTS AND KAPPHAHN[21] was used to determine NAD^+ formed during nitrite reduction.

Determination of nitric oxide

Nitric oxide absorbed by alkaline permanganate solution was assayed by the method of ANDERSON[22].

Mass spectrometry

The incubation was conducted in Rittenberg tubes with the standard reaction mixture except that 2 μmoles of $NaNO_2$ and 2 μmoles of NADH were used. The tubes were rigorously evacuated and then the contents of the two arms mixed and incubated at 30° for 2 h. The gaseous products of the reaction were transferred under high vacuum into an A.E.I. MS-2 mass spectrometer for analysis.

Co-factors and other reagents

NADH, NADPH, FMN, FAD, riboflavin and bovine serum albumin were obtained from Sigma Chemical Corporation, St. Louis (U.S.A.); ^{15}N-labelled nitrite (31.3 atom%) was purchased from the Office National Industriel de l'Azote, France. Benzyl viologen was supplied by British Drug Houses, Poole, England; 2-amino-4-nitrophenol was a gift from Fluka AG, Buchs, Switzerland; 2,4-dinitrophenol piperidine salt and 2,6-dinitrophenol piperidine salt were gifts from Dr. D. WOODCOCK,

Long Ashton Research Station, University of Bristol, England. All inorganic reagents were of analytical grade.

Protein was determined by the Folin method as modified by LOWRY *et al.*[20] using bovine serum albumin as a standard. Protein fractions containing ammonium sulphate were dialysed against a large volume of distilled water to remove ammonium ions before analysis.

RESULTS

In contrast with other denitrifying bacteria, *e.g. Micrococcus denitrificans*, nitrite reductase activity in *P. denitrificans* is not associated with large particles but was found in the supernatant fluid left after centrifuging the crude extract at 144000 × *g* for 2 h as shown in Table I. If the 144000 × *g* supernatant fraction was further centrifuged at 225000 × *g* for 6 h, about 50 % of the activity was then found in the pellet thus indicating that some of the nitrite reductase is associated with very small particles.

TABLE I

DISTRIBUTION OF NITRITE REDUCTASE IN CELL HOMOGENATES

The enzyme activity was assayed anaerobically at 30° in Thunberg tubes. The reaction mixtures were as follows: (i) NADH system: 1 μmole NADH, 0.1 μmole FMN, 1 μmole $NaNO_2$, 0.1 ml enzyme preparation 0.1 M phosphate buffer (pH 6.0) to 1 ml; (ii) reduced benzyl viologen system: 2 μmoles NADH, 1 μmole benzyl viologen, 1 μmole $NaNO_2$, 0.05 ml Azotobacter (P6) particles, 0.05 ml enzyme preparation, 0.1 M phosphate buffer (pH 7.0) to 1 ml.

Fraction	*Total enzyme units (μmoles NO_2^- reduced per 5 min)*	
Hydrogen donor:	*NADH*	*Reduced benzyl viologen*
1. Crude extract left after centrifuging homogenate at 20000 × *g* for 30 min	428	2000
2. 144000 × *g* supernatant fraction left after centrifuging Fraction 1 for 2 h	418 (97 %)	1500 (75 %)
3. 144000 × *g* pellet from Fraction 2	44 (10 %)	300 (15 %)

Purification of the enzyme

The specific activity of the nitrite reductase in the cell-free extract (Fraction I) was increased 160-fold by a fractionation procedure involving differential centrifugation and ammonium sulphate fractionation at pH 6.0 as shown in Table II.

The enzyme is stable at −17° for up to 2 months.

pH optima

Optimal enzyme activity was at pH 6.0 with the NADH–FMN donating system but none was recorded at either pH 4.0 or 9.0. With reduced benzyl viologen as hydrogen donor, the maximal activity was between pH 6.0 and 7.0 with 65 % of the optimum value at pH 5.0 and none at pH 9.0.

Electron donors and co-factors

The use of various electron donors with a range of oxidation–reduction potentials was investigated. Appropriate controls were included to check for the occur-

TABLE II

PURIFICATION OF NITRITE REDUCTASE

Washed cells suspended in 2 vol. 0.05 M Tris–HCl buffer (pH 7.0) were disrupted in a French pressure cell. The enzyme was assayed using reduced benzyl viologen as an electron donor and protein determined by the Folin–Ciocalteau method.

