Biochimica et Biophysica Acta 852 (1986) 119–125
Elsevier

BBA 42141

Inhibition of nitrous-oxide respiration by nitric oxide in the denitrifying bacterium *Pseudomonas perfectomarina*

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(Received 28 May 1986)

Key words: Denitrification; N_2O reduction; Nitrous oxide reductase; Nitric oxide; (*Ps. perfectomarina*)

The respiration of nitrous oxide to dinitrogen was inhibited by nitric oxide in *Pseudomonas perfectomarina* and in other denitrifying bacteria. The sensitivity of N_2O respiration towards NO was correlated with the growth mode under which the denitrifying system was expressed. N_2O respiration was more sensitive to NO in N_2O-grown cells than in nitrate-grown cells, exhibiting apparent K_i values of 0.35 ± 0.1 kPa and 5.5 ± 0.6 kPa, respectively. In both cell types no competitive relation between NO and N_2O was observed. Cells grown under N_2O had only weak NO reductase activity, whereas cells grown with nitrate showed an at least 3-fold higher rate of NO reduction under 10 kPa NO. Oxygen limitation during expression of the denitrifying system also led to a differential expression of individual reaction steps. This, together with inhibition of N_2O respiration by NO, may cause the large variation of the products of denitrification formed under oxygen-limited conditions in several representative denitrifying Pseudomonads. NO reacted with the purple form of purified N_2O reductase, converting its electronic absorption spectrum to the pink form concomitant with a loss of most of the catalytic activity. Removal of NO and subsequent air oxidation of the enzyme largely restored the original spectrum, but not the activity.

Introduction

The reduction of nitrous oxide (N_2O) is one of several energy-yielding steps of bacterial denitrification. Coupling of N_2O reduction to ATP generation was shown by growth-yield measurements of a non-fermentative denitrifier [1] and by means of growth studies with N_2O in the absence of other terminal electron acceptors [2]. More recently, it was shown that the reduction of N_2O is coupled to the formation of a membrane potential [3] and to proton translocation [4–6].

The terminal oxido-reductase for N_2O respiration is a high M_r copper protein in Pseudomonads [7,8]. The enzyme exists in several spectroscopically distinct states due to an unusual chromophore [9].

N_2O respiration by intact cells is inhibited by 2,4-dinitrophenol, azide, cyanide, monoiodoacetate and CO [10], by acetylene [11,12] and by sulphide [13]. Previous observations indicated inhibition also by nitric oxide (NO) [14,15], the putative intermediate of denitrification, antecedent to N_2O in the reaction sequence [16]. In this paper we describe several characteristics of the inhibition of N_2O respiration by NO and identify N_2O reductase as one site of interaction.

Materials and Methods

Organisms and growth conditions. Pseudomonas perfectomarina ATCC 14405 and the other de-

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nitrifying bacteria used in this study were grown anaerobically in an asparagine and citrate-containing, synthetic medium (ACN), which was supplemented with 1 g $NaNO_3$ per l [15]. Growth of cells under N_2O was obtained in the same medium without $NaNO_3$ (AC medium) by sparging 2-l medium in round flasks continuously with 50 ml N_2O per min through a sinter glass disk. Additional agitation was provided by magnetic stirring. *Rhodobacter sphaeroides* (formerly called *Rhodopseudomonas sphaeroides*) forma sp. *denitrificans* was grown as described by Satoh et al. [17]. For oxygen limitation cells were grown in 2-l Erlenmeyer flasks with 1-l ACN medium in a gyratory shaking incubator at 120 rpm. All cultures were started with an inoculum of 10% and incubated at 30°C. The transfer rate of oxygen into shaking cultures was determined by a modification of the Winkler method [18].

Cells were harvested at the end of the exponential growth phase by centrifugation for 15 min at $7000 \times g$ and 4°C. Fresh cell paste was resuspended in approx. the 4-fold volume of 50 mM phosphate buffer (pH 7.0) and kept on ice. This stock suspension was diluted with phosphate buffer immediately before use.

