Hydroxylamine as an Inhibitor and Terminal Acceptor in the Respiratory Chain of the Bacterium *Paracoccus denitrificans*

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Abstract. Three sites of inhibitory action of hydroxylamine were identified in the respiratory chain of anaerobically grown bacterium *Paracoccus denitrificans*. Terminal oxidases were blocked at concentrations of 10^{-4} to 10^{-3} mol . l^{-1}, and the inhibitor competed with artificial donor of electrons N, N, N′, N′-tetramethyl-1, 4-phenylenediamine. In the anaerobic part of the respiratory chain inhibition of nitrite reductase and apparently also nitric oxide reductase occurred, resulting in the increased accumulation of nitric oxide during denitrification. These effects together with the inhibition of terminal oxidases by nitric oxide are probably realized through switching the electron flow from oxygen to nitrogen terminal acceptors in the presence of hydroxylamine. By means of difference spectroscopy, the respiratory inhibitor mucidin and a cytochrome *c* — deficient mutant of *Paracoccus denitrificans*, hydroxylamine could be shown to serve also as a terminal acceptor of the cytochrome *c* region. Reduction of hydroxylamine to ammonia was at the same time accompanied by the formation of transmembrane electrical gradient. Hydroxylamine reductase was purified 123-fold from the periplasmatic cell fraction by FPLC; the product obtained showed the features of respiratory nitrite reductase of the cytochrome cd_1 type.

Key words: Hydroxylamine — Inhibition of terminal oxidases — Inhibition of nitric oxide reductase — Respiratory hydroxylamine reductase — *Paracoccus denitrificans*

Introduction

During anaerobic growth in the presence of nitrate, *Paracoccus denitrificans* synthesizes a branched respiratory chain which enables the utilization of nitrate, nitrite, nitrous oxide and probably also nitric oxide as electron acceptors (Ferguson 1987; Carr et al. 1989). In intact cells the electron flow from substrate to oxygen is strictly preferred to the flow to nitrogen terminal acceptors, consumption of which ceases under the influence of oxygen (John 1977; Alefounder and Ferguson 1982). In our previous experiments nitrate and nitrite underwent reduction also during aerobiosis if hydroxylamine was present at concentrations of the order of 10^{-4} mol.l^{-1} (Kučera et al. 1981, 1987). Under these conditions a potent inhibitor, which prevents oxygen utilization, has been shown to be generated from nitrite (Kučera et al. 1987).

The original aim of the experiments described in this paper was a more detailed study of the hydroxylamine inhibition of respiration and of the mechanism of switching of the electron flow from oxygen to nitrite. However, it turned out that hydroxylamine, besides its inhibitory effect, can also act as a terminal acceptor during anaerobic respiration. Therefore we also directed our attention to identify components of the respiratory chain that are involved in the newly-disclosed activity of hydroxylamine reductase.

Materials and Methods

Microorganisms and growth conditions. Paracoccus denitrificans (NCIB 8944) was obtained from Czechoslovak Collection of Microorganisms (CCM 982). Its anaerobic cultivation was static at 30 °C in an anaerobic growth medium containing 50 mmol.l^{-1} sodium succinate and 10 mmol.l^{-1} nitrate (Kučera et al. 1983). Cytochrome *c* — deficient mutant S 1659, kindly donated by Dr. H. W. van Verseveld, Vrije Universiteit Amsterdam, the Netherlands, was cultivated aerobically in a chemostat in a medium containing no nitrate, at a dilution rate of 0.4 h^{-1} (30 mmol.l^{-1} succinate). As this mutant is not able to grow anaerobically, formation of denitrification enzymes was induced by an additional 4 h incubation of aerobically cultivated cells in an anaerobic growth medium with nitrate (cell concentration 1.1 mg dry weight per ml).

Cell fractionation. Using the method based on lysozyme action and osmotic perturbation of spheroplasts (Witholt et al. 1976; Alefounder and Ferguson 1980) cells were suspended in a medium containing 200 mmol.l^{-1} Tris/HCl (pH 7.3), 0.5 mol.l^{-1} sucrose and 0.5 mmol.l^{-1} EDTA to yield a concentration of 30 mg dry weight per ml. After addition of lysozyme (8 mg per 1 g dry weight of cells) the mixture was twofold diluted with water, incubated for 30 min at 30 °C, and centrifuged (30 min, 35,000 × *g*). The supernatant containing periplasmic proteins was concentrated by ultrafiltration on a modified hemodialyzer (L. Macholán, unpublished). Cytoplasmic and membrane fractions were obtained by full lysis of sedimented spheroplasts in 5 mmol.l^{-1} Tris/HCl and by differential centrifugation (Burnell et al. 1975).

High-performance liquid chromatography. During the separation of hydroxylamine reductase a concentrated solution of periplasmic proteins was applied on a 10 × 14 mm column filled with Spheron Micro DEAE 300, particle size 12 μm (Lachema, Czechoslovakia). The column connected to a "Fast protein liquid chromatography" system (Pharmacia, Sweden) was previously equilibrated with 30 mmol.l^{-1} Tris/HCl (pH 7.3), and after sample application the elution was achieved by a gradient of 0—0.7 mol.l^{-1} NaCl at a flow rate of 3 ml.min^{-1}. Collected fractions containing the enzyme were concentrated on an Amicon apparatus (USA) with a PM 50 membrane and subjected to gel permeation chromatography on a TSK G3000 SWG column (LKB, Sweden), equilibrated with 30 mmol.l^{-1} Tris/HCl and 100 mmol.l^{-1} NaCl at a flow rate of 2.5 ml.min^{-1}. Chromatographic determination of cytochrome c_{550} in periplasmic fractions was performed on a Mono Q 5/5 column (Pharmacia, Sweden) equilibrated with 20 mmol.l^{-1} Tris/HCl (pH 7.3) and eluted by a gradient of 0—0.8 mol.l^{-1} NaCl at a flow rate of 0.6 ml.min^{-1}. The eluate protein concentration was continuously monitored at 280 nm. The cytochrome c_{550} content was proportional to the ratio of the peak area at 415 nm to the total area at 280 nm.