Fraction	*Vol. (ml)*	*Enzyme activity (μmoles NO_2^- reduced per 5 min)*	*Recovery enzyme (%)*	*Protein (mg)*	*Specific activity (μmoles NO_2^- reduced per 5 min per mg protein)*	*Purification*
1. Homogenate	185	—	—	—	—	—
2. Supernatant fraction after centrifuging homogenate at 20000 × *g* for 30 min	150	1500	100	4650	0.32	1
3. Supernatant fraction after centrifuging Fraction 2 at 144000 × *g* for 2 h	110	5400	360	1210	2.46	7.7
4. Fraction 3 diluted with an equal volume of 0.1 M phosphate buffer (pH 6.0) precipitated between 75–80% satn. with $(NH_4)_2SO_4$, dissolved in 0.1 M phosphate buffer (pH 6.0) and dialyzed for 12 h against the same buffer	35	2870	190	56	51.2	160

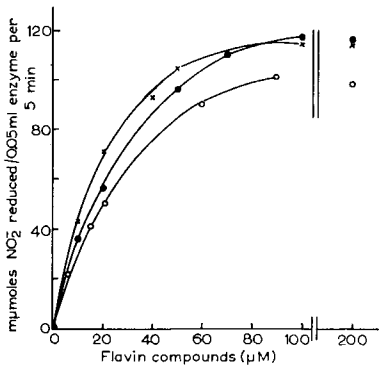


Fig. 1. Effects of graded amounts of various flavin compounds on nitrite reductase. Enzyme activity assayed with NADH as donor, as described in METHODS. ×—×, FAD; ●—●, FMN; ○--○, riboflavin.

rence of a chemical reduction of nitrite. Table III shows that reduced FMN or reduced benzyl viologen are the best hydrogen donors. NADH and NADPH had an absolute requirement for a flavin co-factor for enzyme activity which was met by FAD, FMN, or riboflavin. The effect of added flavins on the rate of nitrite reduction is shown in Fig. 1. The K_m values for the three co-factors calculated according to the method of LINEWEAVER AND BURK[23] were: FAD, $1.1 \cdot 10^{-5}$ M; FMN, $1.7 \cdot 10^{-5}$ M; riboflavin, $2.0 \cdot 10^{-5}$ M.

TABLE III

THE REDUCTION OF NITRITE IN EXTRACTS USING VARIOUS ELECTRON DONORS

The enzyme was assayed anaerobically in Thunberg tubes as described in METHODS.

Electron donor	*Final conc. (M)*	*Relative activity for reduction of nitrite (NADH + FMN = 100)*	*Redox potential (V) at pH 7*
NADH	10^{-3}	0	−0.320
NADH + FMN (10^{-4} M)	10^{-3}	100	
NADPH	10^{-3}	0	−0.320
NADPH + FMN (10^{-4} M)	10^{-3}	100	
Reduced methylene blue	$2 \cdot 10^{-4}$	133	+0.011
Reduced benzyl viologen	$3 \cdot 10^{-3}$	2500	−0.359
$FMNH_2$	10^{-3}	2500	−0.185
Reduced methyl viologen	10^{-3}	Non-enzymic reduction	−0.446
Reduced phenazine methosulphate	10^{-3}	Non-enzymic reduction	−0.080
Hydroxylamine–HCl (pH 7.0)	10^{-3}	0	+0.450

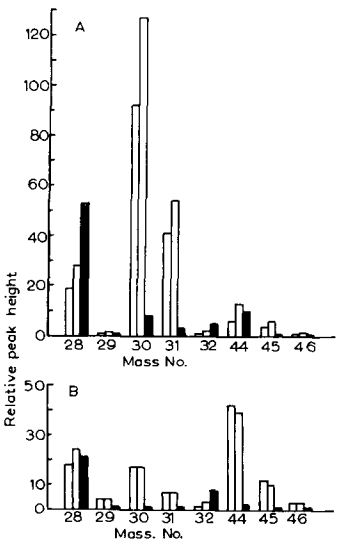


Fig. 2. Reaction products of the reduction of ^{15}N-labelled nitrite by purified enzyme on whole cells. A. Reaction mixture for purified enzyme as in METHODS, but with 2 μmoles ^{15}N-labelled nitrite. B. As for A but 0.1 ml suspension of whole cells used instead of the purified enzyme.