Analytical methods. Nitrite, NO and N_2O reductase activities were measured in 12 ml vials, containing an amount of cells equivalent to approx. 2–4 mg protein, 150 μmol lactate, and 150 μmol phosphate buffer (pH 7.0) in a total volume of 3 ml. The vials were filled with helium and reactions were started by injecting either 50 μmol sodium nitrite or 42 μmol N_2O or NO. The reaction mixtures were incubated at 30°C and 100 oscillations per min in a shaking water bath. Products were measured by gas chromatography [19]. Cytochrome oxidation levels and difference spectra of cell suspensions were recorded with a dual-wavelength spectrophotometer equipped with an end-on photo-multiplier. Cell suspension, in stoppered cuvettes under helium, contained 150 μmol lactate and 100 μmol phosphate buffer (pH 7.0) in 2 ml.

Chemicals and gases. Helium, NO and N_2O were purchased from Linde AG, Höllriegelskreuth, or from Messer Griesheim, Karlsruhe. NO was also prepared in the laboratory from nitrite and acidified iron(II)sulfate, when the N_2O contamination of commercial NO interfered with the enzymatic measurements. Other chemicals were of analytical grade. N_2O reductase from *Ps. perfectomarina* was prepared as published previously [9].

Results

Expression of denitrifying, partial activities in Pseudomonas perfectomarina

The expression of nitrogenous oxide-reducing activities of whole cells was controlled by the terminal electron acceptor supplied during growth (Table I). High nitrite and NO reductase activities were found in anaerobically, nitrate-grown cells. Activities were low in cells grown with N_2O or under oxygen limitation. NO uptake of N_2O-grown cells showed saturation kinetics and had a broad maximum around 8 kPa partial pressure of NO (Fig. 1). In nitrate-grown cells NO reduction exceeded that of N_2O-grown cells about 4-fold and was not yet saturated at 11 kPa NO. In contrast to nitrite- and NO-reducing activities, expression of N_2O reductase activity was found to be less dependent on the electron acceptor available during growth. More than half of its maximal level observed in anaerobically, nitrate-grown cells was present at a transfer rate of oxygen ($K_L aC^*$) into the medium of 0.02 mmol $O_2 \cdot l^{-1} \cdot min^{-1}$ and in the absence of any nitrogenous electron acceptor.

TABLE I

EXPRESSION OF DENITRIFYING, PARTIAL ACTIVITIES BY INTACT CELLS OF *PSEUDOMONAS PERFECTOMARINA*

Activity tests were started by injection of 50 μmol nitrite, 42 μmol NO, or 42 μmol N_2O into anaerobic cell suspensions, containing 150 μmol lactate in 50 mM phosphate buffer (pH 7.0). Activities are expressed with respect to substrate uptake per mg cell protein.

Growth conditions and electron acceptor supplied for growth	Denitrifying activity (nkat·mg^{-1})		
	NO_2^-	NO	N_2O
Nitrate (anaerobic)	2.8	1.2	4.9
N_2O (anaerobic)	0.1	0.23	3.1
Oxygen-limited [a] (without NO_3^-)	0.15	0.34	2.7

[a] $K_L aC^* = 0.02$ mmol $O_2 \cdot l^{-1} \cdot min^{-1}$.

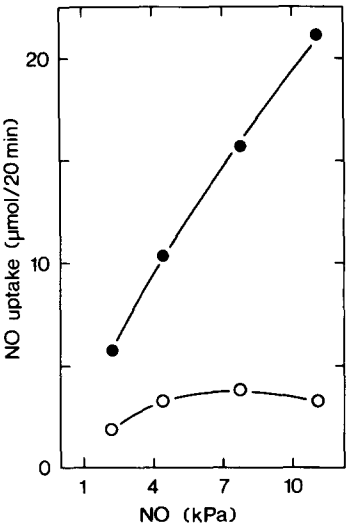

Fig. 1. Reduction of NO by N_2O- or nitrate-grown cells of *Ps. perfectomarina* as a function of the partial pressure of NO. Reactions were started by addition of NO. Differences in the total pressures among individual reaction vials were compensated by injecting a corresponding volume of helium prior to NO. Each assay contained cells equivalent to 9 mg of protein. N_2O-grown cells (○); nitrate-grown cells (●).