Optical measurements. Absorption spectra and absorbance time courses were recorded on a Shimadzu UV 3000 spectrophotometer (Japan). The changes of redox state of intracellular cytochromes c were followed in a dual-wavelength mode at the wavelength pair 550—540 nm. Complete reduction was achieved by the addition of solid dithionite, and 0 % reduction by hexacyanoferrate (III). The trapping of nitric oxide by extracellular cytochrome *c* was monitored as absorbance difference at 563—580 nm (Kučera 1989). 13-Ethylberberine fluorescence reflecting the membrane energization (Mikeš and Kovář 1981; Mikeš and Dadák 1983) was measured in a 1 cm cuvette using an Amin-co-Bowman spectrophotometer (USA) at excitation wavelength 420 nm (2 mm slit) and emission wavelength 520 nm (1 mm slit).

Measurement of enzyme activities. Oxygen consumption was measured with a Clark oxygen electrode in an electromagnetically-stirred closed vessel (volume 2 ml) equilibrated at 30 °C. Reduction of 2,6-dichlorphenolindophenol (DCIP), (measuring DCIP reductase) was monitored as the absorbance decrease at 600 nm (absorption coefficient 21 $mmol^{-1}.l.cm^{-1}$ cf. King 1967), and formation of Wurster's Blue by oxidation of N, N, N', N'-tetramethyl-1, 4-phenylenediamine (TMPD) (measuring TMPD oxidase) as absorbance increase at 563 nm. Hydroxylamine reductase activity of intact cells was determined in closed test tubes filled with nitrogen and containing 2 ml of 0.1 mol.l^{-1} sodium phosphate (pH 7.3), 20 mmol.l^{-1} succinate and bacteria being tested. The reaction was started with hydroxylamine. After incubation at 30 °C proteins were denaturated by 1 ml 10 % trichloroacetic acid, centrifuged and the remaining hydroxylamine was determined in the supernatant using the colorimetric reaction based on "indo-oxine" formation (Berg and Becker 1940) as described elsewhere (Jeter and Ingraham 1984). Reaction with the common Nessler reagent (Health Medical Supply Office, Brno) served for the determination of NH_4^+ ions produced. Hydroxylamine reductase was determined with the artificial donor of electrons in a cuvette covered with a rubber septum containing 4.5 ml of anaerobic 0.1 mol.l^{-1} sodium phosphate buffer (pH 7.3) and 0.5 mmol.l^{-1} benzyl viologen. After reduction to cation-radical by adding several μl of 1 % $Na_2S_2O_4$ solution in 10 % $NaHCO_3$ to reach A_{550} of aprox. 1.0, 5 mmol.l^{-1} hydroxylamine was added followed by the tested sample. The activity was calculated from the observed absorbance decrease using the absorption coefficient of 10.4 $mmol^{-1}.l.cm^{-1}$ (Kristjansson and Hollocher 1980). In the same anaerobic cuvettes the oxidation of 20 μmol.l^{-1} reduced cytochrome *c* (beef heart) was measured at 550—535 nm in the presence of 5 mmol.l^{-1} NH_2OH or 2 mmol.l^{-1} $NaNO_2$ (absorption coefficient 25.3 $mmol^{-1}.l.cm^{-1}$, Berry and Trumpower 1985). Nitrite reductase activity of intact cells was determined from nitrite consumption according to the method described previously (Kučera et al. 1988).

Protein analysis. Soluble proteins were determined by the method of Lowry et al. (1951); the modified biuret method (Szarkowska and Klingenberg 1963) was used for the determination of membrane bound proteins. Polycarylamide gel electrophoresis in the presence of sodium dodecylsulphate was performed according to Laemmli (1970). Gels were stained with 0.04 % solution of Coomassie Brilliant Blue in 25 % 2-propanol and 10 % acetic acid.

Computer programs. The influence of the inhibitor on the initial rates was evaluated using a SAPI I microcomputer and the Cleland program (Cleland 1979); the program was kindly translated from Fortran into Basic by Dr. Peč from the Faculty of Science, Palacký University, Olomouc, Czechoslovakia. A simple program for linear regression of the integrated Michaelis-Menten equation and for the time-dependence of substrate concentration were written by the authors.

Chemicals. TMPD, NADH and lysozyme were from Sigma (USA), cytochrome *c* from Koch-Light (Great Britain), benzyl viologen from Serva (FRG). Reduced cytochrome *c* was prepared by means of dithionite followed by gel filtration on Sephadex G 25 using FPLC. Mucidin was a generous gift of Dr. Musílek from the Microbiological Institute of the Czechoslovak Academy of Sciences, Prague. 13-Ethylberberine was supplied by Dr. Mikeš from our Department. Other chemicals of analytical purity were from Lachema (Czechoslovakia).

