JOURNAL OF BACTERIOLOGY, Mar. 1982, p. 816–823
0021-9193/82/030816-08$02.00/0
Vol. 149, No. 3

Modulation by Copper of the Products of Nitrite Respiration in *Pseudomonas perfectomarinus*

TERUO MATSUBARA, KURT FRUNZKE, AND WALTER G. ZUMFT*
Department of Botany, University Erlangen-Nürnberg, Schlossgarten 4, 8520 Erlangen, Federal Republic of Germany

Received 29 June 1981/Accepted 22 September 1981

A synthetic growth medium was purified with the chelator 1,5-diphenylthiocarbazone to study the effects of copper on partial reactions and product formation of nitrite respiration in *Pseudomonas perfectomarinus*. This organism grew anaerobically in a copper-deficient medium with nitrate or nitrite as the terminal electron acceptor. Copper-deficient cells had high activity for reduction of nitrate, nitrite, and nitric oxide, but little activity for nitrous oxide reduction. High rates of nitrous oxide reduction were observed only in cells grown on a copper-sufficient (1 μM) medium. Copper-deficient cells converted nitrate or nitrite initially to nitrous oxide instead of dinitrogen, the normal end product of nitrite respiration in this organism. In agreement with this was the finding that anaerobic growth of *P. perfectomarinus* with nitrous oxide as the terminal electron acceptor required copper. This requirement was not satisfied by substitution of molybdenum, zinc, nickel, cobalt, or manganese for copper. Reconstitution of nitrous oxide reduction in copper-deficient cells was rapid on addition of a small amount of copper, even though protein synthesis was inhibited. The results indicate an involvement of copper protein(s) in the last step of nitrite respiration in *P. perfectomarinus*. In addition we found that nitric oxide, a presumed intermediate of nitrite respiration, inhibited nitrous oxide reduction.

The anaerobic reduction of nitrate provides an alternative respiratory energy metabolism for facultative and a few obligate anaerobic bacteria (9, 10, 13). The process can be terminated at the level of nitrite or proceed further as nitrite respiration (denitrification) with the formation of dinitrogen. Nitrite respiration is coupled to proton translocation (20, 25) and to electron transport phosphorylation at the intermediate redox pairs nitrite/nitrous oxide (N_2O) and N_2O/dinitrogen (18). Nitric oxide (NO) is also considered an intermediate, before the formation of N_2O (7, 26). This allows a presumed sequence of nitrite respiration according to the formal oxidation state of the reactants (24; for review see reference 27): $NO_2^-(+3) \rightarrow NO(+2) \rightarrow N_2O(+1) \rightarrow N_2(0)$. Alternative pathways have been proposed with the same intermediates, where NO is not in the main sequence (32), is part of a parallel route (36), or is in equilibrium with an enzyme-bound intermediate (7).

Of maximally three terminal oxidoreductases in nitrite respiration, only nitrite reductase has been highly purified and studied as an isolated entity. The enzyme is either a copper protein (EC 1.7.99.3) (15) or a four-heme metalloprotein (EC 1.9.3.2) (34), depending on the organism. The metal requirement of NO reductase is still not established (see below). N_2O reductase presumably is also a metalloenzyme because of its inhibition by azide, cyanide, and CO (19, 24), acetylene (6), and sulfide (31) and the analogy to N_2O-reducing nitrogenase (11). A recent study of metal-dependent growth of *Alcaligenes* sp. NCIB 11015 has extended the role of copper to the level of N_2O reduction (16). Search for possible electron donors for this process showed the involvement of *c*- and *b*-type cytochromes (2, 23). Despite continuing efforts, N_2O reductase itself has proven elusive and difficult to handle in a cell-free system. Cell lysates either were inactive (23) or had only low activities (28), but for osmotically shocked spheroplasts of *Paracoccus denitrificans* high rates of N_2O reduction were recently reported with dithionite-reduced viologens as electron donors (19).

To identify the presumed participation of a transition element in N_2O reduction, we studied the growth of the marine denitrifier *Pseudomonas perfectomarinus* on a metal-deficient medium. We deemed it necessary to find an analytical and spectroscopic tool for the study of this enzyme, even in the absence of catalytic activity, because the published assay methods (19, 28) and other attempts failed to stabilize cell-free N_2O reduction. *P. perfectomarinus* appeared particularly suitable for this study, since it vigorously evolves dinitrogen from nitrate with aspar-

agine as the only oxidizable carbon source (29), and the entire pathway of nitrite respiration has been studied at least to a preliminary extent in this bacterium (1, 4, 5, 28, 37, 38).

In this contribution we identify a requirement of copper for N_2O reduction in *P. perfectomarinus*. Growth with different electron acceptors in the presence or absence of copper permitted specific downshifts of partial reactions of nitrite respiration and dramatically affected the products being formed. Copper-deficient cells thus terminated transiently nitrite respiration at the level of N_2O instead of dinitrogen. A rapid stimulation of the weak residual N_2O-reducing activity by exogenous copper, under conditions of inhibited protein synthesis, indicated ongoing biosynthesis of apoprotein under copper deficiency. We also provide evidence that under certain conditions NO is a potent inhibitor of N_2O reduction.

