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DISCRIMINATION OF ASCORBATE-DEPENDENT NONENZYMATIC AND ENZYMATIC, MEMBRANE-BOUND REDUCTION OF NITRIC OXIDE IN DENITRIFYING *PSEUDOMONAS PERFECTOMARINUS*

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The marine nitrite-respiring (denitrifying) bacterium, *Pseudomonas perfectomarinus*, catalyzes by a membrane-bound enzyme the reduction of nitric oxide to nitrous oxide with ascorbate-reduced phenazine methosulfate as electron donor. The entire nitric oxide-reducing capability of a cell-free system was membrane bound and this process was studied with respect to pH and substrate dependency. The enzymatic process was perturbed by an identical nonenzymatic reduction by iron(II) ascorbate in neutral to alkaline aqueous solution. 2 mol nitric oxide and 1 mol ascorbate were consumed per mol nitrous oxide formed. Enzymatic and nonenzymatic processes were discriminated by their differential behavior towards pH and metal-chelating agents. The pH optimum for the enzymatic and nonenzymatic reaction was 5.2 and greater than 7.0, respectively. EDTA (10 mM) inhibited the nonenzymatic reduction completely without interfering with the membrane-bound activity. The nonenzymatic system mimics the reaction of nitric oxide reductase and could serve as a model to study the formation of the N–N bond in denitrification. Enzymatic generation of nitric oxide by cytochrome *cd* and subsequent nonenzymatic reduction to nitrous oxide simulate an overall quasi-enzymatic nitrous oxide formation by cytochrome *cd*. The nonenzymatic reduction of nitric oxide might have occurred in previous work due to the ubiquitous use of ascorbate in studies on nitrite respiration and the likelihood of adventitious iron in biological samples.

Introduction

Nitrogenous oxides are used by certain facultative anaerobic bacteria as a substitute for oxygen in electron-transport phosphorylation. This requires the synthesis of several terminal oxidoreductases during the transition from aerobic to anaerobic metabolism [1,2]. From a chemical viewpoint, the most interesting reaction of nitrite respiration is the formation of the N–N bond, which is broken again only in nitrogen fixation under considerable expenditure of metabolic energy. There is ample evidence for N_2O as the first stable N–N-bonding obligatory intermediate [3].

The marine nitrite respirer (denitrifier) *Pseudomonas perfectomarinus* reduces nitrite to N_2. However, in the presence of acetylene [4] or under copper deficiency [5], nitrite respiration is terminated at the level of N_2O. Previous work with cell-free preparations has shown the potential of this organism for biochemical studies on nitrite respiration and has detailed the partial reactions, although the corresponding enzymes were not

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Abbreviations: Tricine, *N*-tris(hydroxymethyl)methylglycine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; Tes, *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid.

characterized [6–8]. We have identified cytochrome *cd* (ferrocytochrome c_2:oxygen oxidoreductase, EC 1.9.3.2) in this organism as nitrite reductase [9,10], and have tentatively assigned a membrane-bound activity of N_2O formation from nitrite to this enzyme. The same membrane fraction showed also a high NO-reducing activity [9] and we have further established that the last step of nitrite respiration, the conversion of N_2O to N_2, depends on copper [5].

During attempts to solublize and characterize the membrane-bound nitric oxide reductase, we found that the enzymatic reaction was perturbed by an identical nonenzymatic process. Iron(II) ascorbate in neutral or alkaline solution was a highly efficient system to reduce NO stoichiometrically to N_2O. The nearly exclusive use of ascorbate as electron donor in biochemical studies of nitrite respiration, together with the ubiquity of iron in biological samples, particularly in crude extracts and partially purified fractions, implies a risk in following in part a nonenzymatic reaction. To obtain accurate activity measurements for physiological reactions involving NO, we have studied its chemical reduction by ascorbate and Fe^{2+} in more detail. We describe here basic properties of the membrane-bound enzymatic and the nonenzymatic reaction and provide ways by which the two processes can be discriminated.