Results

Inhibition of membrane oxidase activity by hydroxylamine

Preliminary results showed that NADH and succinate dehydrogenase activities of membrane vesicles prepared from anaerobically grown cells of *P. denitrificans* were inhibited at milimolar concentrations of hydroxylamine. For example, with one of the preparations the specific activity of NADH oxidase (145 nmol $O_2 . min^{-1} . (mg\ protein)^{-1}$, 1 mmol $. l^{-1}$ NADH in 0.1 mol $. l^{-1}$ sodium phosphate pH 7.3, 30 °C) dropped to 80 % under the influence of 0.1 mmol $. l^{-1}$ hydroxylamine, and to 57 % or 34 % of the initial value for inhibitor concentration of 1 mmol $. l^{-1}$ or 10 mmol $. l^{-1}$ respectively. Fig. 1 shows the localization of the sites of the inhibitory action as determined by the method of differential spectrophotometry. In this case, succinate served as the respiratory substrate, the presence of malonate, an inhibitor of succinate dehydrogenase, prevented oxygen consumption during spectrum recording. The parallel reduction of cytochromes *b* and *c* with the increasing inhibitor concentration suggested that hydroxylamine acted in the region of terminal oxidases. The absorbance dependence at 550 nm (read from the obtained difference spectra) on hydroxylamine concentration was hyperbolic (right part of Fig. 1). Half of the maximum absorbance difference was achieved at hydroxylamine concentration of

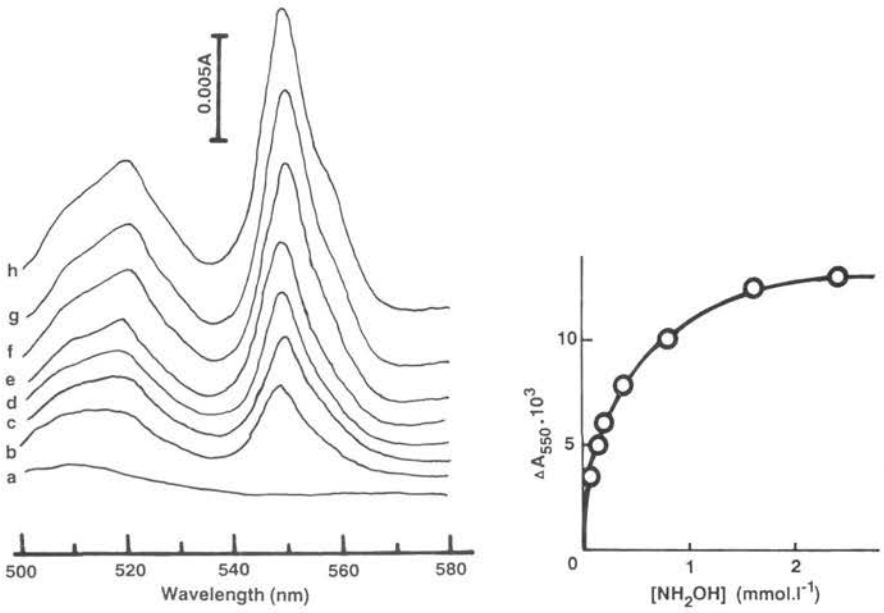

Fig. 1. Spectrophotometric titration of respiring membranes of *P. denitrificans* with hydroxylamine. Spectrophotometer cuvettes contained 3 ml of 0.1 mol.l^{-1} Na phosphate buffer (pH 7.3), 2 mmol.l^{-1} malonate and 2.1 mg membrane protein. Addition of succinate at a concentration of 10 mmol.l^{-1} to both cuvettes started the reaction at a rate of 22.4 nmol O_2.min^{-1} (determined independently by Clark electrode). After base-line recording (*a*), hydroxylamine was added to the measuring cuvette at a final concentration (mmol.l^{-1}) of: 0.07 (*b*), 0.13 (*c*), 0.2 (*d*), 0.4 (*e*), 0.8 (*f*), 1.6 (*g*) or 2.4 (*h*), and the resulting difference spectra were recorded. A_{550} represents the absorbance difference between the maximum at 550 nm and the connecting line of the adjacent minima.

0.26 mmol.l^{-1}. Hydroxylamine inhibited also the oxidation of the artificial donor of electrons TMPD, which supplies redox equivalents specifically to the cytochrome *c* region (Willison and John 1979). The linearized plot in Fig. 2 provides evidence that the inhibition is competitive. The inhibitor constant value, computed by non-linear regression (Cleland 1979), is 0.5 ± 0.1 mmol.l^{-1}.

As preliminary results had not excluded simultaneous action of hydroxylamine on the alternative oxidase of cytochrome *o* type, joining the respiratory chain of *P. denitrificans* presumably in the ubiquinone region (Kučera et al. 1984a, b; Parsonage et al. 1986), the possibility was tested with membranes prepared from the cytochrome *c* — deficient mutant S 1659. These membranes have (in contrast to the preparations of wild strain) high residual activities of NADH and succinate oxidases insensitive to bc_1 inhibitor mucidin. Fig. 3 illu-

