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Cadmium-copper antagonism in the activation of periplasmic nitrous oxide reductase of copper-deficient cells from *Pseudomonas stutzeri*

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Summary. Copper-deficient cells of *Pseudomonas stutzeri* strain ZoBell synthesize catalytically inactive nitrous oxide (N_2O) reductase which is activated by added Cu(II) in the absence of de novo protein synthesis. The apparent K_m for the activation process is 0.13 μM. Activation is temperature-dependent and is inhibited by Cd(II) (K_i 1.27 μM) and less strongly by Zn(II), Ni(II), and Co(II). The same metal ions at 20 μM have little or no effect on N_2O reduction of intact cells. Apo-N_2O reductase of transposon Tn*5*-induced *nos*$^-$ mutants with defective Cu-chromophore biosynthesis is not reactivated by Cu(II). N_2O reductase of Cu-sufficient and Cu-deficient wild type, and of *nos*$^-$ mutants is localized in the periplasm, the latter providing the likely site of metal incorporation into the apoenzyme.

Key words: Nitrous oxide respiration — Copper chromophore biosynthesis — Cytochrome cd_1 — Denitrification — Periplasmic enzyme — *Pseudomonas*

Introduction

Nitrous oxide (N_2O) respiration is an alternative energy-conserving pathway found in many denitrifying bacteria. The process depends on N_2O reductase, a metalloenzyme with approximately 8 Cu atoms per homodimeric molecule of 140 kDa (Zumft and Matsubara 1982; Coyle et al. 1985). The Cu chromophore of N_2O reductase is not readily accomodated within the current classification of Cu proteins. A novel type I center was suggested to be part of the metal chromophore, exhibiting unusual electron paramagnetic resonance (EPR) and resonance Raman properties (Coyle et al. 1985; Dooley et al. 1987).

Evidence from Cu-deficient cells (Matsubara et al. 1982) and transposon Tn*5*-induced mutants (Zumft et al. 1985) indicated that additional gene products are specifically required for the incorporation of Cu into apo-N_2O reductase. The corresponding genes were shown to cluster with the structural gene of the enzyme within a DNA fragment of about 8000 base pairs (Viebrock and Zumft 1987). To gain a better understanding of the functions required for Cu chromophore biosynthesis, we have studied the location of N_2O reductase and the conditions under which Cu is incorporated into the apoprotein found in Cu-deficient cells.

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Materials and methods

Organism and growth conditions. Pseudomonas stutzeri strain ZoBell (formerly *P. perfectomarina* ATCC 14405) was grown in a synthetic medium with 0.1% nitrate and 50 μM $FeCl_3$ (Coyle et al. 1985). Cu-sufficient medium contained 1 μM $CuCl_2$ or the concentrations required for specific purposes. Cu-deficient medium was prepared with deionized and quartz doubled-distilled water. The medium was treated with 1,5-diphenylthiocarbazone and extracted with chloroform to remove Cu (Matsubara et al. 1982). Anaerobic cultures were sealed with a paraffin layer; O_2-limited cultures (transfer rate of approximately 20 μmol $O_2\,l^{-1}\,min^{-1}$) were incubated at 120 rpm on a gyratory shaker at 30° C. *Nos*$^-$ mutants were described previously (Zumft et al. 1985; Viebrock and Zumft 1987).

Reconstitution experiments. Cu-deficient cells were harvested by centrifugation for 10 min at $10410 \times g$. They were washed once with double-distilled water, and suspended in 50 mM phosphate buffer, pH 7.1, containing 50 mM sodium L-(+)-lactate. A sample of the cell suspension, equivalent to approx. 1 mg protein, was placed under He, made 1 μM in $CuCl_2$ and incubated at 30° C in a reciprocal shaker. After an incubation period of 75 min, N_2O was added and its reduction assayed by

gas chromatography. All equipment used for manipulating Cu-deficient cells was acid-cleaned, and rinsed thoroughly with double-distilled water.

