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The Partial Characterization of Purified Nitrite Reductase and Hydroxylamine Oxidase from *Nitrosomonas europaea*

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Nitrite reductase has been separated from cell-free extracts of *Nitrosomonas* and partially purified from hydroxylamine oxidase by polyacrylamide-gel electrophoresis. In its oxidized state the enzyme, which did not contain haem, had an extinction maximum at 590nm, which was abolished on reduction. Sodium diethyldithiocarbamate was a potent inhibitor of nitrite reductase. Enzyme activity was stimulated 2.5-fold when remixed with hydroxylamine oxidase, but was unaffected by mammalian cytochrome *c*. The enzyme also exhibited a low hydroxylamine-dependent nitrite reductase activity. The results suggest that this enzyme is similar to the copper-containing 'denitrifying enzyme' of *Pseudomonas denitrificans*. A dithionite-reduced, 465nm-absorbing haemoprotein was associated with homogeneous preparations of hydroxylamine oxidase. The band at 465nm maximum was not reduced during the oxidation of hydroxylamine although the extinction was abolished on addition of hydroxylamine, NO_2^- or CO. These last-named compounds when added to the oxidized enzyme precluded the appearance of the 465nm-absorption band on addition of dithionite. Several properties of 465nm-absorbing haemoprotein are described.

The nitrifying bacterium *Nitrosomonas europaea* oxidizes NH_3 to NO_2^- with hydroxylamine as an intermediate (Yoshida & Alexander, 1964). During NH_3 oxidation, N_2O is produced and the predominant source of this gas is the end product, NO_2^- (Ritchie & Nicholas, 1972). A hydroxylamine-dependent nitrite reductase has been partially characterized in cell-free extracts of *Nitrosomonas* (Hooper, 1968). The presence of this enzyme has been confirmed in preparations of hydroxylamine oxidase when hydroxylamine was oxidized to yield NO_2^- and N_2O aerobically and N_2O and NO anaerobically (Ritchie & Nicholas, 1972). Although cytochrome oxidase activity was absent, the partially purified hydroxylamine oxidase retained nitrite reductase activity. The identity of the nitrite reductase, either as a separate enzyme or as an activity attributable to pure hydroxylamine oxidase, was considered important since it would clarify the physiological role of the reductase and permit a definition of its involvement with the electron-transport chain.

The present work describes the separation of nitrite reductase from hydroxylamine oxidase. Evidence is presented for the probable identity of nitrite reductase as a copper protein and for its interdependence with hydroxylamine oxidase for full activity of the reductase. During the purification of hydroxylamine oxidase a component concentrated in the enzyme that had an absorption maximum at 465nm after reduction with sodium dithionite. The results of spectral studies on this haem component in pure hydroxylamine oxidase are considered in relation to the properties of a soluble CO-combining haemoprotein from *Nitrosomonas* reported by Erickson & Hooper (1972).

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Experimental

Materials

Phenazine methosulphate, horse-heart cytochrome *c* (type II), β-NADH (grade III), deoxyribonuclease I, lysozyme, dithiothreitol and alcohol dehydrogenase were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.); sodium diethyldithiocarbamate from May and Baker Ltd. (Dagenham, Essex, U.K.); *N*-methylhydroxylamine hydrochloride from Aldrich Chemical Co. (Milwaukee, Wis., U.S.A.); methoxyamine hydrochloride and *NNN'N'*-tetramethylethylenediamine from Eastman Organic Chemicals (Rochester, N.Y., U.S.A.); and αα'-bipyridyl, *o*-phenanthroline, 8-hydroxyquinoline, acrylamide and *NN'*-methylenebisacrylamide from BDH Chemicals Ltd. (Poole, Dorset, U.K.). NH_2OH,HCl (AR)

was recrystallized twice from methanol–water and dried over P_2O_5.

Methods

Culture of organism. N. europaea (kindly supplied by Dr. Jane Meiklejohn of Rothamsted Experimental Station, U.K.) was grown and harvested as described by Ritchie & Nicholas (1972).

Preparation of cell extracts. A lytic procedure was used for the extraction of hydroxylamine oxidase and nitrite reductase. Cell lysis was accomplished by osmotic rupture after an EDTA–lysozyme treatment as described by Rees & Nason (1965).

Chemical methods. (a) Nitrite. This was determined by the Griess–Ilosvay colorimetric method in a final volume of 3.0ml as described by Hewitt & Nicholas (1964). The determination of NO_2^- in the presence of sulphide required the prior removal of sulphide by precipitation as ZnS. A sample (0.2ml) of incubation mixture was added to 1.0ml of 10% (w/v) zinc acetate with shaking. The precipitate was removed by centrifuging at 2000*g* for 10min and a sample (1.0ml) used for NO_2^- determination.

Azide interfered with nitrite determinations by reaction in acid solution (Hewitt & Hallas, 1959). Appropriate chemical controls were therefore included in those experiments.

(*b*) Protein. This was assayed by the method of Warburg & Christian (1941).

Extraction of haem from purified hydroxylamine oxidase. The method of Paul (1950) was used. The haem *c* was dissolved in 1.0ml of methanol and the spectrum recorded. Methanol was removed *in vacuo*, the sample redissolved in 0.5ml of water and the pH adjusted to 11 with NaOH for further spectral analysis.

The aqueous enzyme-protein moiety was redissolved and the silver removed by dialysis against three changes of 5mM-K_2HPO_4–KH_2PO_4 buffer, pH7.5, containing 5mM-NaCl and 1mM-EDTA. The dialysed enzyme was used for spectral analysis. A sample of horse-heart cytochrome *c* (Sigma, type II) was treated by the same procedure for comparative purposes.

Analytical polyacrylamide-gel electrophoresis. Polyacrylamide-gel electrophoresis was carried out at 2°C by using the methods and apparatus described by Reid & Bieleski (1968). For routine protein purity checks the gel buffer used was Tris–HCl, pH8.6 (0.038M-Tris). The electrophoresis buffer was Tris–glycine, pH8.3 (0.025M-Tris) and the gels contained 7.5% (w/v) acrylamide.