MATERIALS AND METHODS

Organism and growth conditions. *P. perfectomarinus* (ATCC 14405) was grown in the following basal medium (components in grams per liter): L-asparagine·H_2O, 2.0; trisodium citrate·$2H_2O$, 7.0; KH_2PO_4, 2.0; $CaCl_2 \cdot 2H_2O$, 0.2; $MgSO_4 \cdot 7H_2O$, 4.0; $FeCl_3 \cdot 6H_2O$, 0.02; and NaCl, 20. The pH was adjusted to 6.6 with 5 N NaOH. The growth temperature was 30°C.

All media used to determine the effect of copper were treated at pH 6.6 with 1,5-diphenylthiocarbazone (dithizone) to chelate copper and, to a certain extent, other contaminating metals (14, 16). A 10-fold-concentrated basal medium from which NaCl was omitted was stirred vigorously for 2 h with 20 mg of dithizone in 3 ml of $CHCl_3$ per liter of medium. Undissolved dithizone was removed by filtration; dithizonates and dissolved, unreacted dithizone were removed by extracting the medium three times with 100 ml of $CHCl_3$ per liter. The medium was then diluted 10-fold and supplemented with NaCl and, if necessary, $NaNO_3$ (1 g/liter), and the entire treatment with dithizone was repeated. When required, copper or other trace elements were added after the second treatment with dithizone at the following final concentrations: $CuCl_2$, 1 μM; $ZnSO_4 \cdot 7H_2O$, 1.05 μM; $MnCl_2 \cdot 4H_2O$, 0.9 μM; $CoCl_2 \cdot 6H_2O$, 1.0 μM; $NiSO_4 \cdot 6H_2O$, 0.99 μM; and $Na_2MoO_4 \cdot 4H_2O$, 0.96 μM.

For growth on N_2O as electron acceptor two loops of bacteria from an agar slant were transferred to 200 ml of nitrate-free, dithizone-treated medium in a 500-ml Erlenmeyer flask. The culture was incubated aerobically for 20 to 24 h on a gyrotory shaker (120 rpm). These cells were used as inoculum for 1.8 liter of chelator-treated medium, with or without copper, in a 2-liter round flask. Before inoculation, the medium was saturated with N_2O. Cultures were stirred magnetically and sparged with N_2O at a flow rate of about 60 ml/min.

For anaerobic growth on nitrate as electron acceptor the experimental procedure was identical to that for the N_2O cultures, except that the dithizone-treated, copper-supplemented and unsupplemented media contained 1 g of $NaNO_3$ per liter instead of N_2O. The cultures were sparged with argon to maintain anaerobic conditions.

Cells were harvested by centrifugation for 15 min at 4,080 × *g*. The cell paste was resuspended in 50 mM potassium phosphate buffer (pH 7.0) and used immediately for experiments.

Assays of partial reactions of nitrite respiration. Nitrite, NO, and N_2O reductions were measured by gas chromatography as gas formation from nitrite, gas formation from NO or N_2O, or as uptake of NO or N_2O, respectively. The conditions for gas analysis were as reported previously (38). An aged column of Porapak Q (80-100 mesh; length, 2.5 m) was used at 40°C, and a helium flow rate of 30 ml/min was used. Reaction mixtures of 3 ml in stoppered 12-ml serum vials contained samples of cell suspensions, 150 μmol of sodium lactate, 135 μmol of potassium phosphate buffer (pH 7.0), and 40 μmol of $NaNO_2$. The vials were filled with helium on a vacuum line and incubated horizontally at 30°C in a reciprocal shaking water bath at 100 oscillations per min. Gas samples of 100 μl were withdrawn at regular intervals from the head space of the vials with a gas-tight syringe and analyzed by gas chromatography. For assays of NO and N_2O reduction, nitrite was replaced by 10% (vol/vol) NO or N_2O in the gas phase of the vials.

Spectrophotometric measurements. Difference spectra of cells grown under various conditions were recorded at room temperature with a microprocessor-equipped, dual-wavelength spectrophotometer operating in the subtraction mode. The reaction mixture of 2 ml in a stoppered cuvette of 1-cm light path contained intact cells (equivalent to approximately 1 mg of protein per ml), 90 μmol of potassium phosphate buffer (pH 7.0), and 100 μmol of sodium lactate. The cuvette was filled with helium on a vacuum line. Twenty microliters of anaerobic 1 M KNO_3 or $NaNO_2$ or 0.5 ml of N_2O was added by syringe to start the reaction.

Analytical procedures. Protein concentration was measured the method of Lowry et al. (21). Nitrite was estimated as azo dye (30). In addition to gas chromatographic evidence, N_2O was identified by its infrared absorption spectrum (courtesy of H.-J. Wunschel, Linde AG).

Materials. N_2O was from Matheson (Belgium) at a minimal purity of 99.5% in the liquid phase. NO was purchased from Linde (Höllriegelskreuth, Germany) and contained approximately 0.5% N_2O. Alternatively, NO was synthesized from $NaNO_2$ in this laboratory and stored in gas-tight glass containers. This gas was virtually free of N_2O. 1,5-Diphenylthiocarbazone was from Sigma Chemical Co. (München). Chloroform was from Merck (Darmstadt) at a maximal level of contaminating metals (Fe) of 0.1 μg/g of solvent. The residual copper level was 10 ng/g of solvent. Copper(II) chloride was obtained from Ventron (Karlsruhe) at 99.999% purity (metals basis). L-Asparagine was of biochemical grade; other chemicals were of analytical grade (Merck, Darmstadt).

