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NITRITE REDUCTION IN *PARACOCCUS HALODENITRIFICANS*: EVIDENCE FOR THE ROLE OF A cd-TYPE CYTOCHROME IN AMMONIA FORMATION

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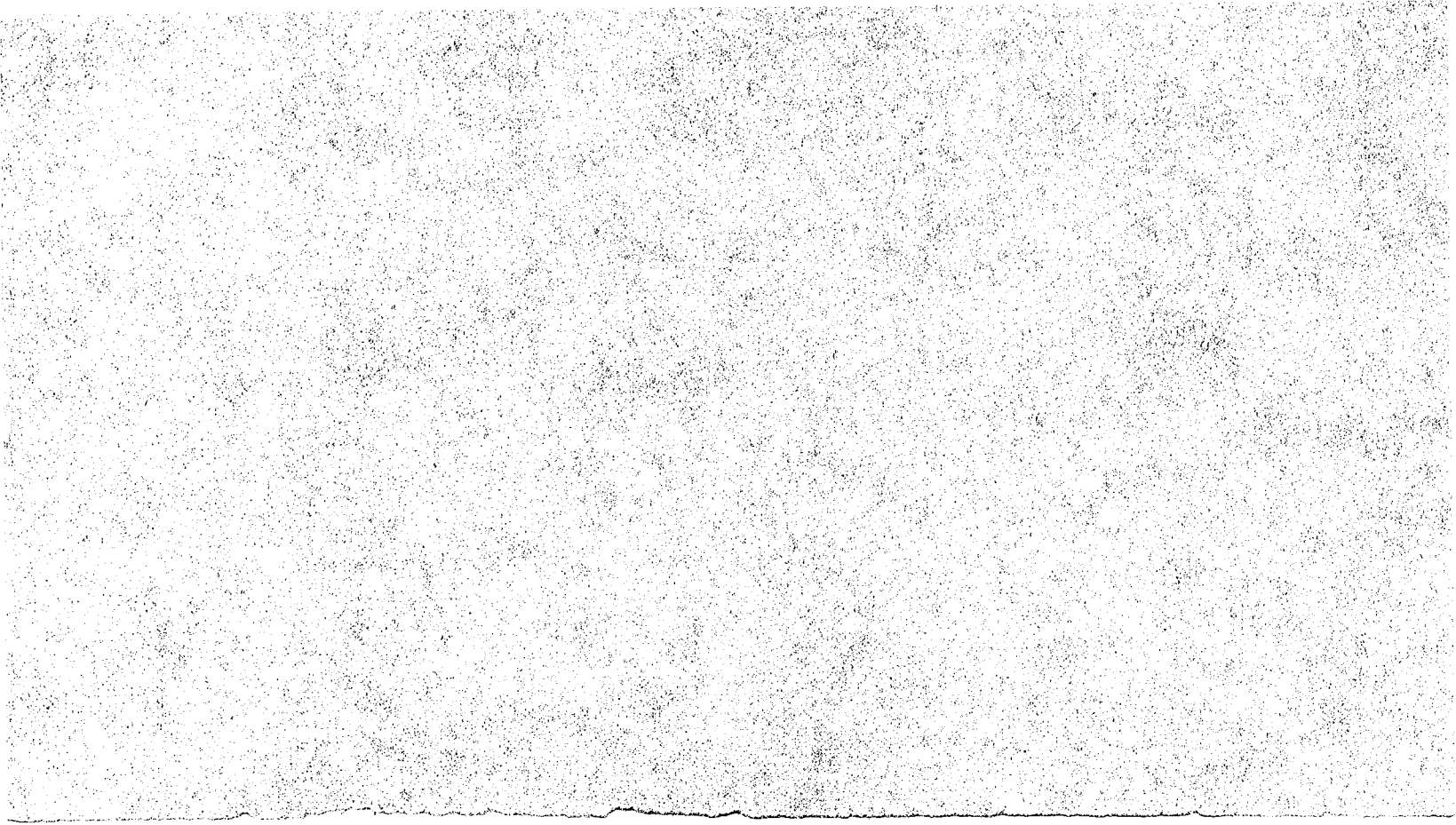
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Summary

Cell-free extracts prepared from *Paracoccus halodenitrificans* catalyzed the reduction of nitrite to ammonia in the presence of dithionite and methyl viologen. Enzyme activity was located in the soluble fraction and was associated with a cytochrome whose spectral properties resembled those of a cd-type cytochrome. Unlike the dissimilatory cd-cytochrome nitrite reductase associated with the membrane fraction of *P. halodenitrificans*, this soluble cd-cytochrome did not reduce nitrite to nitrous oxide.