For electrophoresis of the reduced enzyme the gels were prepared containing 7.5% (w/v) acrylamide, Tris–HCl, pH8.0 (0.06M-HCl), and 0.1% (w/v) sodium dodecyl sulphate. The electrophoresis buffer was Tris–HCl, pH8.0 (0.038M-Tris), containing 0.1% (w/v) sodium dodecyl sulphate. After sample application the electrophoresis was for 4h at 24mA (150V).

The proteins were stained with 0.25% (w/v) Coomassie Blue and the haem components were stained by the method of Newton (1969).

Spectral methods. Absorption measurements were made in a Shimadzu multipurpose recording spectrophotometer, model MPS-50L (Shimadzu Seisakusho Ltd., Tokyo, Japan). Spectra at room temperature were recorded by using 0.2 and 1.0cm silica cuvettes, with a photomultiplier voltage of 400V automatic slit control, and the absorbance ranges 0–1 or −0.1–+0.1. Spectra at the temperature of liquid N_2 were recorded by using the Shimadzu low-temperature spectrophotometry attachment with cuvettes of 0.2cm light-path. Spectra were recorded in cracked-ice samples in the absence of glycerol by using a photomultiplier voltage of 650V and a scan speed of 25nm/min.

Pyridine haemochromogen derivatives were prepared by addition of 1 vol. of a solution of 2M-pyridine in 0.05M-NaOH to 1 vol. of haemoprotein solution. Sample reduction was by addition of a few crystals of sodium dithionite.

Assay of hydroxylamine oxidase. The assay mixture, in a 15cm×2.5cm test-tube, contained 2ml of 0.1M-K_2HPO_4–KH_2PO_4 buffer, pH8.0, 0.1ml (75nmol) of phenazine methosulphate, 0.1ml (22.5μmol) of NH_2OH,HCl dissolved in the above buffer solution, enzyme and water to 3.0ml. The mixture was preincubated for 5min at 30°C and the reaction started by addition of hydroxylamine. Samples (0.2ml) were removed at various time-intervals for 15min and NO_2^- was determined. Specific activity units are defined as μmol of NO_2^- formed/min per mg of protein.

When inhibitors were included in the assay, they were added in ethanol and appropriate ethanol controls were included. The final ethanol concentration did not exceed 0.8% (v/v).

Assay of nitrite reductase. The assay involved measuring NO_2^- utilization under an atmosphere of O_2-free N_2 in the presence of enzyme and an NADH–phenazine methosulphate couple as electron donor. The assay mixture was contained in a 10ml-capacity flask with a side-port closed by a rubber septum (Suba-seal; Wm. Freeman, Barnsley, Yorks., U.K.). The flask was fitted with a ground-glass stopcock. The assay mixture contained 1.5ml of 0.25M-K_2HPO_4–KH_2PO_4 buffer, pH6.0, 0.1ml (2μmol) of $NaNO_2$, 0.05ml (0.1μmol) of phenazine methosulphate, 0.1ml (3μmol) of NADH, enzyme and water to 2ml. The flasks containing enzyme, water, buffer and $NaNO_2$ were evacuated and flushed four times with O_2-free N_2 before a final filling with N_2; the apparatus described by Elleway *et al.* (1971) was used. The phenazine methosulphate was added from a gas-tight micro-syringe and flasks were preincu-

bated at 30°C for 5min before the addition of NADH with the gas-tight micro-syringe. Duplicate samples (0.05ml) were removed after 1min and at two other timed intervals during incubation for the determination of residual NO_2^-. Samples were added to a test-tube containing 0.8ml of water, 0.1ml of 1M-acetaldehyde and 0.1ml of alcohol dehydrogenase (1.75mg/10ml of water). The oxidation of excess of NADH under these conditions was extremely rapid and after 2min the nitrite reagents were added.

During the first minute of the assay there was a chemical reaction between NO_2^- and NADH resulting in the disappearance of some NO_2^-. The extent of this reaction was increased by the presence of phenazine methosulphate and this loss of NO_2^- was partially reversible. The NO_2^- concentration increased slowly after the first minute and reached a concentration similar to that remaining when NO_2^- and NADH were present in the absence of phenazine methosulphate. This effect of phenazine methosulphate was observed by using concentrations of NADH and phenazine methosulphate twice and fifteen times respectively those in the routine assay. Therefore an incubation mixture without enzyme was used to correct for this chemical loss of NO_2^-.

Specific activity units are defined as μmol of NO_2^- reduced/min per mg of protein. A unit of activity was that amount of protein catalysing the reduction of 1μmol of NO_2^-/min.

Purification of nitrite reductase. All procedures were carried out at 2°C. Crude extracts were centrifuged at 10000*g* for 15min to sediment whole cells and cell debris. The supernatant fraction was concentrated by adding solid $(NH_4)_2SO_4$ to 100% (w/v) saturation (69.7g/100ml). The suspension, allowed to stand overnight, was then centrifuged at 10000*g* for 10min. The red precipitate was redissolved in a small vol. (20ml) of 0.025M-K_2HPO_4–KH_2PO_4 buffer, pH7.5, and dialysed for at least 4h against two changes of 2 litres of the same buffer. This concentrated fraction (10S) was stored at −15°C until used for further purification.

Chromatography of nitrite reductase on DEAE-cellulose. A portion of the 10S fraction (250mg of protein) was adsorbed on a DEAE-cellulose column (Whatman DE-11 medium fibrous powder, 3.0cm ×12.0cm) equilibrated with 0.025M-K_2HPO_4–KH_2PO_4 buffer, pH7.5. The column was washed with the same buffer until free of non-adsorbed cytochromes and protein. Hydroxylamine oxidase and nitrite reductase were eluted with a linear gradient of phosphate supplied by mixing 300ml of 0.05M buffer at the same pH. The flow rate was 3ml/min and the fraction volume was 8.0ml. Protein and haem elution were monitored by measurement of E_{280} and E_{410} respectively. The nitrite reductase activity was eluted in the range 0.15–0.21M-potassium phosphate. The most active fractions were combined and concentrated by $(NH_4)_2SO_4$ precipitation at 100% (w/v) saturation. The red precipitate was redissolved in the minimum volume of 0.025M-K_2HPO_4–KH_2PO_4 buffer, pH7.5, and dialysed against 2 litres of the same buffer. The concentrated enzyme preparation was adjusted to 10–15mg of protein/ml before application to a polyacrylamide-gel-electrophoresis column.