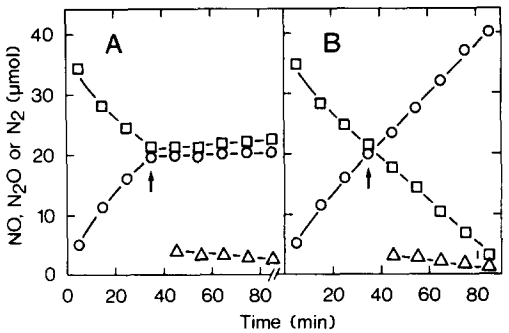

Fig. 2. Differential sensitivity of N_2O reductase towards NO in N_2O- and nitrate-grown cells of *Ps. perfectomarina.* Cells grown under 0.1 MPa N_2O (A) or with 0.1% sodium nitrate (B) were incubated under standard assay conditions. Reactions were started by addition of 42 μmol N_2O. After 35 min (indicated by arrows) 4.2 μmol NO were injected into both vials. Symbols: N_2O (□); N_2 (○); NO (△).

Activities shown in Table I, however, may not reflect true levels of the corresponding enzyme in each case. For N_2O reductase, for instance, it had been shown that nitrate-grown cells express an up to 10-fold higher level of the enzyme, measured immunochemically, than do N_2O-grown cells [20].

Inhibition of N_2O respiration by NO

N_2O reduction of N_2O-grown cells was fully inhibited by 1.1 kPa NO (20 μM in solution) (Fig. 2). The same concentration, however, did not affect the enzymatic activity of nitrate-grown cells. Determination of an apparent inhibition constant for NO gave values of 0.35 ± 0.1 kPa (N_2O-grown cells) and 5.5 ± 0.6 kPa (nitrate grown cells). Azide inhibited N_2O reduction of N_2O-grown cells stronger than of nitrate-grown cells. Other known inhibitors of N_2O reduction did not show such a differential effect for the two cell types (Table II).

The two cell types further differed in the extent to which the inhibition of N_2O reduction by NO was reversible. For the experiment shown in Fig. 3, the rates of N_2O reduction were equalized by using an appropriate amount of cells from each growth mode. The inhibitor concentration was adjusted to obtain full inhibition at the lowest NO concentration for each experimental setup. After removal of substrate and inhibitor by repetitive evacuation and filling of the cuvettes with helium both reactions were started again with N_2O. N_2O-grown cells remained almost completely inhibited, whereas the reduction of N_2O by nitrate-grown cells was restored to about 80% (Fig. 3).

The interaction between NO and N_2O was not competitive (Table III). N_2O-reducing activity of cell samples from either type of growth mode was

TABLE II

APPARENT K_i VALUES FOR VARIOUS INHIBITORS OF N_2O RESPIRATION

The K_i values for NO were measured in samples containing 1.1 mg protein (N_2O-grown cells) and 4.4 mg (nitrate-grown cells). Because of the dependency of the inhibition by NO on the protein concentration the values cannot be conceived as true K_i-values from a kinetic viewpoint.