Introduction

Two types of dissimilatory nitrite reductases have been described. One, a cd-type cytochrome, is present in several bacterial genera [1-6]. The other nitrite reductase is a copper-containing enzyme first found in *Pseudomonas denitrificans* [7] and later in *Achromobacter cycloclastes* [8]. This enzyme contains two copper atoms per mole of enzyme. Sawada and Satoh [9] described another copper-containing enzyme

from *Rhodopseudomonas sphaeroides* which had two subunits, each with a copper atom. Both the copper- and cytochrome-containing enzymes reduce nitrite to nitric and nitrous oxides [8,10,11], although there is some question if the nitrous oxide is produced as a result of a nonenzymatic reduction of nitric oxide [12].

A variety of assimilatory nitrite reductases have also been described including: an FAD-dependent metalloprotein [13]; a reductase which has two molecules of cytochrome-c [14] and another which has six cytochrome-c molecules [15] per mole of enzyme; an NADH-dependent nitrite reductase, which contains FAD, iron, acid-labile sulfur, and siroheme [16]; and ferrodoxin-dependent nitrite reductases [17,18]. All of these enzymes reduce nitrite to ammonia via a 6-electron step reduction.

Paracoccus halodenitrificans grows anaerobically in the presence of nitrate or nitrite and produces nitrous oxide and dinitrogen [19]. Suitably supplemented cell-free extracts prepared from cells grown anaerobically in the presence of nitrite catalyze the disappearance of nitrite. When such extracts are centrifuged so as to sediment the membrane fraction, the sedimented material (Grant and Hochstein, Arch. Microbiol., in press) and the supernatant fraction bring about the disappearance of nitrite. We wish to report on the partial purification of the enzyme found in the supernatant fraction and to describe some of its properties. In addition, we will present evidence that the product of nitrite reduction is ammonia, but that this reaction is brought about by an enzyme whose spectral characteristics are those of a cd-type cytochrome.

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Materials and Methods

Preparation of the soluble enzyme

P. *halodenitrificans* (ATCC 13511) was grown anaerobically in a complex medium [20] which contained 0.25% potassium nitrite and 0.5% sodium lactate instead of glycerol. Nitrite was sterilized by filtration, using a Nalgene 0.2-micron Sterilization Filter Unit, and added to the autoclaved medium after it had cooled to room temperature. The medium was first flushed with nitrogen and then inoculated with an anaerobically grown, nitrite-adapted culture; finally, nitrogen was continuously passed through the medium. The cells were grown at 30°C and harvested shortly after the culture entered the maximum stationary phase, at which time no nitrite was detected in the growth medium. The cells were suspended in 50 mM HEPES*-150 mM NaCl-10 mM $MgCl_2$-100 μM dithiothreitol, pH 7.4 buffer (HD buffer). The cell-to-buffer ratio was adjusted to 0.25 (wet wt/v) and the cells were passed through a cold (4°C) French Pressure Cell operated at 12 000 psi (84 MPa). Any intact cells were removed by centrifuging the suspension at 3000 × g for 30 min at 4°C. The resulting extract was centrifuged at 149 000 × g for 1 hr at 4°C. The dark-green supernatant fraction was decanted, designated as the S_2 fraction, and used as the source of the soluble nitrite reductase.

Assays

Nitrite reductase activity was determined by measuring the disappearance of nitrite from reaction mixtures which contained the following additions in a total volume of 1 ml: 200 μmol sodium borate (pH 8.8),**

5 µmol sodium nitrite, 4 µmol methyl viologen, and 23 µmol of freshly prepared sodium dithionite (dissolved in pH 8.8, 400 mM sodium borate buffer). After preincubating this mixture for 2 min at 30°C, the reaction was initiated by adding the enzyme. The reaction was terminated by diluting a 20-µl aliquot of the reaction mixture with 2.6 ml 0.006% formaldehyde and mixing vigorously. Nitrite was determined by a diazotization reaction [21]. For ammonia determinations, 1 ml 10% trichloroacetic acid was added to the reaction mixture and the acidified mixture was centrifuged at 12 000 × g for 10 min at 4°C. Ammonia was measured by diluting a suitable aliquot of the supernatant to 3.8 ml with 0.008 N NaOH and using the Indophenol procedure described by Verdouw et al. [22]. The rate of ammonia formation was linear for at least 15 min and proportional to the amount of enzyme added when no more than 2 µmol of ammonia were produced. A unit of activity is that amount of enzyme that forms 1 µmol ammonia min^{-1}. Specific activity is units mg protein^{-1}.