RESULTS

Bacterial growth. *P. perfectomarinus* grew well anaerobically with nitrate as the terminal electron acceptor in the dithizone-treated medium with or without added copper (Fig. 1). The

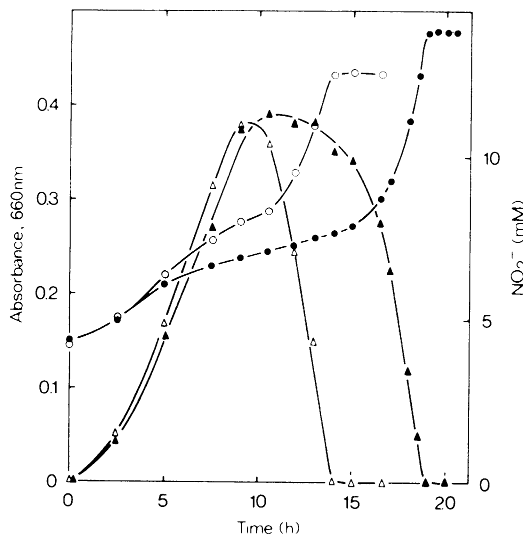

FIG. 1. Comparison of nitrate-dependent growth of *P. perfectomarinus* in copper-containing and copper-deficient media. Symbols: absorbance increase (●) and nitrite concentration (▲) in the copper-containing medium; absorbance increase (○) and nitrite concentration (△) in the copper-deficient culture.

growth curve showed a diauxic phase similar to that of nitrate-respiring *Pseudomonas stutzeri* (17). The first growth phase was due to nitrate reduction, and nitrite accumulated in the medium at a concentration approximately equal to the initial amount of nitrate added. After a lag period, nitrite disappeared from the medium, and a second growth phase occurred. The lag period in the medium with copper was longer than that in the medium without it. Growth in the nitrate-dependent phase also appeared to be slightly inhibited by 1 μM copper, but the final growth yield was greater in the medium with copper. In three experiments with nitrate as electron acceptor we found that the ratio of the growth yields from the medium without copper to that with copper was 0.75 to 0.85.

In contrast to nitrate, *P. perfectomarinus* grew anaerobically on N_2O only when copper was added after the dithizone treatment of the medium (Fig. 2). No growth or only a negligible increase in optical density was observed in a copper-deficient medium. At pH 6.6 the extraction procedure also removes other trace elements to different extents (14). Adding several of these metals or increasing the concentration over their adventitious amount did not initiate anaerobic growth under N_2O.

Levels of respiratory activities. Table 1 shows the activity levels of partial reactions of nitrite respiration in cells which were grown with or without copper on different terminal electron acceptors. The activities of nitrite and NO reduction in nitrate-grown cells were not affected by the absence of copper. In striking contrast to this, the reduction of N_2O was diminished by 80 to 90%. When copper was added to copper-deficient cells, a rapid reconstitution of N_2O reduction occurred under conditions where protein synthesis was inhibited by rifampicin (Table 2). Because the reconstitution was rapid and required extremely small quantities of copper, it is likely that the residual activity of N_2O reduction observed in copper-deficient cells was due to fortuitous incorporation of copper during cell harvest and assay procedures rather than to incomplete removal from the medium (dissociation constant of the secondary copper dithizonate, 10^{-23} mol^2/liter^2; partition coefficient of dithizone between $CHCl_3$ and water 2.5×10^7 [14]).

Cells grown on N_2O had high N_2O reductase activity (Table 1), but reduced nitrite and NO

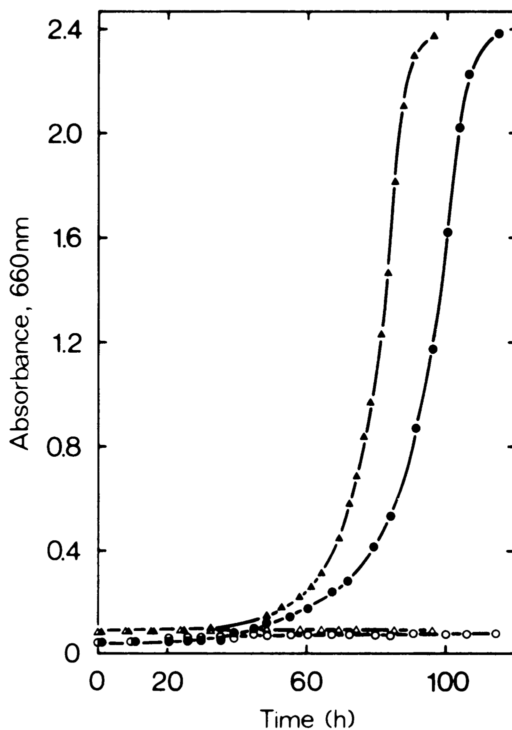

FIG. 2. Effect of cupric ions and other transition metals on the N_2O-dependent growth of *P. perfectomarinus*. A dithizone-treated medium was supplemented with 1 μM $CuCl_2$ (▲) or used without any further additions (△). A second batch of medium was supplemented with copper and additionally with manganese, cobalt, nickel, zinc, and molybdenum (●) or used without copper but with the addition of these trace elements (○). The trace element concentration as well as growth conditions are specified in the text.