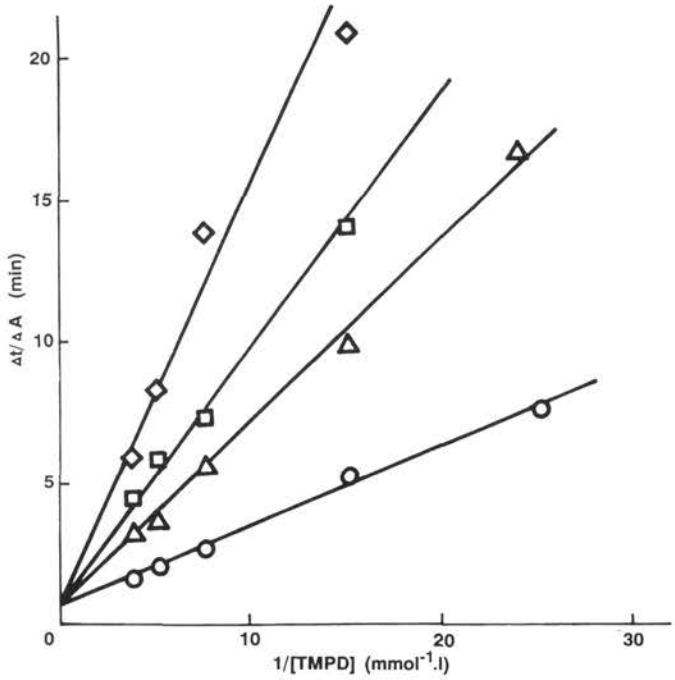

Fig. 2. Inhibition of TMPD oxidase activity of membranes by hydroxylamine. The reaction mixture contained 3 ml of 0.1 mol.l^{-1} Na phosphate (pH 7.3, 30 °C), 0.15 mg membrane proteins (○) and hydroxylamine at a concentration of 0.33 mmol.l^{-1} (△), 1.67 mmol.l^{-1} (□) or 3.3 mmol.l^{-1} (◇). The reaction was started by addition of different TMPD concentration. The initial rates were corrected for the rate of non-enzymic TMPD oxidation in the absence of membranes.

strates the obtained dependence of activities on hydroxylamine concentration. Concentrations corresponding to 50 % inhibition were mutually similar: 11.3 mmol.l^{-1} for NADH oxidase and 10.7 mmol.l^{-1} for succinate oxidase. The activity for NADH:DCIP oxidoreductase was nevertheless considerably less sensitive to hydroxylamine. The results illustrated in Fig. 3 suggest that hydroxylamine at milimolar concentrations inhibits also the alternative oxidase of *P. denitrificans*.

Redistribution of the electron flow from oxygen to nitrite and production of nitric oxide from nitrite under the influence of hydroxylamine

Hydroxylamine can cause preferential use of nitrite to oxygen if it inhibits cells nitrite reductase weaker than it does the oxidase(s). Meeting this condition in

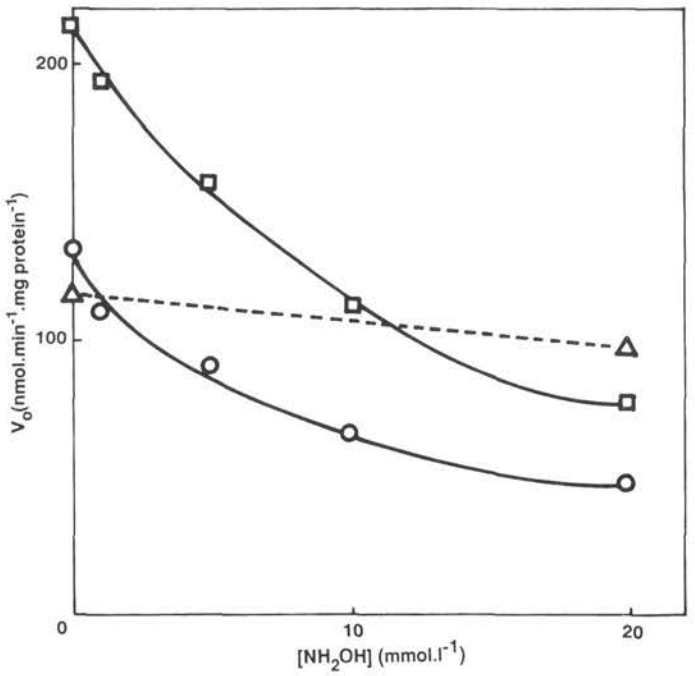

Fig. 3. Effect of hydroxylamine on the respiratory activities of membranes from cytochrome *c*-deficient mutant S 1659. The reaction mixture for measuring NADH oxidase (○) and succinate oxidase (□) contained 2 ml of 0.1 mol.l^{-1} Na phosphate (pH 7.3, 30 °C), 0.36 mg membrane proteins, 5 μg mucidin and 1 mmol.l^{-1} NADH or 10 mmol.l^{-1} succinate. v_o represents the initial rate of O_2 consumption. The activity of NADH:DCIP-oxidoreductase (△) was measured in 2 ml Na phosphate with 0.36 mg protein, 10 mmol.l^{-1} KCN, 33 mmol.l^{-1} DCIP and 1 mmol.l^{-1} NADH. v_o represents the initial rate of DCIP reduction corrected for non-enzymic reaction of DCIP with NADH or hydroxylamine (5.7 or 10 nmol DCIP.min^{-1}).

anaerobically-grown cells of *P. denitrificans* was checked by determination of I_{50} values for the inhibition of oxidase and/or nitrite reductase activities by hydroxylamine; values measured were 0.6 mmol.l^{-1} or 4.5 mmol.l^{-1} (0.1 mol.l^{-1} sodium phosphate (pH 7.3) plus 20 mmol.l^{-1} succinate, 30 °C). A more detailed study showed that the switching of the electron flow from O_2 to NO_2^- under the influence of increasing hydroxylamine concentrations was accompanied by the release of nitric oxide which could be trapped on exogenous cytochrome *c* added to the reaction mixture (Fig. 4). The fact that NO production from NO_2^- can be stopped by blocking the electron flow to nitrite reductase with mucidin (1 μg per mg dry weight of cells, not shown) confirms the enzyme nature of this process.