Preparative polyacrylamide-gel electrophoresis of nitrite reductase. Preparative polyacrylamide-gel electrophoresis of nitrite reductase was carried out in an apparatus (Shandon Scientific Co. Ltd., London N.W.10, U.K.) with an annular gel compartment of 3.9cm^2. The gel and buffer systems were as follows: the resolving gel (28.0ml) consisted of 7.5% (w/v) acrylamide with 0.2% (w/v) *NN'*-methylenebisacrylamide as cross-linking reagent and gave an effective gel height of 7.0cm. The gel was prepared in 0.38M-Tris–HCl buffer, pH8.0, containing 0.125% (w/v) ammonium persulphate. No concentration gel was used. The upper and lower buffer reservoirs contained 0.15M-Tris–acetate buffer, pH8.0. A constant current of 30mA was applied to the gel at 4°C for 2h to remove any E_{280}-absorbing material and catalyst residues, which would interfere with the protein profile. Samples of up to 30mg of protein in 2ml were made viscous by additon of 1g of sucrose and layered on top of the resolving gel. Electrophoresis was carried out at 4°C with a constant-current power supply The current was set at 80mA and eluate was collected in 12ml fractions at a flow rate of 10ml/h.

Purification of hydroxylamine oxidase. All procedures were carried out at 2°C. The purification procedure was that described above for the partial purification of nitrite reductase, including the step of preparative polyacrylamide-gel electrophoresis. The fractions containing hydroxylamine oxidase activity were combined, dialysed overnight against 10vol. of 0.05M-K_2HPO_4–KH_2PO_4 buffer, pH7.5, and stored at −15°C at a protein concentration of 0.4mg/ml. The pure enzyme migrated as a single protein and haem band on electrophoresis at pH8.6 and its specific activity in different preparations was in the range 5–6.4mol of NO_2^- produced/min per mg of protein.

Results

Nitrite reductase activity in whole cells

Under aerobic conditions NO_2^-, the end product of NH_3 oxidation in whole cells, is the predominant source of N_2O (Ritchie & Nicholas, 1972). Exogenous NO_2^- is not reduced except under anaerobic conditions. The specific activity of nitrite reductase under aerobic conditions concomitant with oxidation of NH_3 was 128nmol of NO_2^- reduced/min per mg of

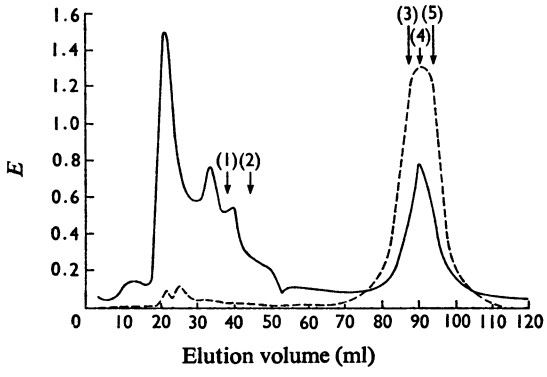

Fig. 1. *Preparative polyacrylamide-gel electrophoresis of nitrite reductase*

The profiles are shown of protein (E_{280}, ——) and haem (E_{410}, – – – –) eluted during electrophoresis. The apparatus, gel preparation and conditions for electrophoresis were as described in the Experimental section. The concentrated protein fraction after DEAE-cellulose chromatography containing both nitrite reductase and hydroxylamine oxidase activities was applied to the resolving gel. Total protein applied was 27mg containing 7.8 units of nitrite reductase and 80 units of hydroxylamine oxidase. After electrophoresis the specific activity of nitrite reductase in the numbered fractions was: (1) 0.027; (2) 0.042; (3) 0.014; (4) 0.021 and (5) 0.009 μmol/min per mg of protein. Combined fractions for nitrite reductase eluted between 38 and 45ml contained 0.041 unit of activity. Combined fractions of hydroxylamine oxidase eluted between 80 and 100ml contained 47 units of hydroxylamine oxidase activity.

cell protein, assuming all N_2O was derived from NO_2^-. Since some N_2O is produced during NH_3 oxidation (Ritchie & Nicholas, 1972), the value for specific activity is higher than that due to nitrite reductase alone.

Purification of nitrite reductase

A complete summary of purification of the enzyme was not possible owing to the lability of the enzyme, especially after preparative polyacrylamide-gel electrophoresis.

Concentrated 10S fractions contained specific activities of about 100nmol of NO_2^-/min per mg of protein. During DEAE-cellulose chromatography nitrite reductase activity was always eluted coincident with hydroxylamine oxidase activity, and their relative specific activities were about 1:15 (nitrite reductase/hydroxylamine oxidase). Purification was always low (approx. 1.5) with this step and recovery was 50%.

The combined fractions after the DEAE-cellulose step were used for preparative polyacrylamide-gel electrophoresis and 3.0–8.0 units of enzyme activity were applied to the gel.

The elution profile of protein (E_{280}) and haemoprotein (E_{410}) during preparative polyacrylamide-gel electrophoresis of a nitrite reductase preparation is shown in Fig. 1. Three haemoproteins were observed to migrate rapidly through the gel closely followed by other proteins, one of which was eluted as a shoulder on the profile and contained nitrite reductase activity. Hydroxylamine oxidase was eluted after 8–10h as a broad red protein band. Nitrite reductase was detectable in low activities in fractions eluted between 38 and 45ml and also in the hydroxylamine oxidase-containing fractions (Fig. 1).

The combined eluates (7ml) containing the nitrite reductase activity, which was not associated with that in the hydroxylamine oxidase, were used for further characterization.