TABLE 1. Activities and associated cytochrome steady-state oxidation levels of partial reactions of nitrite respiration in cells of *P. perfectomarinus* grown on nitrate or N_2O in the presence or absence of copper

Growth conditions (anaerobic)	Terminal electron acceptor					
	Nitrite		NO		N_2O	
	NO_2^--reduction[a]	Oxidation level[b]	NO reduction[a]	Oxidation level[b]	N_2O reduction[a]	Oxidation level[b]
With NO_3^-, with Cu	3.06	44.7	1.88	49.0	5.31	64.0
With NO_3^-, without Cu	3.89	41.8	2.04	46.0	0.92	17.8
With N_2O, with Cu	0.04	7.6	0.60	15.0	2.86	58.5
With N_2O, without Cu	No growth					

[a] Denitrifying activities are expressed as initial rates of substrate reduced in nanokatals per milligram of protein.

[b] The steady-state oxidation levels of total cytochromes are expressed as percent oxidation by the different electron acceptors of lactate-reduced whole cells. Cytochrome oxidation was measured as absorbance at 420 nm minus absorbance at 450 nm. All figures are related to the control value (100%) of dithionite-reduced minus air-oxidized cells.

only at about 20 to 30% of the rate of nitrate-grown cells.

During the reduction of nitrate, nitrite, NO, and N_2O by *P. perfectomarinus*, cytochromes were oxidized as in other nitrite-respiring bacteria (2, 16, 23). Figure 3 shows the spectral changes for nitrate-grown, copper-deficient cells upon addition of different electron acceptors. Nitrate and nitrite caused a substantial oxidation of *c*-, *b*-, and *cd*-type cytochromes in lactate-reduced cell suspensions. The addition of NO did not cause changes typical for cytochrome *cd* in the difference spectrum beyond 600 nm, although the peak at 467 nm was present. There was also a shoulder in the Soret band at 425 nm which was not observed on addition of other substrates. The addition of N_2O oxidized only a very small fraction of *c*- and *b*-type cytochromes and did not oxidize cytochrome *cd*.

Cells grown anaerobically in the presence of copper on nitrate or on N_2O showed spectral changes qualitatively similar to the examples in Fig. 3 on addition of various electron acceptors, but had different activity levels for nitrate and nitrite reduction. To provide a comparative index, the steady-state levels of cytochrome oxi-

TABLE 2. Reconstitution of N_2O reduction by the addition of copper (3 μM) to copper-deficient, nitrate-grown cells

Growth mode of cells	Incubation time with Cu (min)	Sp act[a]	Oxidation level[b]
With Cu		3.83	60
Without Cu	0	0.80	19.7
	10	1.19	ND[c]
	30	1.55	ND
	60	2.44	55.6

[a] N_2O formation from nitrite was measured as described in the text. Rifampicin (30 μg) was included in the assay mixture to inhibit protein synthesis. The specific activity is expressed as average N_2O formation from five experiments as nanokatals per milligram of protein.

[b] Steady-state oxidation levels of total cytochrome content as specified in Table 1.

[c] ND, Not determined.

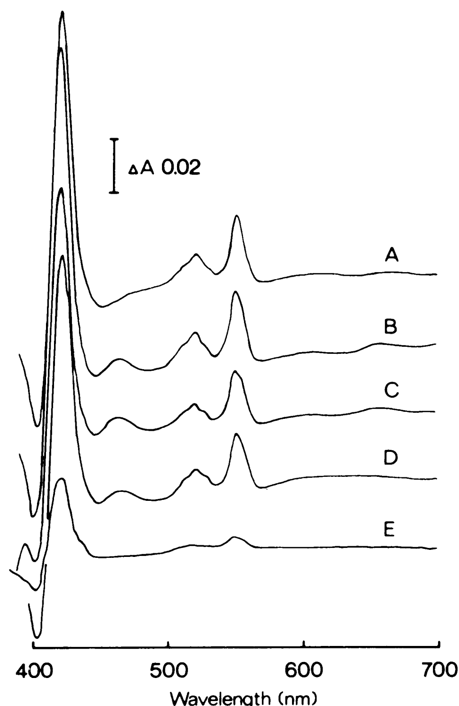

FIG. 3. Reduced minus oxidized difference spectra of whole cells of *P. perfectomarinus*. The cells were grown on nitrate in a copper-deficient medium. A, Dithionite-reduced minus air-oxidized cells; B, lactate-reduced minus nitrate-oxidized cells; C, lactate-reduced minus nitrite-oxidized cells; D, lactate-reduced minus NO-oxidized cells; and E, lactate-reduced minus N_2O-oxidized cells. Spectrum A was attenuated by a factor of 0.5. For experimental details see the text.