Stoichiometry and product of the reaction

The purified enzyme reduced nitrite ($K_m = 1.5 \cdot 10^{-4}$ M) to a gas which had a mass of 30 in the mass spectrometer. The gaseous product was further identified as nitric oxide by its complete absorption into alkaline sulphite or alkaline permanganate solution during Warburg manometry experiments. When the alkaline permanganate was analysed for absorbed NO, the correct stoichiometry for nitrite reduction to NO was observed. For every mole of nitrite reduced 1 mole of NADH was oxidized.

In contrast with the purified enzyme, whole cells or crude extracts of the bacterium reduced nitrite to a mixture of nitrous oxide and nitric oxide. The products of this reaction were identified, in experiments with ^{15}N-labelled nitrite, in the A.E.I. MS-2 mass spectrometer as shown in Fig. 2.

Inhibitor studies

The effects of a range of inhibitors on the purified nitrite reductase (Fraction 4, Table II) when NADH–FMN or reduced benzyl viologen was the hydrogen donor are shown in Table IV. Sodium diethyldithiocarbamate (5 mM) inhibited nitrite reduction 70 % when FMNH was the hydrogen donor. Iodoacetamide and amytal each at 1 mM and NaF; quinine, nitrate, chlorate, thiosulphate, sulphate and sulphite each at 5 mM had no effect on nitrite reduction.

These data suggest the participation of a metal and sulphydryl groups in the enzyme reaction.

TABLE IV

INFLUENCE OF INHIBITORS ON THE PURIFIED NITRITE REDUCTASE

The effect of inhibitors on the enzyme was assayed anaerobically in Thunberg tubes as described in METHODS. The inhibitor was incubated with the enzyme for 15 min before starting the reaction.

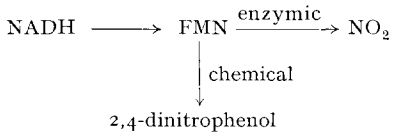
Inhibitor	*Final concn. (mM)*	*% Inhibition*	
	Hydrogen donors:	*NADH–FMN*	*Reduced benzyl viologen*
Sodium diethyldithiocarbamate	5	100	83
KCN	5	60	93
	10	86	93
2,2′-Dipyridyl	5	27	47
o-Phenanthroline	5	40	35
8-Hydroxyquinoline	5	21	52
p-Chloromercuribenzoate	1	82	0
p-Chloromercuribenzoate + glutathione	1 1	27	—
Mepacrine	1	12	0
Hydroxylamine–HCl (pH 7.0)	5	20	

Effect of aromatic nitro compounds

The results shown in Fig. 3 demonstrate that 2,4-dinitrophenol inhibits nitrite reduction competitively whereas the inhibition by 2,6-dinitrophenol is non-competitive.

The first effect was found to be associated with a chemical reduction of 2,4-dinitrophenol to 2-amino-4-nitrophenol. The inhibition thus appears to be the result

of a competition for reducing power by nitrite reductase and by the chemical reduction of 2,4-dinitrophenol:



It was shown that FMNH, produced chemically by using either NADH as hydrogen donor or hydrogen gas and palladised asbestos catalyst, reduced 2,4-dinitrophenol to 2-amino-4-nitrophenol. The reaction product was isolated by thin-layer chromatography on silica gel plates. The product was found to have an R_F value identical with that of authentic 2-amino-4-nitrophenol (R_F = 0.66). Fig. 4 shows that the spectra of the 2-amino-4-nitrophenol and that of the isolated reaction product are also identical.