Materials and Methods

Organism and growth conditions. *Ps. perfectomarinus* (ATCC 14405) was grown in a medium containing (per liter): NaCl, 15 g; $MgSO_4 \cdot 7H_2O$, 5 g; $CaCl_2 \cdot 2H_2O$, 0.76 g; $NaNO_3$, 1.0 g; KCl, 1.0 g; trisodium citrate $\cdot 2H_2O$, 250 mg; tryptone (Difco), 5 g; yeast extract (Merck), 1.5 g; and $FeCl_3 \cdot 6H_2O$, 25 mg. Growth conditions were described in detail by Zumft and Vega [9].

Membrane preparation. Thawed cell paste or fresh cells were resuspended in 2 vol. 100 mM Tris-HCl buffer, pH 7.5. 2 mg DNAase and RNAase were added per 10 g cells to reduce the viscosity on cell breakage. Cells were broken by passing them three times through a French press in the cold at 110 MPa. To remove intact cells the extract was centrifuged at 4°C for 15 min at $10000 \times g$. The supernatant of this centrifugation was spun again at $29000 \times g$ for 60 min. The resulting pellet was discarded and the membrane-containing supernatant was subjected to a high-speed centrifugation at $235800 \times g$ for 120 min. The membrane pellet obtained after this centrifugation was resuspended in 0.1 M Tris-HCl buffer, pH 7.1, and stored at −15°C. No significant difference was observed between freshly prepared membranes and those stored in the frozen state.

Reaction mixture. Standard assay mixtures were prepared in 12 ml reaction vials with a liquid volume of 3.0 ml. They contained sodium ascorbate, 100 μmol; $FeSO_4$, 3 μmol; a buffering component, and when membranes were used, also phenazine methosulfate, 0.5 μmol. The vials were filled with helium on a vacuum line and adjusted to have an overpressure of 15 mbar. All reactions were started by addition of the electron acceptor, i.e., 40 μmol nitrite or 41.7 μmol NO. The vials were incubated horizontally at 30 ± 1°C in a reciprocal shaking water bath at 100 cycles per min.

Gas analysis. To avoid air contamination during sampling and injection, additions of NO were made without pressure equilibration between the inside and outside of the stoppered reaction vials. For the determination of N_2, NO and N_2O, 100 μl of gas from the head space were withdrawn with a gas-tight syringe and injected onto an aged Porapak Q column, 80-100 mesh, of 2.50 m length. Helium was the carrier gas at a flow rate of 30 ml/min. The column temperature was 40°C. The difference between the solubility of NO and N_2O in aqueous solutions, and a small loss of sample due to overpressure were accounted for by making calibrations under the same pressures and concentrations as those for the test samples. N_2O was identified by its infrared absorption spectrum in addition to gas chromatography.

Protein determination. Protein was estimated by the method of Lowry et al. [36]. Membrane samples were digested with 1 M NaOH for at least 10 h, and bovine serum albumin was used as a standard.

Materials. Ascorbic acid was purchased from four commercial sources (Merck, Darmstadt; Serva, Heidelberg; Sigma, München; EGA-Chemie, Steinheim) and neutralized with NaOH. NADH and FMN were from Sigma, München or Serva, Heidelberg. The provenience of these com-

pounds was without influence on the results. Phenazine methosulfate and buffer substances were from Sigma Chemical Co., München; Tricine was purchased from Serva, Heidelberg. N_2O (greater than 99.5% purity in the liquid phase) was from Matheson, Belgium. NO was from Linde (Höllriegelskreuth, F.R.G.) and contained about 0.5% N_2O. For experiments where the N_2O background was interfering, NO was prepared in the laboratory from acidified $FeSO_4$ and $NaNO_2$. This procedure gave NO which did not contain detectable amounts of N_2O.