Analytical polyacrylamide-gel electrophoresis of the nitrite reductase fraction indicated that 95% of the protein was present in two bands, a slower protein, approx. 50%, and a faster one, approx. 45%, together with a faster contaminant protein eluted off the preparative polyacrylamide-gel column between 31 and 36ml. Since both analytical and preparative electrophoresis gels were made at the same pH and with identical acrylamide content, the electrophoretic patterns were equivalent. The protein comprising 45% of the total in the pooled enzyme fraction was identified as nitrite reductase.

Spectrum of nitrite reductase fraction from polyacrylamide-gel electrophoresis

The oxidized protein had an extinction maximum at 275nm, a shoulder at 290nm, a Soret band at 405nm and a further symmetrical absorption band with an extinction maximum at 590nm; the ratio of E_{590}/E_{575} was 0.055. On reduction with sodium dithionite, the extinction at 590nm was abolished completely and the Soret band shifted to 416nm. The ratio $E_{416}/E_{275} = 0.059$ was extremely small. No spectral change was observed when $NaNO_2$ was added to the oxidized enzyme. These results suggest that the Soret absorption was due to contamination by the haemoprotein eluted off the polyacrylamide-gel column between 31 and 36ml.

No spectral changes were observed in the oxidized and reduced spectra of the hydroxylamine oxidase preparation after polyacrylamide-gel electrophoresis.

Properties of purified nitrite reductase

Nitrite reductase activities in the partially purified nitrite reductase fraction and in the hydroxylamine oxidase fraction are shown, together with the effects of some inhibitors, in Table 1. Specific activities were

very low in the recovered enzyme when compared with the activity loaded on the polyacrylamide gel. The enzyme before electrophoresis had a specific activity of 0.290 μmol of NO_2^- reduced/min per mg of protein and 7.8 units were loaded on the gel. The enzyme in the nitrite reductase fraction had a specific activity of 0.015 μmol/min per mg of protein and 0.041 unit was recovered. The nitrite reductase activity in the hydroxylamine oxidase fraction was extremely labile. The activity of the nitrite reductase fraction was stable for at least 1 week under normal storage conditions. When the nitrite reductase fraction and the hydroxylamine oxidase fraction were mixed in equal ratios, a 3.5-fold increase in activity was observed (Table 1). When a stored hydroxylamine oxidase fraction completely devoid of nitrite reductase activity was mixed with the nitrite reductase fraction, a twofold stimulation of nitrite reductase activity was still observed.

The two separated nitrite reductase activities were inhibited by dithiothreitol, although this reagent did not inhibit nitrite reductase recovered from the DEAE-cellulose step. Mepacrine slightly inhibited activity in the nitrite reductase fraction. KCN (0.1 mM) did not inhibit nitrite reductase in preparations from the DEAE-cellulose step of the purification.

Sodium diethyldithiocarbamate produced complete inhibition of activity of the nitrite reductase fraction and of the stimulated activity observed on mixing the two enzyme fractions. It was also a potent inhibitor of the enzyme in unpurified systems. When mammalian horse-heart cytochrome *c* was added to the incubation mixture in catalytic amounts, it was immediately reduced by the reduced phenazine methosulphate. The presence of this reduced cytochrome *c* did not, however, significantly stimulate nitrite reductase activity in the enzyme eluted from DEAE-cellulose or in the fractions obtained by electrophoresis either assayed separately or when mixed (Table 1).

Hydroxylamine-dependent nitrite reductase

Hydroxylamine was an effective electron donor for nitrite reductase in preparations after DEAE-cellulose chromatography, without the mediation of phenazine methosulphate (Table 2). The enzymic rate was dependent on the concentration of hydroxylamine, and with equimolar concentrations of NO_2^- and hydroxylamine (1 mM) in the incubation mixture the specific activity was 0.017 μmol/min per mg of protein. Specific activity, when the NADH–phenazine methosulphate couple provided the reducing power, was 0.155 μmol/min per mg of protein. The results indicate that in the presence of catalytic amounts of phenazine methosulphate, hydroxylamine-dependent nitrite reductase activity was stimulated tenfold. Since the enzyme preparation contained hydroxylamine oxidase, the presence of the added electron carrier mediated the anaerobic oxidation of hydroxylamine and the concomitant reduction of NO_2^- by nitrite reductase.

Table 1. *Properties of nitrite reductase purified by preparative polyacrylamide-gel electrophoresis*

Assay conditions were as described in the Experimental section. Enzyme preparations were as follows: nitrite reductase fraction, 203 μg of protein; hydroxylamine oxidase fraction (a haemoprotein containing hydroxylamine oxidase activity and eluted after 9 h), 195 μg of protein. Reagent additions to the reaction mixture were made before the flasks were flushed with N_2.

Enzyme fraction	Reagent addition and concn. (mM)	Reaction rate (nmol/min per mg of protein)	Inhibition (I) or stimulation (S) (%)
Nitrite reductase	None	2.69	—
Hydroxylamine oxidase	None	0.76	—
Nitrite reductase+hydroxylamine oxidase (1:1)	None	9.60	256 (S)
Nitrite reductase	Dithiothreitol (0.5)	0.52	80 (I)
Nitrite reductase	Cytochrome *c* (0.05)	1.83	32 (I)
Nitrite reductase	Mepacrine (0.5)	2.00	25 (I)
Nitrite reductase	Sodium diethyldithiocarbamate (0.5)	0	100 (I)
Hydroxylamine oxidase	Dithiothreitol (0.5)	0	100 (I)
Hydroxylamine oxidase	Cytochrome *c* (0.05)	0.83	10 (S)
Hydroxylamine oxidase	Mepacrine (0.5)	1.38	82 (S)
Nitrite reductase+hydroxylamine oxidase (1:1)	Cytochrome *c* (0.05)	11.7	335 (S)
Nitrite reductase+hydroxylamine oxidase (1:1)	Sodium diethyldithiocarbamate (0.5)	0	100 (I)

Table 2. *Hydroxylamine-dependent nitrite reductase*

The assay conditions were the same as described in the Experimental section but NADH and phenazine methosulphate were omitted and the assay was initiated by the addition of hydroxylamine. The enzyme preparation containing both nitrite reductase and hydroxylamine oxidase was that obtained from the DEAE-cellulose step (280 μg of protein/assay).