Proteins were measured by the method of Bradford [23], using Sigma bovine serum albumin (Fraction V) as the standard.

Pyridine hemochromogens were prepared as described by Jacobs and Wolin [24]. All spectra were determined at room temperature, using an Aminco DW-2A Spectrophotometer operated at a slit width of 1 nm and at a scan rate of 2 nm sec^{-1}.

Gel electrophoresis

Electrophoresis was performed according to the procedure of Davis [25]. Samples were diluted with 40% glycerol, applied to

7.5% gels, and electrophoresis was carried out at a current setting of 2.5 mA per gel using tris-glycine buffer (pH 8.9). The following procedure was used to obtain sufficient material by gel electrophoresis. Fifty μl of the G-100 fraction (120 μg protein) was applied to each of 11 gels and electrophoresis was carried out for 2 hr. Afterward, the visible yellowish-green bands were eluted by triturating each gel with 50 mM HEPES-1.6 M NaCl buffer (pH 7.4) and filtering the resulting suspension through a Millipore fritted glass base. The eluate contained 552 μg protein in a total volume of 1.2 ml.

Nitrite reductase activity was detected using a modification of the method of Greenbaum et al. [26]. The gels were immersed in a solution similar to the one used for the enzyme assay, except that the dithionite concentration was 6 mM, and incubated for 15 min at room temperature. The gels were rinsed with distilled water and immersed in a 1.25% solution of neotetrazolium red. Nitrite reductase activity appeared as a clear zone in contrast to the red-colored gel.

Results

Purification of nitrite reductase

Unless stated otherwise, all operations were carried out at 4°C. The S_2 fraction was brought to room temperature (ca. 22°C) and adjusted to a protein concentration of 7 mg ml^{-1} with HD buffer. The S_2 fraction was made 60% saturated with respect to ammonium sulfate by slowly adding 39 g of the salt/100 ml solution. Fifteen minutes after the ammonium sulfate had been added, the suspension was centrifuged for 15 min at 10 400 × g. The dark-green supernatant was applied to a

column containing Octyl-Sepharose (2 cm × 6.4 cm) equilibrated with 50 mM HEPES-150 mM NaCl buffer, pH 7.4 (HN buffer) that was 60% saturated with respect to ammonium sulfate. The column was washed with 200 ml of the 60% ammonium sulfate-HN buffer, and nitrite reductase activity was eluted when the HN buffer was made 40% saturated with respect to ammonium sulfate. Those fractions containing nitrite reductase activity were combined, concentrated by ultrafiltration using an Amicon PM-10 membrane, and ammonium sulfate removed by passing the concentrated Octyl-Sepharose fraction through a Pharmacia PD-10 column equilibrated with HN buffer. This "low salt" fraction was applied to a 1.5 cm × 25 cm DEAE-BioGel A column equilibrated with HN buffer. The gel was washed with 250 ml HN buffer followed by a 300-ml linear gradient, constructed using HN buffer in the mixing chamber and 50 mM HEPES-350 mM NaCl (pH 7.4) buffer in the reservoir. Nitrite reductase activity was eluted between 260 mM and 280 mM NaCl. Those fractions containing nitrite reductase were combined and concentrated by ultrafiltration. The concentrated DEAE fraction was applied to a Sephadex G-100 column (1.5 cm × 85 cm) equilibrated with HN buffer. The enzyme was eluted using the same buffer and appeared at a Ve/Vo of 1.25. The specific activity of the G-100 fraction was 22 μmol ammonia min^{-1} mg protein^{-1}, which represented a 35-fold purification with a 75% recovery of the units present in the S_2 fraction (Table 1).