Inhibitor	K_i value	
	N_2O-grown cells	Nitrate-grown cells
Nitric oxide	0.35 ± 0.1 kPa	5.5 ± 0.6 kPa
Acetylene	0.1 ± 0.05 kPa	0.15 ± 0.05 kPa
Azide	40 μM	100 μM
Cyanide	6 μM	5 μM
2,4-Dinitrophenol	35 μM	35 μM
Oxygen	2 kPa	2 kPa

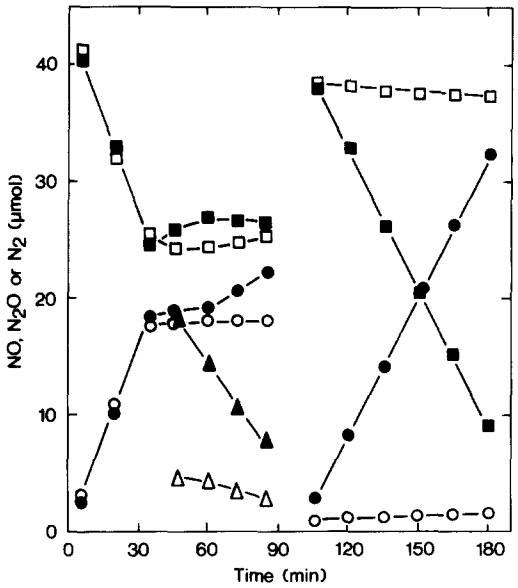

Fig. 3. Reversibility of NO inhibition in nitrate-grown cells of *Ps. perfectomarina*. Nitrate-grown (solid symbols, 2.1 mg cell protein) and N_2O-grown cells (open symbols, 5.3 mg cell protein) were incubated under standard assay conditions as described in Materials and Methods. The cell samples were adjusted to obtain equal N_2O reduction rates for both cell types. The reactions were started by addition of 42 µmol N_2O. After 35 min NO was injected into both vials (5.5 kPa and 1.1 kPa for nitrate- and N_2O-grown cells, respectively). After 90 min the headspaces were evacuated and filled with helium five times, to remove substrate and inhibitor. Reactions were immediately started again by injecting once more 42 µmol of N_2O. Symbols: N_2O (■, □); N_2 (●, ○); NO (▲, △).

TABLE III

INHIBITION OF N_2O REDUCTION BY NO IN RELATION TO N_2O PARTIAL PRESSURE

Separate samples for each condition were incubated for 5 min under standard conditions in the presence of 0.33 kPa NO (N_2O-grown cells) and 3.3 kPa NO (nitrate-grown cells) before starting the reactions with N_2O. The assay with N_2O-grown cells contained 2.4 mg protein, that with nitrate-grown cells, 3.1 mg.

Partial pressure of N_2O (kPa)	N_2O uptake (nkat)	
	N_2O-grown cells	Nitrate-grown cells
11.2 (control without NO)	3.36	8.81
5.6	1.74	3.72
11.1	1.76	3.89
17.0	1.64	–
22.4	1.66	4.83

inhibited in this experiment by NO to 50%. A change in the ratio of NO to N_2O from 1 : 17 to 1 : 68 for N_2O-grown cells did not alleviate NO inhibition, i.e., the rate of N_2O uptake remained at its 50% inhibition level. A change in the ratio of the two gases over the range 1 : 1.7 to 1 : 6.8 for nitrate-grown cells increased the rate of N_2O reduction at the highest N_2O concentration only slightly (Table III). However, this small increase might be due to the decrease in NO concentration from 3.3 kPa to 2.5 kPa during the experiment, because the nitrate-grown cells produced 1.5 µmol N_2O from the added NO.

Inhibition of N_2O reduction in either cell type was reciprocally proportional to cell mass. Table IV shows the results obtained with N_2O-grown cells; data for nitrate-grown cells (not shown) were similar. At each doubling of the cell mass, with the NO concentration constant in the assay vials, the inhibition of N_2O reduction was reduced by half. This indicated covalent interaction of NO with intact cells, e.g., at the site of N_2O reduction. In addition to that, interaction with a heme component might occur. Lactate-reduced cells responded to N_2O addition with a partial oxidation of its bulk cellular cytochromes as evidenced from an absorbance increase at 550 nm vs. 576 nm. Adding NO to this cell suspension locked the cytochrome pool in a fully reduced state (N_2O-grown cells) or partially oxidized state below that attainable with N_2O (nitrate-grown cells). This state could not be shifted for either cell type by further additions of N_2O (data not shown).