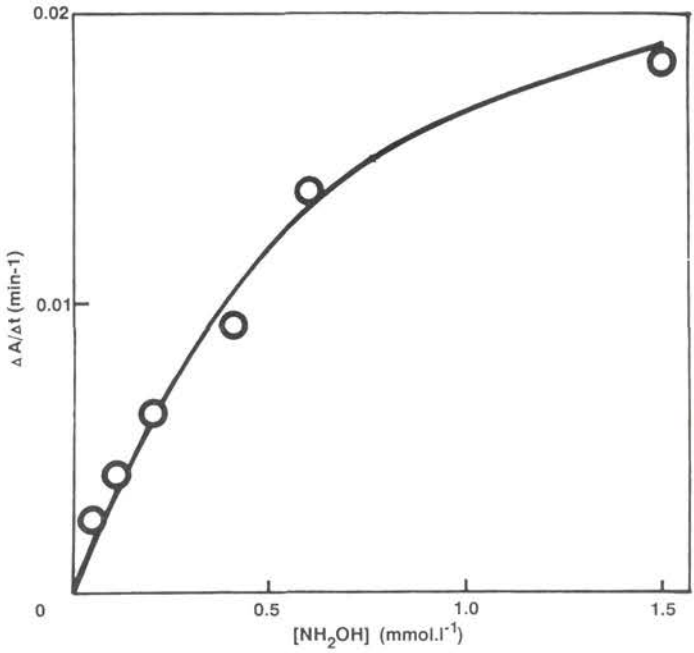

Fig. 4. The dependence of aerobic production of nitric oxide on hydroxylamine concentration. The reaction mixture (3 ml) contained 0.1 mol.l^{-1} Na phosphate (pH 7.3, 30 °C), 6.7 mmol.l^{-1} sodium succinate, 0.1 mmol.l^{-1} $NaNO_2$, 10.5 μmol.l^{-1} cytochrome *c* and the given concentration of hydroxylamine. The reaction was started by the addition of a suspension of anaerobically grown cells of *P. denitrificans* (0.9 mg dry weight). The rate of the absorbance increase at the wavelength pair 563–580 nm, which reflects formation of cytochrome *c* -NO complex, has been plotted on the vertical axis.

We considered it useful to check if the observed nitric oxide production was a mere consequence of the restricted flow to oxygen after the inhibition of terminal oxidases with hydroxylamine. In a positive case the formation of the complex measured could be expected to be even more intensive in the absence of oxygen while hydroxylamine would have no stimulating effect. These assumptions were not confirmed experimentally. From the record shown in Fig. 5 it can be seen that the maximum concentration of cytochrome *c* — NO complex formed during anaerobic nitrite reduction was approximately 5-times that measured in the presence of 1 mmol.l^{-1} hydroxylamine. Thus hydroxylamine affects the production of free nitric oxide also in another way than by inhibition of terminal oxidases, probably by simultaneous inhibition of nitric oxide reductase.

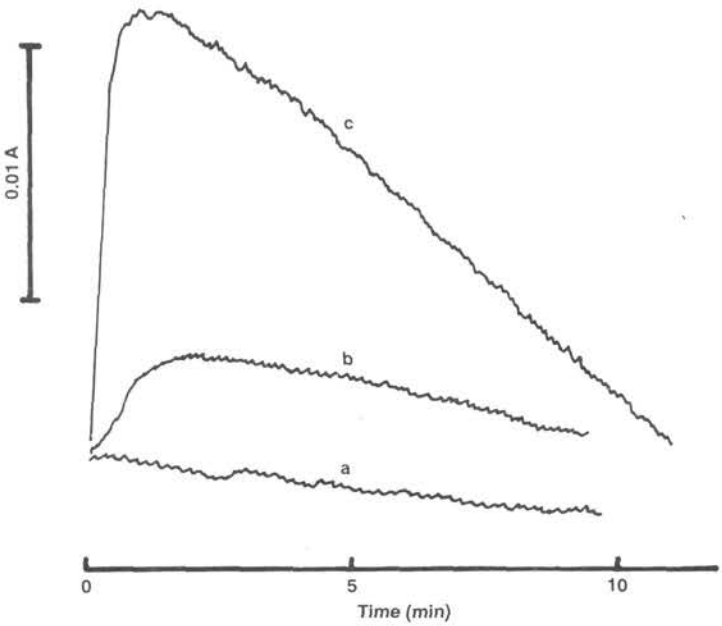

Fig. 5. Nitric oxide release during anaerobic denitrification in the presence of hydroxylamine. Both compounds were added to the closed cuvettes filled with nitrogen and containing 2 ml of 0.1 mol . l^{-1} phosphate buffer (pH 7.3) with 10 mmol . l^{-1} succinate, 27 μmol . l^{-1} cytochrome *c* and 7 mg dry weight of anaerobically-grown cells, at zero time (*a*) 2 μmol . l^{-1} hydroxylamine, (*b*) or 1.5 μmol . l^{-1} $NaNO_2$ (*c*), and the absorbance was measured at the wavelength pair 563—580 nm.