Electron donor and concn. (mM)	Other additions and concn. (mM)	Reaction rate (nmol of NO_2^- reduced/min)
NADH (1.5)+phenazine methosulphate (0.05)	—	43.2
NH_2OH (5.0)	Boiled enzyme	0
NH_2OH (10.0)	—	15.6
NH_2OH (5.0)	—	10.3
NH_2OH (1.0)	—	4.7
NH_2OH (0.5)	—	1.8
NH_2OH (5.0)	Sodium diethyldithiocarbamate (0.5)	0
NH_2OH (5.0)	Phenazine methosulphate (0.05)	28.1
NH_2OH (1.0)	Phenazine methosulphate (0.05)	24.3

The hydroxylamine-dependent nitrite reductase activity was completely inhibited by sodium diethyldithiocarbamate, which suggested that hydroxylamine, in the absence of added electron carrier, was donating electrons for NO_2^- reduction by using the same mechanism as the NADH–phenazine methosulphate couple. The apparent K_m value for hydroxylamine in the nitrite reductase reaction with 1 mM-nitrite was 4×10^{-3} M and for hydroxylamine in the hydroxylamine oxidase reaction, 5×10^{-5} M.

Difference spectra of whole cells

Immediately on addition of NH_3 to a suspension of washed cells the reduced α- and β-bands of cytochrome *c* (552 and 524 nm), cytochrome *b* (558 and 529 nm) and cytochrome *a* (600 and shoulder at 446 nm) appeared. The complex asymmetrical Soret band had maxima at 422 and 424 nm. Under anaerobic conditions NH_3 did not produce a difference spectrum, and on addition of dithionite cytochromes *b* and *c* were fully reduced together with the appearance of an absorption maximum at 463 nm. When $NaNO_2$ was added to the cell suspension, the cytochromes were completely reoxidized and the band at 463 nm maximum was abolished. Further addition of dithionite again reduced the cytochromes but failed to produce the 463 nm-absorbing band. The maximum absorption of the 463 nm-absorbing band in whole cells was measured as the ratio $E_{463}-E_{445}/E_{552}-E_{570}=0.48$.

Spectra of pure hydroxylamine oxidase

The dithionite reduced-minus-oxidized difference spectrum of the pure hydroxylamine oxidase is shown in Fig. 2. In addition to the presence of absorption peaks of cytochromes *b* and *c*, there was a strong absorption band with a maximum at 465 nm. When a slight excess of $NaNO_2$ was added to the dithionite-reduced sample, there was a small oxidation of the *b* and *c* cytochromes and the 465 nm-absorption peak was abolished.

When the oxidized enzyme was treated with $NaNO_2$, there was no change in the difference spectrum between 350 and 700 nm. On subsequent reduction with excess of dithionite, there was no absorption at 465 nm but the other cytochromes were fully reduced.

Addition of the substrate hydroxylamine to the enzyme as a routine resulted in a partial reduction of the cytochromes (approx. 50% reduction) but there was no absorption between 450 and 480 nm (Fig. 3). Subsequent dithionite reduction did not produce the band at 465 nm maximum.

Addition of hydroxylamine to dithionite-reduced enzyme resulted in abolition of the 465 nm absorption peak, although the reduced bands of the cytochromes *b* and *c* were unaffected.

CO binding to hydroxylamine oxidase

Dithionite-reduced enzyme was treated with CO and the reduced-plus-CO-minus-oxidized difference spectrum showed that the band absorbing at 465 nm maximum was immediately abolished. The other absorption bands were unchanged. The reduced-plus-CO-minus-dithionite-reduced difference spectrum showed a trough at 465 nm and a broad shoulder at 447–449 nm. This latter absorption band corresponded to that observed at 450 nm in similar spectra of cytoplasmic fractions by Rees & Nason (1965). When oxidized enzyme was treated with CO, the oxidized-plus-CO-minus-oxidized difference spectrum was identical with the oxidized base-line; however, the 465 nm-absorbing peak was absent and on subsequent dithionite reduction, only the *b* and *c* cytochromes were reduced. The effect of CO on the reduced enzyme was not reversed by prolonged exposure to light.

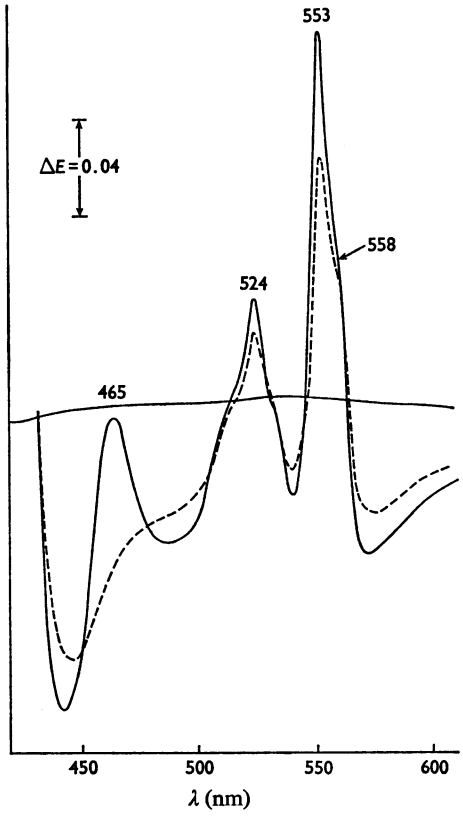

Fig. 2. *Difference spectra of hydroxylamine oxidase*

Each 1.0cm cuvette contained oxidized enzyme (248μg of protein) in 50mM-K_2HPO_4–KH_2PO_4 buffer, pH7.5. After recording the base-line, solid dithionite (0.5mg) was added to the sample cuvette and the reduced-minus-oxidized difference spectrum was recorded (——). Excess of $NaNO_2$ was then added to both cuvettes and the reduced-plus-nitrite-minus-oxidized difference spectrum was recorded (----).