Cell extracts and enzyme preparations. Cell suspensions were broken by one passage through a microcell (3 ml) at 130 MPa of a high-pressure homogenizer. The extract was centrifuged at $29000 \times g$ for 30 min and used for activity reconstitution. The purification procedures for N_2O reductase (Coyle et al. 1985) and nitrite reductase (cytochrome cd_1) (Zumft et al. 1988) were previously described. Periplasmic and cytoplasmic cell compartments were fractionated from spheroplasts (Pages et al. 1984). Alternatively, periplasmic proteins were extracted from chloroform-treated cell suspensions (Ferro-Luzzi Ames et al. 1984). Before the disruption of spheroplasts by freezing and thawing, 0.05 mg each of DNase and RNase was added/ml cell suspension. Periplasmic and cytoplasmic fractions were concentrated by ultrafiltration.

Activity assays. N_2O reduction of intact cells was followed by gas chromatography (Frunzke and Zumft 1986). Cell-free preparation were assayed for N_2O reductase with photochemically reduced benzyl viologen (Coyle et al. 1985). One activity unit (U) of N_2O reductase is the amount which catalyzes the reduction of 1 μmol N_2O min^{-1} (mg protein)$^{-1}$. The assay for malate dehydrogenase followed a standard procedure (Mosbach and Mattiasson 1976).

Analytical methods and chemicals. Cu was determined by atomic absorption spectroscopy at 324.7 nm. Protein was estimated by the Lowry procedure (Lowry et al. 1951). The conditions for Laurell electroimmunoassay were as previously described (Zumft et al. 1985). $CuCl_2$ at 99.999% purity was from Ventron Alfa Products (Karlsruhe, FRG); N_2O (99.5%) was purchased from Hoechst AG (Frankfurt, FRG); dithizone was from Sigma Chemical Co. (Deisenhofen, FRG).

Results

Effect of copper and cadmium on growth of P. stutzeri

P. stutzeri grew best under denitrifiying conditions with 1–5 μM Cu(II) in the growth medium. We observed at these concentrations also the highest specific N_2O-reducing activity (around 0.2 U). At millimolar concentrations of Cu(II), growth and N_2O-reducing activity started to be impaired. Under anaerobic, *i.e.* denitrifying conditions, the growth rate at 1 mM Cu(II) was less than half of that at 1 μM. However, under O_2-limiting conditions and 5 mM Cu(II), which still allowed expression of the denitrifying system, the growth rate was diminished only by one third. *P. stutzeri*, thus, showed a considerable tolerance towards Cu, and exhibited a differential sensitivity depending on the presence of O_2. Cadmium was inhibitory for the growth of *P. stutzeri*. Its minimal inhibitory concentration was 10 μM under either aerobic or anaerobic growth conditions (media supplemented also with 1 μM Cu^{2+}).

Reactivation of apo-N_2O reductase by copper

Cells of *P. stutzeri* grow anaerobically without Cu(II) in nitrate-containing medium, but temporarily terminate respiration under those conditions at the level of nitrous oxide (Matsubara et al. 1982). Cu-deficient cells showed a very low rate for N_2O reduction which could be increased in a short-term process by added Cu(II) (Table 1). The degree of activation was variable and depended on the physiological state of the culture. Cells were usually harvested at a low density ($A_{660} = 0.4$), and activation factors ranged from 5 to 10. Occasionally, when severe Cu-deficiency was achieved, activation could be as high as about 35-fold. Cells grown in medium not treated with the chelator and not supplemented with Cu(II) had considerable N_2O-reducing activity and activation by added Cu(II) was low. This indicates that traces of Cu in the medium are used efficiently by *P. stutzeri* and become incorporated into N_2O reductase. An outer-membrane protein was found in *P. stutzeri* JM300 which might be involved in this process. Its mutational loss results in strains synthesizing the apoenzyme only (Mokhele et al. 1987).

An apparent K_m value of 0.13 μM was estimated for Cu(II) in the activation process. Addition of rifampicin or chloramphenicol to the reactivation system did not prevent reactivation (Table 1), strengthening our previous suggestion that de novo protein synthesis is not required (Matsubara et al. 1982). At 0–10 °C the activation process proceeded very slowly, if at all. In vitro activation of N_2O reductase was attempted by adding Cu(II)

Table 1. Reactivation by added copper of N_2O reductase in Cu-deficient intact cells of *P. stutzeri*

Conditions	N_2O-reductase activity (U)
Without Cu	0.019
With Cu	0.132
With Cu, with rifampicin	0.126[a]
	0.125[b]
With Cu, with chloramphenicol	0.109[a]
	0.122[b]