Hydroxylamine as a terminal acceptor during the anaerobic respiration

As we have repeatedly observed, anaerobically grown cells of *P. denitrificans* are able to metabolize external hydroxylamine. This activity has an average value of 30 nmol NH_2OH . min^{-1} . (mg dry weight of cells)$^{-1}$ (0.1 mol . l^{-1} sodium phosphate (pH 7.3) plus 20 mmol . l^{-1} sodium succinate, 30 °C), and the Michaelis constant of the order of 0.1 mmol . l^{-1} (Fig. 6). It is strongly inhibited by oxygen and does not occur in aerobically grown cells nor in membrane preparations. An analysis of the reaction mixture following the termination of the reaction revealed the presence of ammonia ions in a 1:1 ratio to the initial amount of hydroxylamine; this suggested some hydroxylamine-reductase activity. The following results support the connection of this activity with the respiratory chain:

(i) Hydroxylamine consumption was substantially inhibited by respiratory

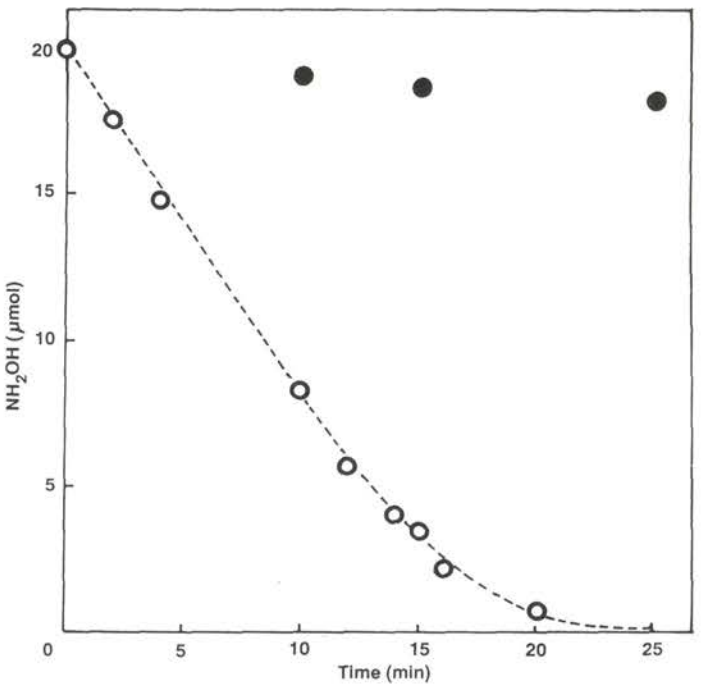

Fig. 6. The time course of hydroxylamine consumption and the effect of mucidin. The experiment was carried out in closed test tubes containing 4.9 mg dry weight of anaerobically grown cells (see Materials and Methods). The calculated curve drawn through experimental points corresponds to kinetic parameters K_M (NH_2OH) = 0.12 mmol . l^{-1} and V_{max} = 142 nmol NH_2OH . min^{-1}. Experimental data represented by the filled points were obtained in the presence of 10 μg mucidin.

inhibitors which block the flow of redox equivalents through the bc_1 segment of the respiratory chain, e. g by mucidin (Fig. 6).

(ii) Cytochrome *c* involved in the respiratory chain of *P. denitrificans*, is obviously essential for hydroxylamine reduction by physiological respiratory substrates. It was shown that cells of mutant S 1659, grown aerobically and then adapted in a growth medium with nitrate, did not reduce hydroxylamine with succinate but only with the artificial donor of electrons, the cation-radical of benzyl viologen (specific activity 17 nmol BV^+ per minute per mg dry weight). According to the results of chromatographic analysis the periplasmic fraction obtained from mutant cells had a hundred times less content of soluble cytochrome c_{550} compared with the periplasmic fraction from anaerobically grown cells of the wild strain.

(iii) Hydroxylamine caused reoxidation of cytochromes previously reduced by anaerobic incubation of cells in the presence of a respiratory substrate

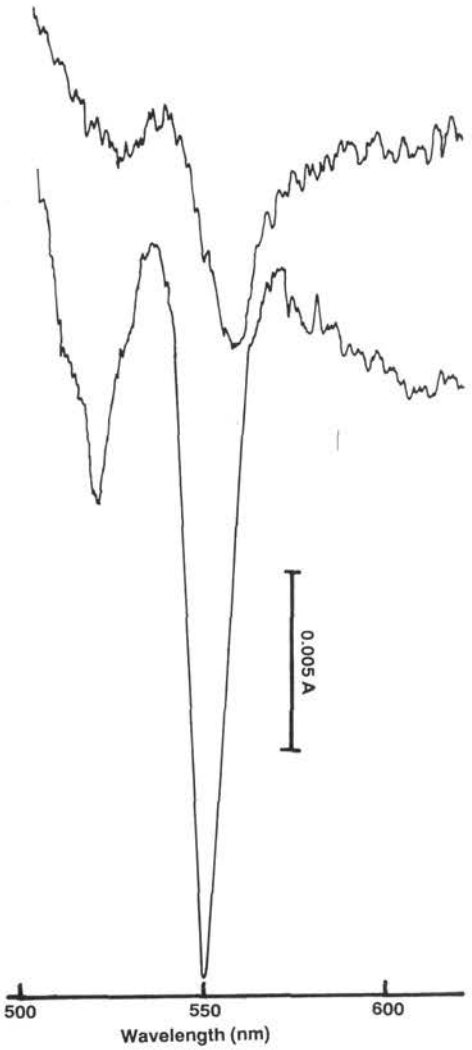

Fig. 7. Reoxidation of bacterial cytochromes of *P. denitrificans* by hydroxylamine. Both cuvettes contained 3 ml of 0.1 mol . l^{-1} Na phosphate buffer (pH 7.3) with 20 mmol . l^{-1} succinate and 5.6 mg anaerobically-grown cells. After oxygen consumption and cytochrome reduction the final spectrum was stored in the memory and the difference spectrum was recorded at a rate of 10 nm . min^{-1} (record *a*) after the addition of 3 μmol hydroxylamine. Record *b* was obtained after the addition of 20 μg mucidin.