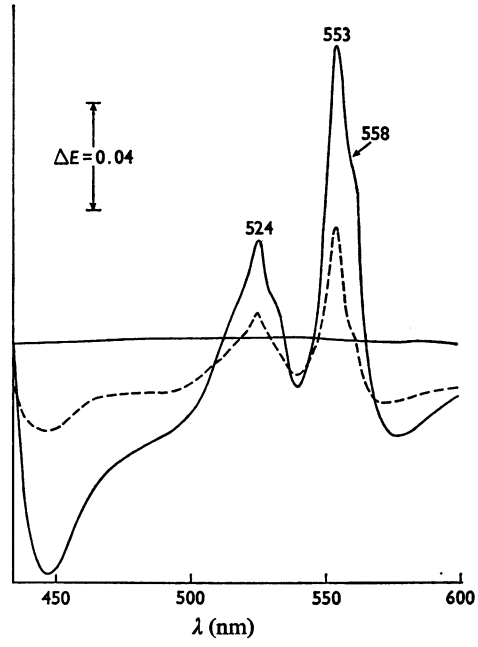

Fig. 3. *Difference spectra of hydroxylamine oxidase*

Each 1.0cm cuvette contained oxidized enzyme (248μg of protein) in 50mM-K_2HPO_4–KH_2PO_4 buffer, pH7.5. After recording the baseline, NH_2OH,HCl (1.0mg) was added to the sample cuvette and the NH_2OH reduced-minus-oxidized difference spectrum was recorded (----). Excess of dithionite was then added to the sample cuvette and the NH_2OH reduced-plus-dithionite-minus-oxidized difference spectrum was recorded (——).

Effects of azide, KCN and NO on hydroxylamine oxidase

Sodium azide added to oxidized enzyme produced no spectral change and did not affect subsequent dithionite reduction. The 465nm band was formed and could be abolished by $NaNO_2$.

KCN added to oxidized enzyme produced no spectral change. Difference spectra indicated that CN^- had no effect upon the reduced 465nm peak or on its abolition by NO_2^-.

Oxidized enzyme in buffer at pH7.4 was treated with NO in an anaerobic cuvette. The oxidized-plus-NO-minus-oxidized difference spectrum showed that the *c*-type cytochrome of the enzyme was not converted into the ferrocytochrome–nitrosyl ion complex. Both mammalian cytochrome *c* and soluble cytochrome *c* present in crude extracts of *Nitrosomonas* were rapidly converted into the complex with absorption peaks at 530 and 564nm. Reduction of the NO-treated enzyme with dithionite gave full reduction of the cytochrome and the 465nm peak.

Effects of various reducing agents on hydroxylamine oxidase

The extent of reduction of the cytochromes was not increased above 50% when hydroxylamine was added to enzyme in an argon atmosphere. Therefore the partial reduction was not due to the presence of an oxidase activity in the enzyme. The only compound other than dithionite to reduce the enzyme instantaneously to the same extent as hydroxylamine was *N*-methylhydroxylamine. Another analogue, *O*-methylhydroxylamine, did not reduce the enzyme. The NADH–phenazine methosulphate couple used

in the nitrite reductase assay was an effective electron donor.

Neither the enzyme nor the 465nm maximum was reduced in the presence of any of the following compounds: $(NH_4)_2SO_4$, K_4FeCN_6, $NaBH_4$, Na_2SO_3, NaS, ferrous oxalate, NADH, SO_2, NO or ascorbic acid plus dichlorophenol-indophenol. A gradual reduction of the *b* and *c* cytochromes was observed in the presence of 2-mercaptoethanol.

The relative height of the peak at 465nm compared with the reduced α-peak of the cytochrome *c* at 552nm varied with different preparations, being greatest in intact cells. The extinction at 465nm was progressively decreased as the enzyme aged during storage at 2°C; moreover, full reduction after aging was attained only after it was left in the presence of dithionite for a few minutes at room temperature. The absorption band did not appear to be autoxidizable under aerobic conditions.

Low-temperature difference spectra of purified hydroxylamine oxidase

The hydroxylamine reduced-minus-oxidized difference spectrum of enzyme (620μg/ml) showed the presence of the following absorption maxima: 415, 523, 549.5 (shoulder), 550.5 and 557nm. The extent of the reduction on the basis of the 550.5nm peak absorption was 35% of the fully reduced enzyme. The absorption maxima for the dithionite reduced-minus-oxidized difference spectrum of the enzyme were 419.5, 422.5, 465, 521, 548.5, 549.5 (shoulder) and 555.5nm. The ratio $E_{465}/E_{548.5}$ was 0.30 at the temperature of liquid N_2, and the equivalent extinction ratio for the bands at 465 and 552nm at room temperature was 0.29.

Polyacrylamide-gel electrophoresis

A sample of electrophoretically homogeneous hydroxylamine oxidase was treated with sodium dodecyl sulphate and 2-mercaptoethanol and subjected to electrophoresis at pH8.0. The electrophoretogram indicated that the unfolded enzyme particle was separated into at least three polypeptides, of which two contained haem prosthetic groups.

Inhibition of hydroxylamine oxidase

The effect of several respiratory inhibitors and transition metal-chelating agents is shown in Table 3. KCN was a potent inhibitor of NO_2^- production during hydroxylamine oxidation. CO was not inhibitory when present in the gas phase during the incubation at 50% (v/v) with O_2. The chelating agents were inhibitory at similar concentrations in the assay but sodium diethyldithiocarbamate at 0.10mM and EDTA (sodium salt) at 10mM were without effect.

Table 3. *Inhibition of purified hydroxylamine oxidase*

Each inhibitor was included in the assay over the range 10mM–1μM. Assay conditions were as described in the Experimental section. Purified enzyme 4μg of protein/assay. NH_2OH,HCl was 7.5mM. Control enzyme reaction rate = 26nmol of NO_2^- produced/min.