TABLE IV

INHIBITION OF N_2O RESPIRATION BY NO IN N_2O-GROWN CELLS RELATED TO CELL MASS

The assays were run under standard reaction conditions. The NO partial pressure was 0.33 kPa where indicated. Reduction of NO was negligible. All reactions were started by injection of 42 µmol N_2O.

Cell protein (mg)	N_2O uptake (nkat)		Inhibition (%)
	without NO	with NO	
0.8	1.89	0.19	90
1.6	3.58	1.92	46
3.2	6.94	5.39	22

Binding of NO to N_2O reductase

N_2O reductase is isolated in two forms, designated as 'pink' and 'purple', under aerobic and anaerobic conditions, respectively. These forms are distinguished by their enzymatic activity and absorption characteristics, with the purple form apparently representing the more oxidized state and having a higher catalytic activity [9]. The purple form of N_2O reductase reacted with NO and decreased its activity to about one tenth (0.2–0.4 μmol N_2O reduced per min per mg protein) of its original value. The electronic spectrum changed to a new pattern with the qualitative characteristics of the pink form of the enzyme (Fig. 4). The spectral features of the purple form, but not its original activity, were nearly restored upon removal of NO and air oxidation of the protein. The pink form of the enzyme, as isolated, was different from the NO-complexed state in that it showed a lower absorptivity over the visible range of the spectrum and that it was not convertible to the purple form by oxidants [9].

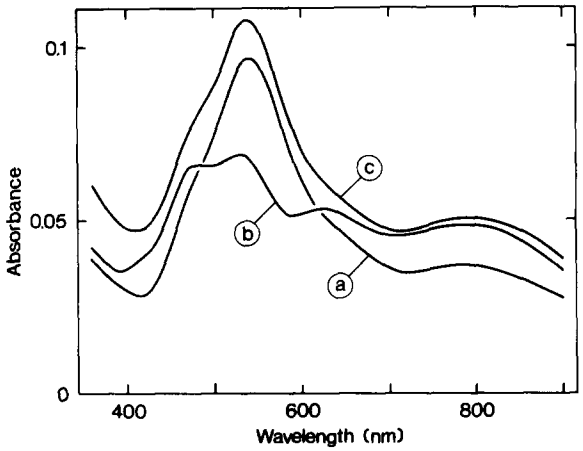

Fig. 4. NO-induced absorption changes of purified N_2O reductase of *Ps. perfectomarina*. The purple form of the enzyme (1.4 mg) was dissolved in 2 ml 50 mM Tris-HCl buffer (pH 7.5) and incubated under argon. (a) Electronic absorption spectrum of the purple form before addition of NO; (b) spectrum of the protein after addition of 50 μl NO (2.5 kPa partial pressure in the cuvette); (c) spectrum after removal of NO by repetitive evacuation and subsequent air oxidation of the sample.

Inhibition of N_2O reduction by NO in representative members of denitrifying bacteria

Inhibition of N_2O reduction by NO was found in other denitrifying bacteria such as *Pseudomonas stutzeri* JM 300, *Pseudomonas fluorescens* DSM 50415, *Pseudomonas aeruginosa* DSM 50071, and *Pseudomonas mendocina* DSM 50017, with *P. aeruginosa* having the least sensitive N_2O-reducing system of the Pseudomonads. The inhibition by 5.5 kPa NO of whole cells grown anaerobically in the presence of nitrate ranged from 65 (*Ps. aeruginosa*) to 95% (*Ps. stutzeri*). The photo-denitrifier

TABLE V

DISTRIBUTION OF THE PRODUCTS OF DENITRIFICATION IN VARIOUS PSEUDOMONADS

Cells were grown in ACN medium under oxygen-limiting conditions. The distribution of gases in the headspace of reaction vials was determined after a reaction time of 40 min. The electron donor for the reaction was lactate; the initial amounts of substrates were as stated in Table I.