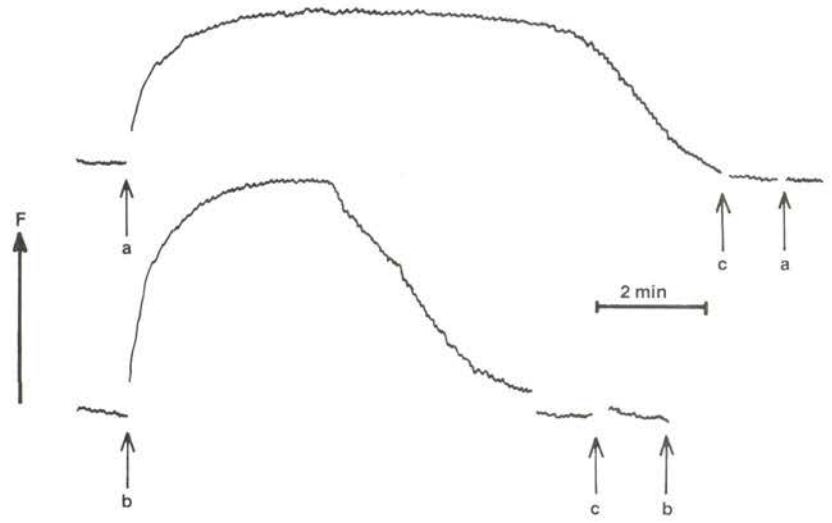

Fig. 8. Energization of cells of *P. denitrificans* during the consumption of hydroxylamine and/or nitrite. The mixture (3 ml) in the closed cuvette contained 0.1 mol.l^{-1} Na phosphate (pH 7.3), 20 mmol.l^{-1} succinate, 20 μmol.l^{-1} 13-ethylberberine and 4.8 mg dry weight of anaerobically-grown cells. The additions (arrows) were: (*a*) 0.75 μmol hydroxylamine, (*b*) 0.75 μmol $NaNO_2$ and (*c*) 10 nmol carbonyl cyanide *m*-chlorophenylhydrazone. For conditions of fluorescence (*F*) recording see Materials and Methods.

(succinate). Fig. 7 shows that subsequent additions of mucidin considerably deepened the reoxidation of cytochromes *c* (at a wavelength of 550 nm). This behavior of hydroxylamine is reminiscent of nitrite which acts in *P. denitrificans* as a terminal acceptor for cytochrome *c* region (Lam and Nicholas 1969a). The difference between redox changes of cytochromes caused by both substances is only of quantitative nature. As was found by measuring reduction time-courses in dual-wavelength mode, cytochromes *c* were reduced by 88 % during utilization of hydroxylamine, whereas the respective figure for nitrite utilization was only 35 % (not shown).

(iv) Reduction of hydroxylamine by *P. denitrificans* cells is accompanied by the formation of a transmembrane potential gradient (inside negative) which can be recorded with the positively charged fluorescent probe 13-ethylberberine. From the record in Fig. 8 it follows that the fluorescent response caused by hydroxylamine was two thirds of that of nitrite. Due to the linear dependence of the probe fluorescence on transmembrane potential (Mikeš and Kovář 1981; Mikeš and Dadák 1983) it can be assumed that a similar quantitative relation

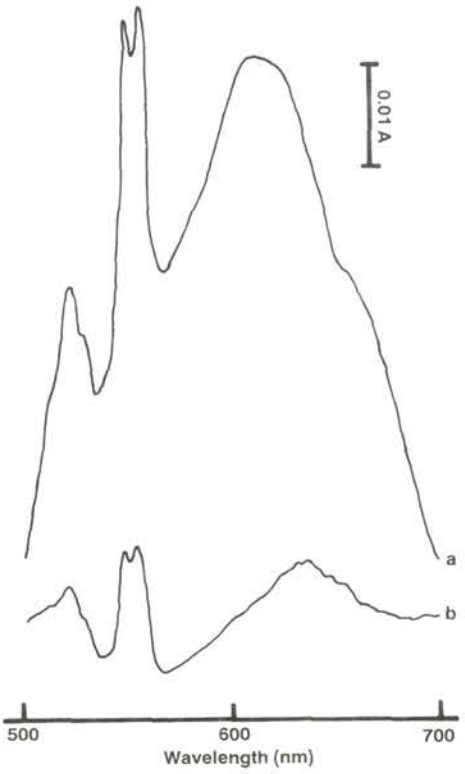

Fig. 9. Reoxidation of haems *c* and *d* of isolated hydroxylamine reductase by hydroxylamine. The difference spectrum (*a*) was recorded with the spectrum of the oxidized enzyme (0.24 mg protein in 1 ml of 0.1 mol . l^{-1} Na phosphate) stored in the memory, on bubbling the cuvette with nitrogen and reduction of its content by 10 μl of 2 % $Na_2S_2O_4$ in 10 % $NaHCO_3$. Spectrum *b* was recorded after the addition of 20 μmol hydroxylamine to the same cuvette. The control experiment showed that subsequent addition of dithionite did not change the shape of spectrum *b* (not shown).

holds also between the values of both potentials. The fluorescent changes were abolished by addition of the uncoupler (Fig. 8) which, however, did not affect reoxidation of cytochromes tested with the accpetors (not shown). Thus, the possibility of a probe response to changes in redox state or conformation of electron transporting components was ruled out.