Cells were grown anaerobically with nitrate in Cu-deficient medium. When Cu(II) was added, the cell suspension was made 1 μM in $CuCl_2$ and incubated for 60 min before assaying for N_2O reductase. Rifampicin was added at 10 μg/ml cell suspension; chloramphenicol at 8.1 μg/ml suspension

[a] Antibiotics were added 10 min before Cu was added

[b] Antibiotics were added after the 60-min incubation with Cu

to a crude extract of Cu-deficient cells and incubating the mixture at 30° C. No activation comparable to that found in the whole-cell system was manifest when assaying spectrophotometrically for N_2O reductase.

The previously characterized class I of respiratory mutants of *P. stutzeri* exhibiting the *nos*$^-$ phenotype, synthesize and regulate the amount of N_2O reductase like the wild type. The mutant proteins, however, are devoid of Cu or contain only a residual amount (Viebrock and Zumft 1987; Zumft et al. 1985). Three representative mutant strains of this class, MK404, MK416, and MK417, each synthesizing apo-N_2O reductase, could not be reactivated with Cu(II) under the conditions used for Cu-deficient cells of the wild type.

The metal ions Cd(II), Zn(II), Ni(II), and Co(II) inhibited the activation of N_2O reductase by Cu(II) of Cu-deficient cells (Table 2). The most pronounced effect was found with Cd(II), for which an apparent K_i of 1.27 μM was estimated. Mg(II), Mn(II), and Mo(VI)O_4 were only slightly inhibitory in concentrations up to 20 μM. On the other hand, most of these metal ions at 20 μM concentration, including Cd(II), did not inhibit N_2O reduction of Cu-sufficient, intact cells; only Ni(II) and Co(II) at 20 μM were weakly inhibitory (Table 2).

Inhibition of N_2O reductase activation by Cd(II) was counteracted when Cu(II) was added within the first 5 min after exposure of the Cu-deficient cells to Cd(II), but less so upon longer exposure (Fig. 1). Irreversible formation of an initial species in the metal-incorporating system with a half-life of about 10 min is suggested from these data. A similar effect was observed with Zn(II), Co(II), and Ni(II). When Cu(II) was added together with Cd(II), we observed nearly full activation (80%) indicating, in conformity with the low K_m for Cu, a preference of Cu utilization over Cd.

The inhibitory effect of Cd(II) was also temperature-dependent. Incubation of Cu-deficient cells with 5 μM Cd(II) at 30° C for 20 min decreased in a representative experiment the subsequent activation by Cu(II) to 19.8%. However, when the incubation with Cd(II) was done at 0° C, activation by Cu(II) was 80.4%, similar to that achieved by the simultaneous addition of both metal ions (81.4%).

Table 2. Effect of transition metal ions on the activation of N_2O reduction of Cu-deficient cells and on N_2O reduction of Cu-sufficient, intact cells

Metal ion	Concn (μM)	Activation of Cu-deficient cells (%)	Activity of Cu-sufficient cells (%)
Cd(II)	10	3.9	—
	20	1.8	100
Zn(II)	10	35.3	—
	20	28.1	100
Ni(II)	10	45.6	93.5
	20	—	82.7
Co(II)	10	54.4	98.4
	20	—	88.6
Mg(II)	20	84.5	100
Mn(II)	20	94.1	100
Mo(VI)O_4	20	90.2	100

Cu-deficient cells were incubated with the transition metal ions at the indicated concentrations for 20 min at 30° C; the cell suspension was then made 1 μM in $CuCl_2$ and incubated for another 90 min. N_2O reductase activity was measured by gas chromatography. N_2O reduction before activation was 0.012 U; the activity after activation by Cu(II) was 0.116 U (= 100%). For measuring the effect of metal ions on N_2O reduction of Cu-sufficient whole cells, the standard assay contained the indicated metal ions and concentrations. Control activity (100%) in this assay was around 0.2 U
— = not determined