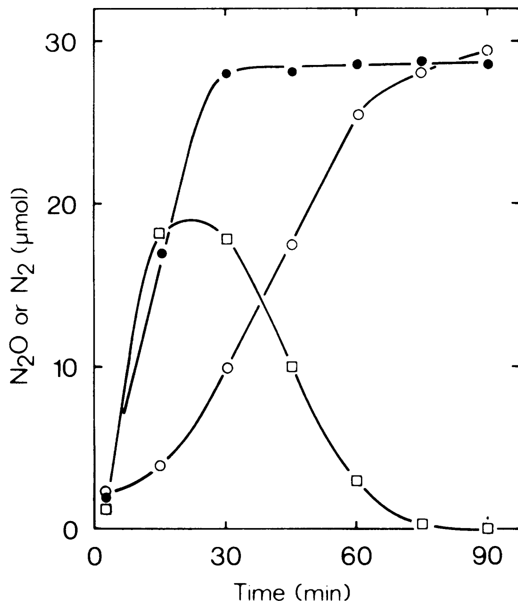

FIG. 4. Production of N_2O from nitrite by copper-deficient cells of *P. perfectomarinus*. Cells were grown anaerobically on nitrate in a copper-deficient medium. A sample of cell suspension, corresponding to 12.6 mg of protein, was supplied with 135 μmol of phosphate buffer (pH 7.0), 150 μmol of sodium lactate, and 50 μmol of sodium nitrite. The evolved gasses were identified and quantitated by gas chromatography. In addition, N_2O was identified by its infrared spectrum in the gas phase (ν = 2,224 cm^{-1}). Symbols: (□) N_2O; (○) dinitrogen; (●) dinitrogen evolution as the only gas from nitrite by a cell suspension (12.7 mg of protein) which was grown on nitrate in the presence of copper.

dation are listed in Table 1. It is evident that the attainable levels of oxidation by the different substrates paralleled the enzymic activities measured by gas chromatography. We further found that the total content of cytochromes in nitrate-grown cells was unaffected by copper. N_2O-grown cells, however, had a conspicuously low content of cytochrome *cd*, as evidenced by the absence of any long-wavelength absorption and the peak around 470 nm (spectrum not shown).

Products of nitrite respiration. *P. perfectomarinus* grown on nitrate in the presence of copper reduced nitrite completely to dinitrogen. During this process no gaseous nitrogenous oxides detectable by gas chromatography were liberated from a cell suspension (Fig. 4). In contrast to this was the pathway of nitrite respiration in copper-deficient cells. Approximately 75% of nitrite supplied to those cells was initially converted to N_2O and accumulated in the reaction vial (Fig. 4). Dinitrogen evolution had a lag phase and proceeded then at a much lower rate than in copper-sufficient cells until all of the N_2O was consumed.

Nitrate-grown, copper-deficient and -sufficient cells reduced NO with identical rates, but yielded different products (Fig. 5). Dinitrogen was the product of NO reduction in cells grown with copper, whereas predominantly N_2O was evolved from copper-deficient cells.

Inhibition of N_2O reduction by NO. Cells grown on N_2O in the presence of copper had low activities for nitrite and NO reduction, but high activity for N_2O reduction (cf. Table 1) and surprisingly discriminated between nitrite and NO in the formation of different products. Nitrite was converted to dinitrogen, whereas NO was reduced only to N_2O. This effect was due to inhibition of N_2O reduction by NO in this particular type of cells (Fig. 6). A concentration of 1% NO in the gas phase (<20 μM in the liquid phase at 30°C) inhibited N_2O reduction completely and irreversibly. This strong inhibition by NO was observed only in cells with a low activity level of NO reduction. When the activities of nitrite and NO reduction were high, as in cells grown

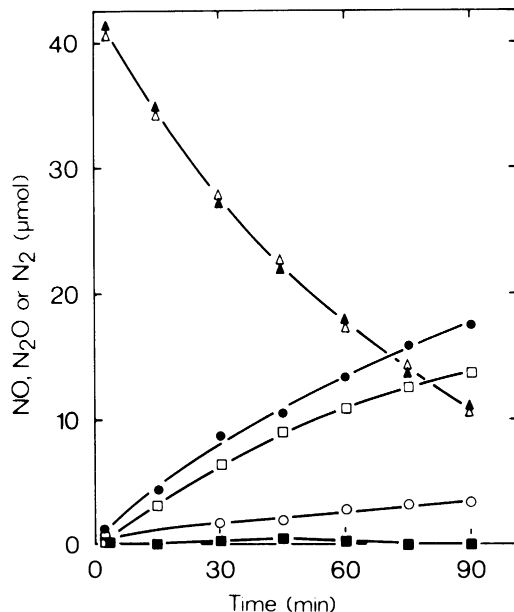

FIG. 5. Kinetics of the reduction of NO by *P. perfectomarinus* grown with or without copper. Cells were derived from an anaerobic nitrate culture in the absence (△, □, ○) or in the presence (▲, ■, ●) of copper. The disappearance of NO (△, ▲) in both cell types was followed concomitantly with the production of N_2O (□, ■) and dinitrogen (○, ●). The reaction was started by the addition of 41.7 μmol of NO, and the first measurements were taken 2 min after the start of the reaction. The cell mass in each assay was equivalent to 9 mg of protein. Other conditions were as described in the text.