Polyacrylamide gel electrophoresis

Following electrophoresis, several components were detected when the gels were stained with coomassie brilliant blue (Fig. 1a). Band 1 consisted of a diffuse staining region. Band 2 corresponded to a greenish-yellow material that could be seen on the gel before staining and exhibited nitrite reductase activity (Fig. 1b). Bands 1 and 2 contained benzidine-reactive hemes (data not shown) when stained by the method of McDonnel and Staehelin [27], indicating the presence of c-type cytochromes. When the greenish-yellow material was eluted from the gel and examined electrophoretically once again, the patterns observed in Fig. 1c were obtained. Only the band corresponding to the original Band 2 reacted with the nitrite reductase activity stain, although both reacted with benzidine (data not shown). These results suggested that Band 1 arose from the nitrite reductase, either during sample preparation or electrophoresis.

Spectral properties

The absorption spectrum of the material from Band 2 was examined from 400 to 700 nm. The oxidized spectrum had a maximum at 410 nm, poorly defined features between 500 and 600 nm, and another absorption maximum at 636 nm (Fig. 2a). After reduction with dithionite, the absorption maximum at 636 nm was replaced with one at 655 nm. Two distinct maxima, located at 554 and 549 nm, were also present. In addition, an absorption band was observed at 460 nm, and the Soret band at 410 nm shifted to 419 nm (Fig. 2a). Isobestic points associated with the alpha bands (dithionite-reduced minus "oxidized")

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were located at the following wavelengths: 646, 627, 559, and 545 nm.

The dithionite-reduced pyridine hemochromogen of the HCl-acetone soluble heme had a maximum at 616 nm, whereas that of the HCl-acetone insoluble residue exhibited a maximum at 551 nm (Fig. 2c). The location of these maxima and the apparent covalent linkage of the one heme is typical of d- and c-type cytochromes [28].

The material associated with Band 1 was eluted from the gel and also examined spectrally. No features were found between 600 and 700 nm in the spectrum of the oxidized or the dithionite-reduced material. The dithionite-reduced material exhibited maxima at 549 and 554 nm (Fig. 2b), suggesting that the material in Band 1 arose from the nitrite reductase by loss of the d-heme. This is consistent with observations that the d-heme of cd-cytochrome nitrite reductase is labile [29]. The material associated with Band 3 exhibited no spectral features either in the oxidized or reduced form when scanned from 400 to 700 nm.

Requirements for ammonia formation

The disappearance of nitrite was accompanied by the production of an equivalent amount of ammonia (Table 2). Although some nitrite disappeared in the absence of dithionite or methyl viologen, very little, if any, ammonia was produced. Small amounts of ammonia were also detected when the enzyme was omitted, or when a heat-denatured enzyme was used. When nitrite was replaced with ammonium chloride, virtually all of the added ammonium was recovered (Table 2). In other experiments, no ammonia was produced from nitrite when the

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cd-cytochrome was replaced with ferrous sulfate, hemoglobin, or horse
heart cytochrome-c.

Properties of the enzyme

Ammonia production (as well as the amount of nitrite lost)
decreased gradually upon storage at 4°C in 50 mM HEPES buffer (pH 7.4)
containing either 1 M or 150 mM NaCl. Storage at room temperature
(ca. 22°C) increased inactivation, whereas only 15% of the initial
activity was lost after 3 months of storage at -19°C. Maximum activity
was observed when the enzyme was assayed in the absence of added sodium
chloride (Na^+ = 62 mM). Higher concentrations of sodium chloride
inhibited ammonia production (Fig. 3b) (and the amount of nitrite lost),
so that the rate of ammonia formation in the presence of 600 mM NaCl
was 29% of that observed in the presence of 50 mM NaCl. This inhibi-
tion occurred at NaCl concentrations approximating the intracellular
monovalent ion concentration of *P. halodenitrificans* [20], and is
similar to the concentration of NaCl that inhibits several other
enzymes [20], including the NADH-cytochrome-c reductase from
P. halodenitrificans [30].

The maximum rate of ammonia formation was observed between
pH 8.8 and 9.2 when assayed in the presence of borate buffer.
Lineweaver-Burke plots for methyl viologen were linear. The apparent
Km for methyl viologen was 400 μM. Similar plots for nitrite were
nonlinear. The concentration of nitrite at half the extrapolated
maximum velocity was 1.2 mM, but the velocity at 2.5 mM nitrite was
96% of the extrapolated maximum velocity.

Ammonia formation was inhibited by cyanide and carbon monoxide. Diethyldithiocarbamate produced slight inhibition at relatively high concentrations (Table 3). Little, if any, inhibition was produced by azide (10 mM) or sulfite (2.0 mM).