Inhibitor	Concn. giving 50% inhibition of enzyme rate (mM)
KCN	0.07
Sodium azide	3.2
NaS	0.3
αα-Bipyridyl	0.1
8-Hydroxyquinoline	0.2
o-Phenanthroline	0.12

Discussion

Nitrite reductase

Ritchie & Nicholas (1972) have shown that in partially purified preparations there is a close association between hydroxylamine oxidase and nitrite reductase. The results in the present paper show conclusively that nitrite reductase is a separate protein. Although the best preparation of nitrite reductase was not more than 45% pure, its spectral properties and its sensitivity to sodium diethyldithiocarbamate indicated a similarity to the purified 'denitrifying enzyme' of *Pseudomonas denitrificans* (Iwasaki *et al.*, 1963). The 'denitrifying enzyme' is a copper-containing protein and has a ratio E_{594}/E_{280} of 0.035, which is very close to that of the nitrite reductase of *Nitrosomonas*. A nitrite reductase containing two copper atoms per molecule has been purified from *Achromobacter cycloclastes* (Iwasaki & Matsubara, 1972); it has similar properties to both the 'denitrifying enzyme' and the *Nitrosomonas* enzyme.

It has been shown that *c*-type cytochromes occur as prosthetic groups in some bacterial nitrite reductases and are involved in electron transfer to NO_2^- (Yamanaka & Okunuki, 1963; Newton, 1969; Lam & Nicholas, 1969; Prakash & Sadana, 1972). Mammalian cytochrome *c* did not stimulate nitrite reductase activity in *Nitrosomonas*. It is possible that the stimulation of activity obtained after addition of hydroxylamine oxidase back to the nitrite reductase fraction was due to the involvement of either *b*- or *c*-type cytochromes, which are prosthetic groups in hydroxylamine oxidase (Rees, 1968). The close association of hydroxylamine oxidase and nitrite reductase is an apparent requirement for maintaining the full activity of the reductase, though not for the oxidase. The specific activity of the purified nitrite reductase was only 5% that of the enzyme before electrophoresis and the stimulation of specific activity when the two enzymes were remixed is significant. It

is suggested that the separation of the two enzymes destroyed a specific relationship between these proteins necessary for full activity of the reductase.

The hydroxylamine-dependent nitrite reductase activity was similar to that found in the *Pseudomonas* 'denitrifying enzyme' (Iwasaki *et al.*, 1963) and also to that reported in *Corynebacterium nephridii* (Renner & Becker, 1970). This activity may result from a lack of specificity of the nitrite reductase for one of its substrates. Free hydroxylamine has been detected in cell suspensions of *Nitrosomonas* only in the presence of an inhibitor of hydroxylamine oxidase (Yoshida & Alexander, 1964). The values of the apparent K_m for hydroxylamine provided evidence that hydroxylamine-dependent nitrite reductase activity did not interfere significantly in the anaerobic production of N_2O by the phenazine methosulphate-coupled activities of hydroxylamine oxidase and nitrite reductase as reported by Ritchie & Nicholas (1972).

Hydroxylamine oxidase

The addition of dithionite to whole cells resulted in full reduction of the cytochromes, together with the appearance of a band with an absorption maximum at 463nm. It has been shown that this organism can reduce NO_2^- under both aerobic and anaerobic conditions (Ritchie & Nicholas, 1972); thus the addition of $NaNO_2$ to dithionite-reduced cells under anaerobic conditions resulted in the immediate collapse of the 463nm-absorbing band and full reoxidation of the cytochromes. The band at 463nm maximum was not observed in cell suspensions or extracts under any condition other than when dithionite was present. The possibility that the 463nm-absorbing band might be a cytochrome *d* (Appleby, 1968) was excluded, since the partially pure nitrite reductase is devoid of haem.

The band at 463nm was purified along with hydroxylamine oxidase, and the electrophoretically pure enzyme particle contained this dithionite-reduced maximum, although its absorption peak was shifted to 465nm. The addition of NO_2^- to dithionite-reduced hydroxylamine oxidase produced an immediate collapse of the band at 465nm, as it did in whole cells, but only a partial reoxidation of the cytochromes *b* and *c* present in the enzyme. By using pure hydroxylamine oxidase, it was found that hydroxylamine itself and CO both resulted in the immediate collapse of the 465nm-absorbing band without reoxidation of the cytochromes *b* and *c* absorption peaks. The addition of either of these compounds, or NO_2^-, to the oxidized enzyme precluded the appearance of the reduced 465nm-absorbing band on subsequent addition of dithionite.

The ligands, CN^-, azide and NO, affected neither the formation of the band at 465nm maximum after the addition of dithionite nor its collapse on addition of NO_2^-. However, it was unusual that NO did not form the ferrocytochrome–nitrosyl ion with the cytochrome *c* component of the enzyme. This may be due to a protection of the haem by the tertiary structure of the enzyme.

Consideration of the effects of hydroxylamine, NO_2^- and CO on the band at 465nm suggests that the enzyme occurs in two states, one existing in the presence of substrate, product or CO in which the 465nm-absorbing band is precluded before, or abolished after, full reduction, and another sustained in the absence of substrate or product, when the band at 465nm can be reduced only by dithionite. CN^-, azide and NO do not affect the enzyme in either state. It appears that the change in state is associated with the binding of the nitrogenous substrate or product, or of CO. The observation that CO combined with oxidized enzyme to preclude the formation of the 465nm absorption band on reduction was most unusual, since no oxidized haemoprotein has to date been shown to combine with CO. It is possible that CO is not only combining with an enzyme-bound haem but may also be acting as if it were an enzyme intermediate causing a change of state, as discussed above, by virtue of its similarity to N_2O_2 and NO (Leigh, 1971).

The absorption maxima of the enzyme at the temperature of liquid N_2 were identical with those reported for the enzyme by Rees (1968). However, the peak at 465nm was not apparent in Rees's (1968) published dithionite-reduced-minus-oxidized difference spectrum. In the present spectral results the intensity of the band at 465nm maximum compared with that of the α-peak of cytochrome *c* was the same as the relative intensity at room temperature. The equivalent intensities may be taken as indirect evidence that the band at 465nm is that of a haemoprotein, since cytochrome absorbances are greatly intensified at low temperatures (Kawai, 1968).