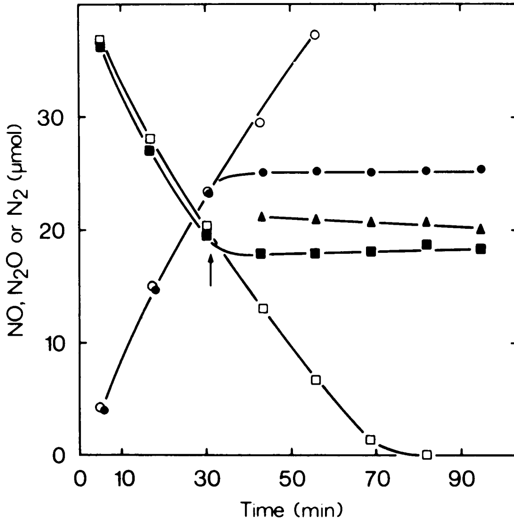

FIG. 6. Inhibition of N_2O reduction by NO in whole cells of *P. perfectomarinus*. Cells were grown anaerobically in the presence of copper and other trace elements at the expense of N_2O as electron acceptor. The reaction mixture contained a cell suspension which was equivalent to 4.0 mg of protein. The time course of N_2O reduction (■) and that of dinitrogen evolution (●) was followed by gas chromatography. The first gas sample was taken at 5 min. At 31 min (arrow), 4.17 μmol of NO (▲) was injected into the reaction vial, and the change in concentration of this gas in the head space was also monitored. In a parallel assay where no NO was added, N_2O reduction (□) and N_2 evolution (○) proceeded unaffected.

anaerobically with or without copper on nitrate (cf. Table 1), the inhibitory effect was less pronounced, and more than 10-fold-higher concentrations of NO were required for complete inhibition of N_2O reduction.

DISCUSSION

If one tentatively accepts the linear reaction sequence outlined above, three terminal oxidoreductases participate in nitrite respiration with N_2 as the end product. *P. perfectomarinus* contains soluble and membrane-bound cytochrome *cd*, and not a copper-type protein, as nitrite reductase (37, 38). Furthermore, we did not detect azurin, a low-molecular-weight copper protein, in this organism (data not shown); azurin is assumed to be interchangeable with cytochrome c_{551} as a physiological electron donor for cytochrome *cd* (12). Yamanaka et al. (33) observed doubling of the level of cytochrome *cd* in *Pseudomonas aeruginosa* on addition of copper to an anaerobic growth medium. Since aeration of the medium without copper produced cells with a still higher level of this enzyme, a specific requirement for copper appears not to be established for this reaction step in organisms depending on cytochrome *cd*.

P. perfectomarinus also has a membrane-bound NO reductase (38). A soluble enzyme was reported to be associated with heme-iron (4, 5). Earlier, the enzyme was claimed to depend on copper in *Pseudomonas stutzeri* (3), but NO reductase activity of *P. aeruginosa* was unaffected by this metal (C. A. Fewson and D. J. D. Nicholas, Biochem. J. **78**:9P–10P, 1961). We have shown that in *P. perfectomarinus* the rates of NO reduction are the same in cells grown with or without copper, and therefore we consider an involvement of this element in NO reduction unlikely. We thus have a case where two of three possible reactions of nitrite respiration are independent of copper, which allows a nearly unequivocal assignment of the copper-requiring step.

Iwasaki et al. (16) have recently established a requirement for copper for N_2O reduction in *Alcaligenes* sp., which has a copper-containing nitrite reductase, and thus depends on at least two partial reactions of nitrite respiration on the same element. These authors also found that *Alcaligenes faecalis* IAM 1015, a cytochrome *cd*-containing nitrite respirer, did not grow at the expense of N_2O in the absence of copper. The strict requirement for copper for N_2O reduction in *P. perfectomarinus* was established by the following observations: (i) no growth without copper on N_2O; (ii) substantial decrease of activity for N_2O reduction in nitrate-grown, copper-deficient cells; (iii) lower growth yield under copper deficiency; (iv) initial formation of N_2O, instead of N_2, from nitrite or NO by nitrate-grown, copper-deficient cells; and (v) rapid reconstitution of N_2O reduction in copper-deficient cells on addition of small amounts of copper. To the best of our knowledge, a clearly defined shift in the products of nitrite respiration by variation of a single physiological growth factor has not been shown previously for any nitrite-respiring species. Nevertheless, there is a wide variety of general factors that influence the relative amount of N_2O produced during nitrite reduction (8; J. M. Tiedje et al., Adv. Chem. Ser., in press). As a more discriminatory factor, the accumulation of N_2O in the presence of acetylene was recently shown with *P. perfectomarinus* (1, 35), strengthening the position of N_2O as an obligate intermediate of nitrite respiration in this organism.

The requirement of copper for N_2O reduction was specific, as also observed with *Alcaligenes* sp. (16). It should be emphasized that molybdenum was ineffective, because the only other identified enzyme (nitrogenase) that reduces N_2O is a molybdoprotein (11). Impairment of the last step of nitrite respiration in the absence of

copper led to accumulation or transient formation of N_2O from nitrite and NO in nitrate-grown, copper-deficient cells. These cells exhibited high activities of the partial reactions of nitrite respiration before N_2O reduction. On addition of copper, N_2O-reducing capability was rapidly restored in a process that did not require protein synthesis. This suggested that catalytically inactive apoprotein was present. The requirement of N_2O reduction for copper could reside in N_2O reductase itself or in a copper-containing electron carrier. Two soluble copper proteins (molecular weight >40,000) and membrane-bound copper were found in *P. perfectomarinus* (data not shown). The possible identity of one of them with N_2O reductase is under investigation.