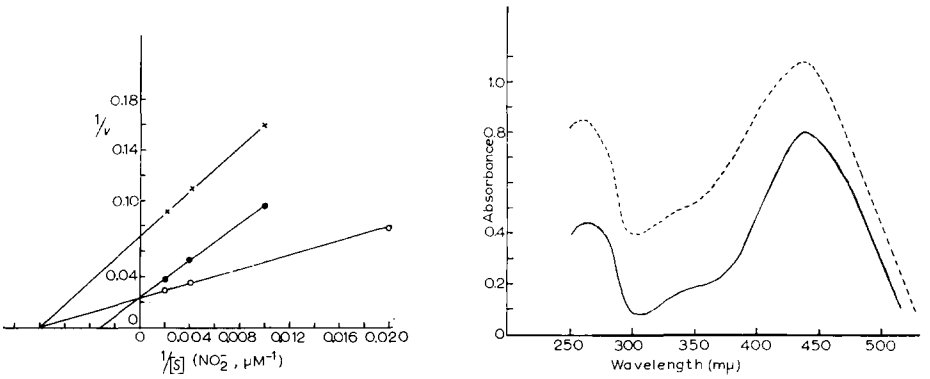
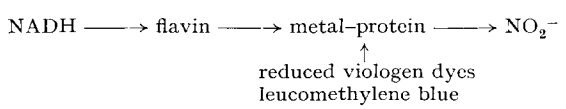


Fig. 3. Double reciprocal plot of the rate of nitrite reduction by purified enzyme with and without nitrophenol inhibitors. Reaction mixture as described in METHODS with addition of the piperidine salts of either 2,4-dinitrophenol or 2,6-dinitrophenol (10^{-3} M). Inhibitors incubated with the enzyme for 15 min before starting the reaction. ○—○, no inhibitor; ●—●, 10^{-3} M 2,4-dinitrophenol piperidine salt; ×—×, 2,6-dinitrophenol piperidine salt.

Fig. 4. Spectra of authentic 2-amino-4-nitrophenol and of the reduction product from the reaction of 2,4-dinitrophenol and FMNH. ———, authentic 2-amino-4-nitrophenol; -----, reduction product eluted from the thin-layer chromatogram. Compounds dissolved in 0.1 ml phosphate buffer (pH 7.5).

DISCUSSION

In the present work a partially purified nitrite reductase prepared from *P. denitrificans* which reduces nitrite to nitric oxide has been characterized. A suggested scheme for electron transfer is as follows:



The significance of inhibition of the nitrite reductase system by 2,4-dinitrophenol does not lie in the well-known uncoupling activity of this compound since it

has been shown that this enzyme is not coupled to oxidative phosphorylation as are nitrite reductases from some other denitrifying bacteria, *e.g. M. denitrificans*[24]. Instead, this inhibition is due to the effect of a competition for reducing power at the reduced flavin level whereby 2,4-dinitrophenol is reduced chemically to 2-amino-4-nitrophenol. Similar reactions have been demonstrated in the reduction of 2,4-dinitrophenol by benzyl or methyl viologen which were reduced by Azotobacter particles[25], by hydrogenase from *Clostridium pasteurianum* with FMN, benzyl viologen or ferredoxin as carriers[26], by formic acid dehydrogenase with benzyl viologen as carrier, and by xanthine oxidase[27].

YAGI, OZAWA AND OKADA[28] have shown by fluorescence quenching studies that phenol derivatives, including 2,4-dinitrophenol, form complexes with FAD. This was also confirmed in these studies since preliminary results indicate that this also occurs with FMN.

The enzyme is found, not in association with large particles, but in the supernatant fraction left after centrifuging at 144000 $\times$ *g* for 2 h. Only 50% of the nitrite reductase activity is located in the pellet formed by centrifuging at 225000 $\times$ *g* for 6 h thus indicating that some of the enzyme is associated with very small particles. This is probably not the result of mechanical disruption of larger complexes since the method for breaking the cells in a French press is relatively gentle.

SUZUKI AND IWASAKI[29] and IWASAKI *et al.*[30] have isolated a nitrite reductase from *P. denitrificans* which reduced nitrite to nitrous oxide using hydroxylamine as an electron donor. The enzyme described in this paper is different since it does not utilize hydroxylamine and indeed, this compound was slightly inhibitory when NADH was used as a hydrogen donor.

Inhibitor studies indicate the participation of sulphydryl groups and possibly one or more metals. The metals involved might be copper and iron, by analogy with other nitrite reductases[4,6–8,13,19] and from the results of inhibitor studies described therein. Further work is required to identify the metals involved.

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