The splitting and extraction of haem *c* and protohaem from the enzyme were only partially successful since the extracted protein moiety was not enzymically active, and the spectrophotometric determination of the band at 465nm and any other previously masked absorption peaks was not possible owing to non-specific absorption in the visible region. The haem *c* of the enzyme was shown to be extractable, and the spectra in methanol and dilute NaOH were identical with those of haemin and the ferri- and ferro-haematins respectively of horse-heart cytochrome *c*. There was no spectral evidence for the extraction of any other type of haem.

Spectra of the alkaline pyridine haemochromogen of the enzyme did not indicate the presence of absorption maxima that may be attributable to a derivative of the band material at 465nm.

The effects of the respiratory inhibitors and the chelating agents were similar to those reported for

partially pure hydroxylamine oxidase by Aleem & Lees (1963) and were consistent with the involvement of haem iron in the enzyme reaction. Neither CN^- nor azide produced spectral changes although they were both strong inhibitors of enzyme activity.

After this work was completed, Erickson & Hooper (1972) reported the preliminary characterization of a variant CO-binding haemoprotein, which was partially purified from the soluble fraction of *Nitrosomonas* cells. The existence of a 465nm-absorbing CO-combining haemoprotein in fractions containing soluble cytochrome *c* has been independently confirmed by Tronson *et al.* (1973).

There were noteworthy comparisons between the reported effects of hydroxylamine, azide and CN^- on the CO-combining haemoprotein (Erickson & Hooper, 1972) and their effects on hydroxylamine oxidase. No absorption at 465nm was apparent in dithionite-reduced spectra of either protein after prior addition of hydroxylamine. Azide had no effect on oxidized or reduced spectra of either protein. CN^- had no effect on spectra of hydroxylamine oxidase but caused shifts in both oxidized and reduced spectra of the CO-combining haemoprotein. NO_2^- had a profound effect on the formation and collapse of the band at 465nm maximum in hydroxylamine oxidase although it was stated not to be active as a ligand with the CO-combining haemoprotein (Erickson & Hooper, 1972). Similarity between the two proteins was noted with respect to the effects of aging, freezing and thawing and pH on the diminution of the band at 465nm, but the reduced band at 465nm maximum in hydroxylamine oxidase was not autoxidizable.

On the basis of available evidence it is possible that the soluble CO-combining haemoprotein of Erickson & Hooper (1972) is an uncombined form of the haemoprotein found in the hydroxylamine oxidase particle.

It is suggested that hydroxylamine oxidase is an enzyme particle possessing other, as yet unidentified, metabolic functions. Evidence to support this view is threefold: hydroxylamine only produces half-reduction of the cytochromes *b* and *c* in the enzyme; the enzyme has been separated by electrophoresis into at least three polypeptides, two containing haem, after treatment with a reducing agent in the presence of sodium dodecyl sulphate; finally, the presence of the dithionite-reduced 465nm-absorbing band in the enzyme cannot be said at present to be directly concerned with the oxidation of hydroxylamine since no natural reductant has as yet been found for it.

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References

Aleem, M. I. H. & Lees, H. (1963) *Can. J. Biochem.* **41**, 763–778

Appleby, C. A. (1968) in *Structure and Function of Cytochromes* (Okunuki, K., Kamen, M. D. & Sekuzu, I., eds.), p. 666, University Park Press, Baltimore

Elleway, R. F., Sabine, J. R. & Nicholas, D. J. D. (1971) *Arch. Mikrobiol.* **76**, 277–291

Erickson, R. H. & Hooper, A. B. (1972) *Biochim. Biophys. Acta* **275**, 231–244

Hewitt, E. J. & Hallas, D. G. (1959) *Nature (London)* **184**, 1485

Hewitt, E. J. & Nicholas, D. J. D. (1964) in *Modern Methods of Plant Analysis* (Linskens, H. F., Sanwal, B. D. & Tracey, M. V., eds.), vol. 7, p. 67, Springer-Verlag, Berlin

Hooper, A. B. (1968) *Biochim. Biophys. Acta* **162**, 49–54

Iwasaki, H. & Matsubara, T. (1972) *J. Biochem. (Tokyo)* **71**, 645–652

Iwasaki, H., Shidara, S., Suzuki, H. & Mori, T. (1963) *J. Biochem. (Tokyo)* **53**, 299–303

Kawai, K. (1968) *Anal. Biochem.* **25**, 264–270

Lam, Y. & Nicholas, D. J. D. (1969) *Biochim. Biophys. Acta* **180**, 459–472

Leigh, G. J. (1971) in *The Chemistry and Biochemistry of Nitrogen Fixation* (Postgate, J. R., ed.), p. 19, Plenum Press, London and New York

Newton, N. (1969) *Biochim. Biophys. Acta* **185**, 316–331

Paul, K.-G. (1950) *Acta Chem. Scand.* **4**, 239–244

Prakash, O. & Sadana, J. C. (1972) *Arch. Biochem. Biophys.* **148**, 614–632

Rees, M. K. (1968) *Biochemistry* **7**, 366–372

Rees, M. K. & Nason, A. (1965) *Biochem. Biophys. Res. Commun.* **21**, 248–256

Reid, M. S. & Bieleski, R. L. (1968) *Anal. Biochem.* **22**, 374–381

Renner, E. D. & Becker, G. E. (1970) *J. Bacteriol.* **101**, 821–826

Ritchie, G. A. F. & Nicholas, D. J. D. (1972) *Biochem. J.* **126**, 1181–1191

Tronson, D., Ritchie, G. A. F. & Nicholas, D. J. D. (1973) *Biochim. Biophys. Acta* **310**, 331–343

Warburg, O. & Christian, W. (1941) *Biochem. Z.* **310**, 384–421

Yamanaka, T. & Okunuki, K. (1963) *Biochim. Biophys. Acta* **67**, 379–393

Yoshida, T. & Alexander, M. (1964) *Can. J. Microbiol.* **10**, 923–926