An unexpected finding was the definite termination of NO reduction at the level of N_2O in copper-sufficient, N_2O-grown cells. Although copper-deficient, nitrate-grown cells preferentially produced N_2O from NO, accumulation was transient and slow reduction to N_2 occurred. In both types of cells, however, NO was found to be inhibitory for N_2O reduction, though much stronger in N_2O-grown cells. A weak suppression by NO of N_2 formation from N_2O (22), and the presence of a lag phase in N_2 formation from NO, but not from nitrite (28), could be previous indications of the inhibitory nature of NO for N_2O reduction.

The diauxic growth curve on nitrate clearly divided nitrate from nitrite-dependent growth (see also reference 38). In the presence of copper, cultures reached a higher final optical density, and the growth yield was greater than without copper. Koike and Hattori (18) indicated that in *P. denitrificans* the molar growth yields for nitrogenous oxides were equivalent to their formal oxidation number. Our ratio of growth yields for the process nitrate $\rightarrow$ N_2O (absence of copper) to nitrate $\rightarrow$ N_2 (presence of copper) of 0.75 to 0.85 (expected value, 0.8) agrees with their findings and suggests phosphorylation to occur at the intermediate reaction steps nitrate $\rightarrow$ nitrite, nitrite $\rightarrow$ N_2O, and N_2O $\rightarrow$ N_2. Our data are not compatible with the previous inference (4) that due to the presumed soluble nature of the enzymes, the reduction of nitrite to N_2O might not be coupled to phosphorylation.

ACKNOWLEDGMENTS

T.M. is an A. von Humboldt fellow on leave of absence from Nagoya University, Japan. We would like to thank D. J. Arp for a critical reading of and comments on the manuscript and S. Mümmler for preparing the illustrations. J. M. Tiedje kindly provided a manuscript before publication.

We gratefully acknowledge financial support from the Deutsche Forschungsgemeinschaft.

LITERATURE CITED

1. **Balderston, W. L., B. Sherr, and W. J. Payne.** 1976. Blockage by acetylene of nitrous oxide reduction in *Pseudomonas perfectomarinus*. Appl. Environ. Microbiol. **31**:504–508.
2. **Boogerd, F. C., H. W. van Verseveld, and A. H. Stouthamer.** 1980. Electron transport to nitrous oxide in *Paracoccus denitrificans*. FEBS Lett. **113**:279–284.
3. **Chung, C. W., and V. A. Najjar.** 1956. Cofactor requirements for enzymatic denitrification. II. Nitric oxide reductase. J. Biol. Chem. **218**:627–632.
4. **Cox, C. D., Jr., and W. J. Payne.** 1973. Separation of soluble denitrifying enzymes and cytochromes from *Pseudomonas perfectomarinus*. Can. J. Microbiol. **19**:861–872.
5. **Cox, C. D., Jr., W. J. Payne, and D. V. DerVartanian.** 1971. Electron paramagnetic resonance studies on the nature of hemoproteins in nitrite and nitric oxide reduction. Biochim. Biophys. Acta **253**:290–294.
6. **Fedorova, R. I., E. I. Milekhina, and N. I. Il'Yukhina.** 1973. Evaluation of the method of "gas metabolism" for detecting extraterrestrial life. Identification of nitrogen-fixing microorganisms. Izv. Akad. Nauk SSSR Ser. Biol. **6**:797–806.
7. **Firestone, M. K., R. B. Firestone, and J. M. Tiedje.** 1979. Nitric oxide as an intermediate in denitrification: Evidence from nitrogen-13 isotope exchange. Biochem. Biophys. Res. Commun. **91**:10–16.
8. **Firestone, M. K., R. B. Firestone, and J. M. Tiedje.** 1980. Nitrous oxide from soil denitrification: factors controlling its biological production. Science **208**:749–751.
9. **Haddock, B., and C. W. Jones.** 1977. Bacterial respiration. Bacteriol. Rev. **41**:47–99.
10. **Hall, J. B.** 1978. Nitrate-reducing bacteria, p. 296–298. *In* D. Schlessinger (ed.), Microbiology—1978. American Society for Microbiology, Washington, D.C.
11. **Hardy, R. W. F., and E. Knight, Jr.** 1966. Reduction of N_2O by biological N_2-fixing systems. Biochem. Biophys. Res. Commun. **23**:409–414.
12. **Horio, T.** 1958. Terminal oxidation system in bacteria. II. Some physical and physiological properties of purified cytochromes of *Pseudomonas aeruginosa*. J. Biochem. **45**:267–279.
13. **Ingraham, J. L.** 1981. Microbiology and genetics of denitrifiers, p. 45–65. *In* C. C. Delwiche (ed.), Denitrification, nitrification, and atmospheric nitrous oxide. John Wiley & Sons, Inc., New York.
14. **Iwantscheff, G.** 1972. Das Dithizon und seine Anwendung in der Mikro- und Spurenanalyse, Vlg. Chemie, Weinheim.
15. **Iwasaki, H., and T. Matsubara.** 1972. A nitrite reductase from *Achromobacter cycloclastes*. J. Biochem. **71**:645–652.
16. **Iwasaki, H., T. Saigo, and T. Matsubara.** 1981. Copper as a controlling factor of anaerobic growth under N_2O and biosynthesis of N_2O reductase in denitrifying bacteria. Plant Cell Physiol. **21**:1573–1584.
17. **Kodama, T., K. Shimada, and T. Mori.** 1969. Studies on anaerobic biphasic growth of a denitrifying bacterium, *Pseudomonas stutzeri*. Plant Cell Physiol. **10**:855–865.
18. **Koike, I., and A. Hattori.** 1975. Energy yield of denitrification: An estimate from growth yield in continuous cultures of *Pseudomonas denitrificans* under nitrate-, nitrite-, and nitrous oxide-limited conditions. J. Gen. Microbiol. **88**:11–19.
19. **Kristjansson, J. K., and T. C. Hollocher.** 1980. First practical assay for soluble nitrous oxide reductase of denitrifying bacteria and a partial kinetic characterization. J. Biol. Chem. **255**:704–707.
20. **Kristjansson, J. K., B. Walter, and T. C. Hollocher.** 1978. Respiration-dependent proton translocation and the transport of nitrate and nitrite in *Paracoccus denitrificans* and other denitrifying bacteria. Biochemistry **17**:5014–5019.
21. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol