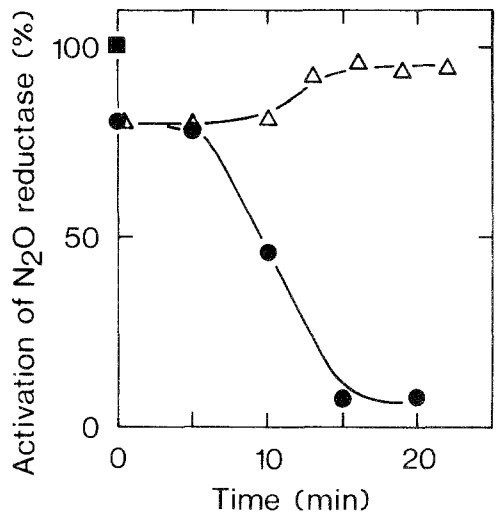

Fig. 1. Antagonism between Cu and Cd as function of time in the activation process of Cu-deficient cells. (●) The reaction mixture for N_2O reductase reactivation was made 5 μM in Cd(II). At the indicated times Cu(II) was added to bring the mixture to 1 μM. After 75 min of further incubation the cell suspension was assayed for N_2O reduction by gas chromatography. (△) The mixture for N_2O reductase reactivation was first made 1 μM in Cu(II) followed at the indicated times by 5 μM Cd(II). N_2O-reducing activity of Cu-deficient cells was 0.003 U before activation; the control activity (■) of Cu-activated cells without added Cu(II) was 0.138 U

Localization of N_2O reductase

N_2O reductase from *P. stutzeri* is a soluble enzyme which is purified from the supernatant of crude extracts after high-speed centrifugation (Zumft and Matsubara 1982; Coyle et al. 1985). The discrimination of N_2O reductase location within the soluble cell compartments was achieved both with the chloroform method (Ferro-Luzzi Ames et al. 1984) and the subcellular fractionation of periplasm and cytoplasm (Pages et al. 1984).

Figure 2 illustrates the distribution of N_2O reductase within the two cell compartments. The periplasmic fraction of the wild type clearly contained the major part of the enzyme. Also, the N_2O reductase protein of the apoenzyme-synthesizing *nos*$^-$ mutants, MK404, MK416, and MK417, was found predominantly within the periplasm. Nitrite reductase (cytochrome cd_1) was chosen as marker which, because of the orientation of its proton-consuming site, is suggested to be periplasmic in *Pseudomonas aeruginosa* (Wood 1978), and *Paracoccus denitrificans* (Meijer et al. 1979). Direct fractionation, as shown here (Fig. 2), provided evidence for a periplasmic location also in *P. stutzeri* strain 224 (Pettigrew and Moore 1987). These conclusions were further supported by the distribution of the cytoplasmic marker enzyme malate dehydrogenase (Mosbach and Mattiasson 1976). Activity of this enzyme was not detected in the periplasmic fraction; the cytoplasmic fraction had an activity of 8.5 nmol min^{-1} (mg protein)$^{-1}$.

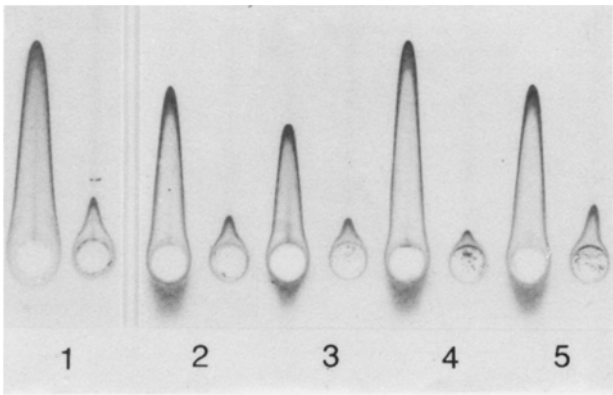

Fig. 2. Electroimmunoassay for N_2O reductase and cytochrome cd_1 in the periplasmic and cytoplasmic subcellular fractions of wild-type *P. stutzeri* strain ZoBell and transposon Tn*5*-induced *nos*$^-$ mutants. Periplasmic and cytoplasmic cell compartments were isolated according to Pages et al. (1984), and subjected to quantitative immunoelectrophoresis. Lane 1, wild type assayed for cytochrome cd_1; 2, wild type, assayed for N_2O reductase; 3, 4, and 5, mutant strains MK404, MK416, and MK417, respectively, each assayed for N_2O reductase. The left track for each sample represents the periplasmic (protein concn 4 mg/ml), the right track the cytoplasmic (protein concn 24 mg/ml) cell compartment. Each well was charged with 3 µl