Hydroxylamine reductase activity of dissimilatory nitrite reductase

In an effort to identify the respiratory component with hydroxylamine reductase activity the distribution of this activity was studied in subcellular fractions with reduced mammalian cytochrome *c* or benzyl viologen serving as the electron

donors (see Materials and Methods). Hydroxylamine reduction was catalyzed exclusively by the periplasmic fraction which was therefore subjected to further separation by ionex chromatography and gel filtration. In this way a single final fraction with a specific activity of hydroxylamine reductase of 3.4 nmol cytochrome $c \cdot s^{-1} \cdot (\text{mg protein})^{-1}$ or 49.7 nmol benzyl viologen $\cdot s^{-1} \cdot (\text{mg protein})^{-1}$ was obtained. The purification factor (the ratio of specific activities of hydroxylamine reductase in a final fraction and in the initial mixture of periplasmic proteins) was 123. The product of isolation showed at the same time comparable enhancement of the specific activity of nitrite reductase. Electrophoresis in polyacrylamide gel with SDS yielded only one major fraction corresponding to the molecular weight of 64,000. The result of gradual tiration with dithionite was a difference spectrum of the nitrite reductase of cytochrome cd_1 type (see Newton 1967). As it is clear from Fig. 9, hydroxylamine caused reoxidation of haemes *c* and *d*, suggesting its simultaneous reduction by the enzyme.

Discussion

The results presented herein enable reliable identification of two regions of hydroxylamine interaction with the respiratory chain of the bacterium *P. denitrificans*: terminal oxidase and nitrite reductase. Additionally, another interaction in the denitrification pathway can be assumed, probably at the nitric oxide reductase level.

Inhibition of membrane oxidases by hydroxylamine (Figs. 1 – 3) is unlikely to be unique to the bacterial system being studied. With isolated mitochondrial cytochrome oxidase in oxidized state (ferricytochrome aa_3) the redox reaction with hydroxylamine, forming ferrocytochrome a_3-NO complex (Blokzijl-Homan and van Gelder 1971), was proved by EPR. The observed competition of hydroxylamine with the electron donor has been interpreted as pointing to the binding of both substances to the oxidized enzyme form, and the sensitivity of membrane TMPD oxidase activity of to inhibition with nitric oxide (Kučera et al. 1986) suggests a similar inhibitory mechanism in *P. denitrificans*. Definitive evidence for the postulated redox mechanism of oxidase interaction with hydroxylamine requires a more detailed physico-chemical study of isolated bacterial enzymes.

The conversion of hydroxylamine to ammonia is usually connected with enzymes which catalyze the six-electron reduction of nitrite or sulphite, e. g. sulphite reductase (Siegel and Kamin 1971), NADH-dependent nitrite reductase (Jackson et al. 1982) and six haem cytochrome c_{552} (Kajie and Anraku 1986),

synthesized by the bacterium *E. coli*. With copper-containing dissimilatory nitrite reductases, which normally reduce nitrite to nitric oxide, hydroxylamine can act as the electron donor; on its reaction with nitrite, nitrous oxide is formed as the sole nitrogenous product (Iwasaki and Mori 1958; Iwasaki et al. 1963; Zumft et al. 1987). On the other hand, with dissimilatory nitrite reductases of cytochrome cd_1 type, such as that present in the denitrification pathway of the bacterium *P. denitrificans* (Newton 1967; 1969; Lam and Nicholas 1969b), hydroxylamine acts as an inhibitor of the reaction of the physiological donor (cytochrome *c*) with nitrite (Lam and Nicholas 1969b; compare with the inhibition observed in the present paper) and also as an electron acceptor (this paper; for a similar enzyme from *Pseudomonas aeruginosa* see Singh 1973). Thus both kinds of the dissimilatory nitrite reductases seem to exhibit marked specificity towards hydroxylamine as the substrate. Apparently, this specificity is related to the difference in the prosthetic groups of the enzymes operating at different redox potentials during the catalytic turnover.

With the known interaction sites of hydroxylamine with the respiratory chain of *P. denitrificans*, individual steps leading to the switching of the electron flow from oxygen to nitrite and to aerobic denitrification (Kučera et al. 1987) can be discussed in more detail. According to the results illustrated in Fig. 1, inhibition of terminal oxidases by hydroxylamine raises the degree of reduction of cytochromes *c*, and thus also the influx of redox equivalents to nitrite reductase which joins the respiratory chain in this region (Lam and Nicholas 1969a). As hydroxylamine probably also inhibits nitric oxide reductase (see above), larger amounts of nitric oxide formed during nitrite reductase reaction are accumulated in the medium (Fig. 4). Terminal oxidase of the respiratory chain is strongly influenced already by micromolar concentrations of NO (Kučera et al. 1986), with the result that the electron flow to oxygen is soon completely blocked and only reduction of nitrogen terminal acceptors can occur.

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Final version accepted April 9, 1990