Discussion

The evidence presented in this paper demonstrates that the reduction of nitrite to ammonia occurred in the presence of a cytochrome-containing protein fraction. The spectral properties of this fraction did not resemble those of an assimilatory nitrite reductase [13-18]. Except for some minor differences in the location of the maximum [1,4], the spectral properties of the *P. halodenitrificans* cytochrome, including those of the two pyridine hemochromogens, were consistent with those of cd-cytochrome. The electrophoretic data indicated the presence of a single nitrite-reducing activity in the partially purified enzyme from *P. halodenitrificans*. Furthermore, since the ratio of nitrite disappearance to ammonia formation was 1 (Table 2), and the nitrite-reducing activity eluted from the gels had spectral properties characteristic of cd-cytochrome, we propose that the reduction of nitrite to ammonia is catalyzed by that cytochrome. The possibility that ammonia was produced by a chemical reduction of nitrite could not be confirmed by using a heat-denatured enzyme or various iron-containing compounds. In addition, membranes from *P. halodenitrificans*, which contain a cd-cytochrome nitrite reductase and which produce nitrous oxide from nitrite (Grant and Hochstein, Arch. Microbiol., in press), do not form ammonia from nitrite.

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Singh [31] reported that an electrophoretically homogeneous cyto-chrome oxidase from *Pseudomonas aeruginosa* [32] converted hydroxylamine to ammonia using either reduced methylene blue or pyocyanine. Ammonia formation was inhibited by cyanide and carbon monoxide, but not by azide. We have prepared crude extracts from *P. aeruginosa* (strain JM 42)[+] grown anaerobically in the presence of nitrate, which reduced nitrite and produced 0.2 μmol ammonia min^{-1} mg $protein^{-1}$. Interestingly, the G-100 fraction from *P. halodenitrificans* also reduced hydroxylamine to ammonia when hydroxylamine replaced nitrite. The rate of ammonia forma-tion was 36 μmol ammonia min^{-1} mg $protein^{-1}$. As with nitrite, ammonia formation did not take place unless methyl viologen and dithionite were present.

The role of the ammonia-forming cd-cytochrome is not clear, since *P. halodenitrificans* cannot use nitrite as a sole source of nitrogen when grown in a synthetic medium (L. I. Hochstein and G. A. Tomlinson, in prep.). We were unable to demonstrate that this enzyme was a sulfite reductase, which also reduces nitrite to ammonia [33]. Since *P. halodenitrificans* also contains a membrane-bound dissimilatory nitrite reductase, the possibility exists that the soluble enzyme is normally membrane-bound, but like the enzymes from *Pseudomonas perfectomarinus* [34,35] or *Pseudomonas aeruginosa* [36] is easily detached during cellular disruption. This raises the possibility that when membrane-bound, the cd-cytochrome from *P. halodenitrificans* reduces nitrite to nitrous oxide, but when detached converts nitrite to ammonia.

Cole and Brown [37] point out that the reduction of nitrite to
ammonia provides an efficient method for disposing electrons during
oxidant-limited growth. The reduction of nitrite to dinitrogen, for
example, requires three electrons, compared with the six required for
reduction to ammonia. Thus, during conditions of oxidant limitation,
the ability to direct electron flow to ammonia represents a potential
sink for disposing excess electrons. The ability to reduce nitrite to
ammonia could thus be an important adaptive mechanism for
P. halodenitrificans. Unlike those organisms in which the reduction of
nitrite to ammonia was demonstrated [37], *P. halodenitrificans* is not
a fermentative organism [19] and has no means for regenerating oxidized
electron carriers (i.e., NAD) during anaerobic growth other than by
denitrification. Since the cells used in these studies were grown in
complex medium, and no nitrite was detected in the spent growth medium,
we assume that growth occurred under conditions that would favor the
reduction of nitrite to ammonia via an electron sink pathway. Presum-
ably, the synthesis of this putative ammonia-forming cd-cytochrome
nitrite reductase should be subject to manipulation by varying the ratio
of nitrite to lactate in the growth medium. The recent availability of
a synthetic mineral-salts medium that supports the anaerobic growth of
P. halodenitrificans will allow us to determine if the level of
ammonia-forming activity can be set by the concentrations of nitrite
and carbon source.