Table 3. Distribution of N_2O reductase protein within the periplasmic and cytoplasmic fractions of Cu-sufficient and Cu-deficient cells of *P. stutzeri*

Fraction	Expt	Cu-sufficient		Cu-deficient	
		total protein (mg/ml)	N_2O reductase (%)	total protein (mg/ml)	N_2O reductase (%)
Periplasm	1	0.55	2.6	0.46	2.8
	2	0.52	2.9	0.35	3.3
Cytoplasm	1	1.99	0.1	1.98	0.08
	2	1.95	0.09	2.20	0.09

The content of N_2O reductase within the periplasmic and cytoplasmic fractions was determined immunochemically. N_2O reductase activity of Cu-sufficient cells was 0.19 U; activity of Cu-deficient cells was 0.0174 U before and 0.172 U after activation

Chloroform treatment of wild-type cells and subsequent quantitative immunoelectrophoresis of an aqueous Tris-buffered extract showed that the major part of N_2O reductase was present in the extract and that enzyme liberation from the cell did not require cell breakage (data not shown). Since the activation process of Cu-deficient wild-type cells did not require protein synthesis, the presence of apoprotein was suggested (see above). Table 3 shows that these cells synthesized immunochemically detectable enzyme protein in amounts comparable to that of Cu-sufficient wild type. Again, this apoenzyme was found in the periplasm.

Discussion

Nitrous oxide reductase of *P. stutzeri* is a periplasmic enzyme. This conclusion is supported by an NH_2-terminal export signal sequence which is predicted from an analysis of the amino acid sequence of the enzyme (Viebrock and Zumft 1988). The same enzyme of *Rhodobacter capsulatus* (McEwan et al. 1985) and the N_2O-reducing activities of *Rhodobacter sphaeroides* forma sp. *denitrificans* (Urata et al. 1982) and *Pa. denitrificans* (Boogerd et al. 1981; Alefounder et al. 1983) were previously concluded to be periplasmic.

Nitrous oxide reductase was immunochemically detected within the periplasm of the three cell types studied: Cu-sufficient and Cu-deficient wild-type cells, and mutants possessing the apoenzyme only. The protein of Cu-deficient cells could be activated by exogenous Cu(II) in the absence of protein synthesis, indicating the incorporation of the metal into extant apoprotein. In contrast, the enzyme from mutants, though still periplasmic, could not be activated. These mutants have Tn*5* insertions within a region of approximately 3500 pairs of the *nos* gene cluster contiguous to the structural gene for N_2O reductase. This region was suggested to have a function in Cu-chromophore biosynthesis (Zumft et al. 1985; Viebrock and Zumft 1987; Zumft et al. 1987).

The available evidence, thus, depicts a situation where export of N_2O reductase into the periplasm precedes Cu-chromophore biosynthesis within the same compartment. The latter process, rather than being spontaneous, appears to be catalyzed by components which form part of the *nos* gene cluster. The mechanism of Cu insertion presumably is complex, when we consider the likely requirement of several gene products (Viebrock and Zumft 1987) and additional evidence for the involvement of a 61-kDa outer-membrane protein in *P. stutzeri* strain JM300 (Mokhele et al. 1987). The inability to obtain enzyme reconstitution in a cell-free system, though perhaps due to unfavorable experimental conditions, may also indicate topological requirements for this process, which cannot be emulated by a cellular homogenate.

The activation process of apoenzyme was strongly inhibited by Cd(II). On the other hand, N_2O-reducing activity of intact cells was not inhibited by comparable concentrations of Cd(II). Our experiments did not determine whether the metal ions found inhibitory for the activation process, Cd(II), Zn(II), Ni(II), and Co(II) would interfere with the system for Cu-chromophore biosynthesis, or whether they would be incorporated by this system in lieu of Cu, yielding inactive, metal-substituted N_2O reductase. In the light of substitutions involving some of these metal ions in different metalloproteins (e.g. Morpurgo et al. 1983; Zeppezauer et al. 1984; Beltramini et al. 1984), the latter is an intriguing possibility.

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