Organism	Specific activity (nkat·mg^{-1})			Products of NO_2^- reduction (%)		
	NO_2^- uptake	NO uptake	N_2O uptake	NO	N_2O	N_2
Ps. perfectomarina ATCC 14405	0.83	0.53	3.1	0	0	100
Ps. stutzeri ATCC 17588	0.45	0.54	2.3	4	96	0
Ps. stutzeri JM 300	0.72	–	2.9	88	11	1
Ps. aeruginosa ATCC 10145	0.30	0.42	2.7	12	78	10
Ps. fluorescens PJ 187	1.10	–	5.5	85	10	5
Ps. fluorescens PJ 188	0.47	0.08	0.11	90	2	8
Ps. fluorescens DSM 50111	0.55	0.13	6.2	100	0	0
Ps. chlororaphis B 560	0.22	–	0	88	12	0
Ps. chlororaphis B 561	1.08	–	0	10	90	0
Ps. aureofaciens ATCC 13985	0.58	0.6	0	100	0	0

Rhodobacter sphaeroides forma sp. *denitrificans* and *Alcaligenes* sp. strain NCIB 11015 showed inhibitions of 55 and 70% by 5.5 kPa NO in the assay system, respectively.

When the denitrifying system of Pseudomonads was expressed in the presence of nitrate under oxygen-limiting conditions ($K_L aC^*$ value as above), we observed a large variation in the distribution of end products of denitrification (Table V). The extremes were represented by *Ps. perfectomarina, Ps. stutzeri* ATCC 17588, and *Ps. fluorescens* DSM 50111, with their exclusive or nearly exclusive products under those particular conditions being N_2, N_2O, or NO, respectively. *Pseudomonas chlororaphis* and *Pseudomonas aureofaciens* showed no N_2O uptake or N_2 production due to the constitutive lack of N_2O reduction.

Discussion

Respiration of N_2O was inhibited by NO in *Ps. perfectomarina* and several other species of denitrifying bacteria. The sensitivity of the N_2O respiratory system to NO was correlated to the activity of NO reductase, whose expression depended on the terminal electron acceptor available during growth. Unlike other inhibitors of N_2O reduction, NO is involved in a yet to be clarified manner in the reaction sequence of bacterial denitrification.

Poor expression of NO reductase in cells grown with N_2O or under oxygen limitation together with the inhibition of N_2O reduction by NO may cause the premature termination of denitrification at the level of NO or N_2O in otherwise dinitrogen-evolving bacteria. N_2O-grown *Alcaligenes* sp. produced from nitrite mainly NO, even though high N_2O reductase activity was present [21]. In their natural environment, denitrifying bacteria may be exposed to similarly restrictive conditions, again causing the liberation of NO or N_2O [22,23].

The protein dependence and non-competitive nature of NO inhibition indicated covalent interaction within the N_2O respiratory system. One site was identified as N_2O reductase itself. N_2O reductase from *Ps. perfectomarina* was converted by NO from the purple form to a spectroscopically pink state. After removing NO, the enzyme was reconstituted by air oxidation nearly to its original purple form, although the catalytic activity was recovered only partially. EPR spectra of N_2O reductase indicated the presence of type I copper in this multi-copper protein [9], with which NO is likely to react. Although, at present, only preliminary structural data are available for N_2O reductase, some parallels are suggested to the reactivity of type I copper centers in ceruloplasmin. Ceruloplasmin, a well-studied blue multi-copper protein, has two non-equivalent type I copper centers [24,25]. They are differently affected on reduction by NO [25]. The reduction of the type I copper centers is reversible on removal of NO and reoxidation of ceruloplasmin by air, similar to that observed with N_2O reductase. More detailed studies of the binding and reaction kinetics of this bacterial copper enzyme with NO and other ligands are in progress.

Acknowledgement

This work was supported by the Deutsche Forschungsgemeinschaft.

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