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Footnotes

*HEPES; N-2-Hydroxyethylpiperazine-N'-2 ethane sulfonic acid.

**The borate buffer was prepared by titrating boric acid (H_3BO_3) with NaOH to pH 8.8.

†Strain JM 42 of *Pseudomonas aeruginosa* was obtained from Dr. C. Carlson, Department of Bacteriology, University of California, Davis.

TABLE 1

PURIFICATION OF THE AMMONIA-FORMING CYTOCHROME

Fraction	Units (total)	Yield (%)	Protein (total) (mg)	Sp. act.	Purification
S_2	614	100	970	0.63	1.0
Octyl Sepharose	504	82	118	4.3	6.8
DEAE Biogel A	469	76	28	17.0	27.0
G-100 Sephadex	458	75	21	22.0	35.0

TABLE 2

REQUIREMENTS FOR AMMONIA FORMATION

Additions	$-\Delta$ NO_2	$+\Delta$ NH_3
	(μmol/10 min)	(μmol/10 min)
Complete + enzyme	1.14	1.09
Complete – MV	0.14	0.02
Complete – dithionite	0.10	0
Complete – NO_2	–	0
Complete – enzyme	0.04	0.02
Complete – NO_2, + NH_4^+ [a]	–	0.98
Complete + boiled enzyme	0.04	0.04

The G-100 fraction was used as the source of the enzyme
and where added its concentration was 5 μg protein ml^{-1}.
The boiled enzyme was prepared by diluting the G-100
fraction (containing 2.5 mg protein ml^{-1}) 1:10 in
150 mM NaCl–50 mM HEPES (pH 7.4) buffer, boiling for
5 min and, after cooling rapidly to 4°C, assaying 20 μl.

[a] 1 μmol of NH_4Cl was added in place of nitrite
so that the reported value represents the amount of
ammonia recovered after 10 min incubation.

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TABLE 3

INHIBITION OF AMMONIA FORMATION

Inhibitor	Addition	% Inhibition
KCN		
	10 μM	25
	50 μM	57
	100 μM	75
	1 mM	87
CO		
	100% saturated	55
Diethyldithiocarbamate	1.0 mM	9
	2.5 mM	22
	5.0 mM	34