reagent. J. Biol. Chem. **193**:265–275.

22. **Matsubara, T.** 1970. Studies on denitrification. XII. Gas production from amines and nitrite. J. Biochem. **67**:229–235.
23. **Matsubara, T.** 1975. The participation of cytochromes in the reduction of N_2O to N_2 by a denitrifying bacterium. J. Biochem. **77**:627–632.
24. **Matsubara, T., and T. Mori.** 1968. Studies on denitrification. IX. Nitrous oxide, its production and reduction to nitrogen. J. Biochem. **64**:863–871.
25. **Meijer, E. M., J. W. Van der Zwaan, and A. H. Stouthamer.** 1979. Location of the proton-consuming site in nitrite reduction and stoichiometries for proton pumping in anaerobically grown *Paracoccus denitrificans*. FEMS Microbiol. Lett. **5**:369–372.
26. **Miyata, M., T. Matsubara, and T. Mori.** 1969. Studies on denitrification. XI. Some properties of nitric oxide reductase. J. Biochem. **66**:759–765.
27. **Payne, W. J.** 1973. Reduction of nitrogenous oxides by microorganisms. Bacteriol. Rev. **37**:409–452.
28. **Payne, W. J., P. S. Riley, and C. D. Cox, Jr.** 1971. Separate nitrite, nitric oxide, and nitrous oxide reducing fractions from *Pseudomonas perfectomarinus*. J. Bacteriol. **106**:356–361.
29. **Rhodes, M., A. Best, and W. J. Payne.** 1963. Electron donors and cofactors for denitrification by *Pseudomonas perfectomarinus*. Can. J. Microbiol. **9**:799–807.
30. **Snell, F. D., and C. T. Snell.** 1949. Colorimetric methods of analysis, vol. 2. Van Nostrand, New York.
31. **Sorensen, J., J. M. Tiedje, and R. B. Firestone.** 1980. Inhibition by sulfide of nitric and nitrous oxide reduction by denitrifying *Pseudomonas fluorescens*. Appl. Environ. Microbiol. **39**:105–108.
32. **St. John, R. T., and T. C. Hollocher.** 1977. Nitrogen 15 tracer studies on the pathways of denitrification in *Pseudomonas aeruginosa*. J. Biol. Chem. **252**:212–218.
33. **Yamanaka, T., S. Kijimoto, and K. Okunuki.** 1963. Biological significance of *Pseudomonas* cytochrome oxidase in *Pseudomonas aeruginosa*. J. Biochem. **53**:416–421.
34. **Yamanaka, T., and K. Okunuki.** 1974. Cytochromes, p. 349–400. *In* J. B. Neilands (ed.), Microbial iron metabolism. Academic Press, Inc., New York.
35. **Yoshinari, T., and R. Knowles.** 1976. Acetylene inhibition of nitrous oxide reduction by denitrifying bacteria. Biochem. Biophys. Res. Commun. **69**:705–710.
36. **Zumft, W. G., and J. Cárdenas.** 1979. The inorganic biochemistry of nitrogen. Bioenergetic processes. Naturwissenschaften **66**:81–88.
37. **Zumft, W. G., B. F. Sherr, and W. J. Payne.** 1979. A reappraisal of the nitric oxide-binding protein of denitrifying *Pseudomonas*. Biochem. Biophys. Res. Commun. **88**:1230–1236.
38. **Zumft, W. G., and J. M. Vega.** 1979. Reduction of nitrite to nitrous oxide by a cytoplasmic membrane fraction from the marine denitrifier *Pseudomonas perfectomarinus*. Biochim. Biophys. Acta **548**:484–499.