Ammonia formation was determined as described in the
Materials and Methods section. The G-100 fraction
(5 μg) was added to start the reaction. The inhibi-
tion by CO was carried out in vials sealed with rubber
septa. The reaction mixture was saturated with CO
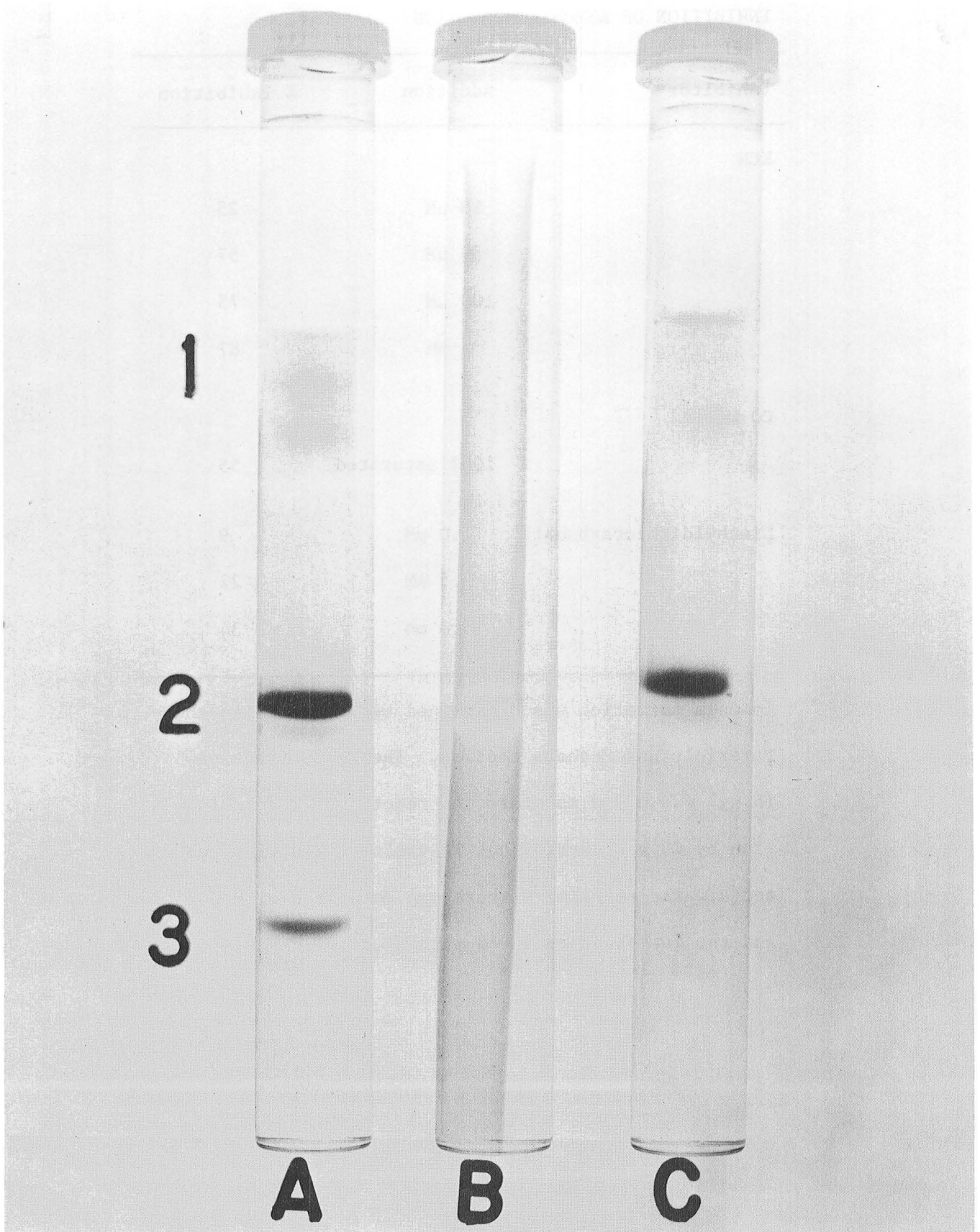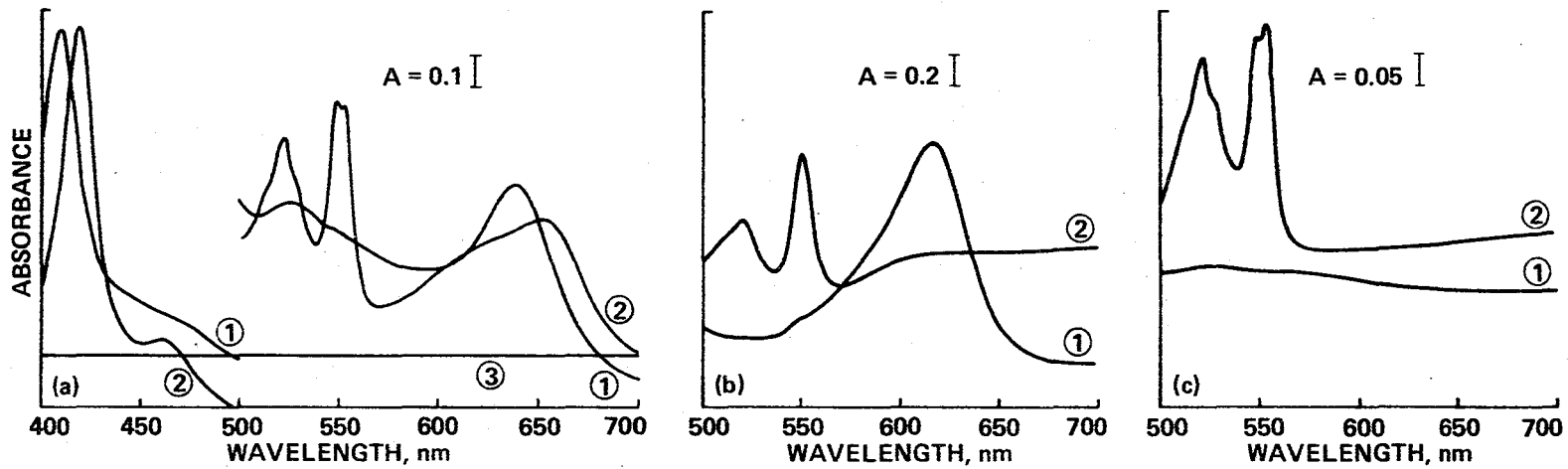and the gas phase replaced with CO.
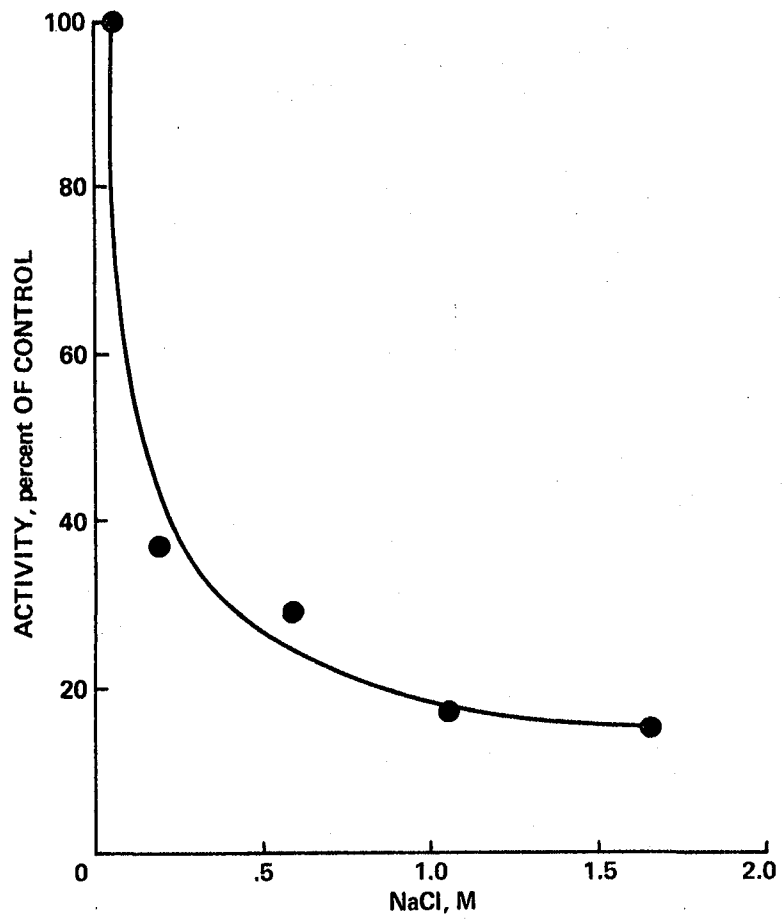
Fig. 1

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Figure 2

Figure 3

Figure Captions

Fig. 1. Polyacrylamide gel electrophoresis of the G-100 Sephadex
fraction. Polyacrylamide gel electrophoresis was performed using
a volume of enzyme that contained 12.5 µg protein. A: The G-100
fraction before electrophoresis, stained with coomassie blue. B: The
G-100 fraction before electrophoresis, stained for
nitrite reductase activity. C: The G-100 fraction after
gel electrophoresis and elution, as described in the Materials and
Methods section, stained with coomassie blue.

Fig. 2. Spectral properties of the ammonia-forming enzyme. Spectra
were determined from 400 to 700 nm using 50 mM HEPES-150 mM NaCl
(pH 7.4 buffer) as the blank. A: Absolute spectra of the G-100
fraction (250 µg protein ml^{-1} in 50 mM HEPES-150 mM NaCl, pH 7.4
buffer) with spectrum 1 representing the oxidized and spectrum 2
the dithionite-reduced spectra, respectively. B: The dithionite-
reduced pyridine hemochromogen spectra of the acid-acetone soluble
heme (spectrum 1) and the residue after acid-acetone extraction
(spectrum 2). C: The absolute spectra of the material from Band 1.
The material was eluted from the gel using 50 mM HEPES-1.6 M NaCl
(pH 7.4 buffer). Absolute spectra were taken of material containing
80 µg ml^{-1} protein. (1) Oxidized sample; (2) dithionite-reduced sample.

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16. Abstract

Cell-free extracts prepared from Paracoccus halodenitrificans catalyzed the reduction of nitrate to ammonia in the presence of dithionite and methyl viologen. Enzyme activity was located in the soluble fraction and was associated with a cytochrome whose spectral properties resembled those of a cd-type cytochrome. Unlike the dissimilatory cd-cytochrome nitrate reductase associated with the membrane fraction of P. halodenitrificans, this soluble cd-cytochrome did not reduce nitrite to nitrous oxide.

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