Results

Enzymatic and nonenzymatic reduction of NO

The properties of respiratory nitrite reduction by a cytoplasmic membrane fraction from *Ps. perfectomarinus* were previously described [9]. At the expense of ascorbate/phenazine methosulfate or NADH-FMN these membranes reduced both nitrite and NO to N_2O. We have found now that the rate of N_2O formation from NO could be enhanced by addition of small amounts of iron,

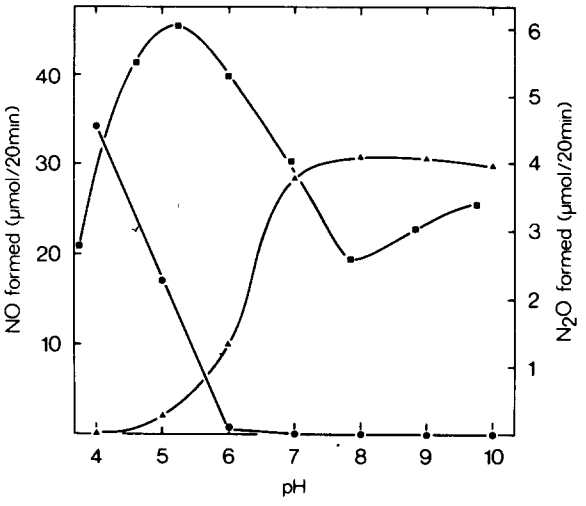

Fig. 1. pH profiles for iron(II) ascorbate-dependent reduction of nitrite to NO, and NO to N_2O, and enzymatic reduction of NO by membranes. The reaction mixture (3 ml) for the nonenzymatic process contained a mixed buffering system of 200 μmol each of glycine, Tes, Tris and potassium phthalate, 3 μmol $FeSO_4$ and 100 μmol sodium ascorbate. The reaction mixture for the enzymatic process contained 200 μmol each of glycine, potassium phthtalate, Hepes, imidazole and potassium phosphate, in addition to the standard amounts of ascorbate and phenazine methosulfate. The protein content was 4.1 mg. The desired pH values were adjusted with NaOH or HCl. The reactions were started by the addition of 100 μmol $NaNO_2$ or 41.7 μmol NO. (●——●) Nonenzymatic formation of NO from $NaNO_2$, (▲——▲) nonenzymatic formation of N_2O from NO, (■——■) enzymatic formation of N_2O from NO by membranes.

TABLE I

ENZYMATIC AND NONENZYMATIC REDUCTION OF NO TO N_2O

The reaction mixtures contained in 3 ml: 400 μmol Tris-HCl (pH 7.5); 50 μmol sodium ascorbate; 0.5 μmol phenazine methosulfate (PMS); or 50 μmol NADH together with 1 mg FMN. Where stated, 3 μmol $FeSO_4$ were added. The protein content of the membrane-containing samples was 11 mg.

Reaction mixtures	N_2O produced (nmol/min)
PMS	0
Ascorbate	6
Ascorbate + PMS	6
Ascorbate + PMS + Fe	83
Ascorbate + PMS + membranes	152
Ascorbate + PMS + Fe + membranes	228
NADH	0
FMN	0
NADH + FMN	130
NADH + FMN + Fe	378
NADH + FMN + membranes	329
NADH + FMN + Fe + membranes	688

and that on the other hand NO reduction proceeded to a considerable extent with these electron donors also in the absence of membranes (Table I). Enzymatic and nonenzymatic processes superimposed on each other additively. The magnitude of the nonenzymatic process at neutral to alkaline pH and in a homogeneous system appears not to have been recognized previously and requires procedures to separate it from enzymatic reduction of NO. Because the reduction of NO by iron(II) ascorbate is closer to mechanistic understanding, we have studied it in more detail than that by NADH-FMN.

Characterization of NO reduction by iron(II) ascorbate: Minimal requirements

NO is readily reduced to N_2O in aqueous solu-

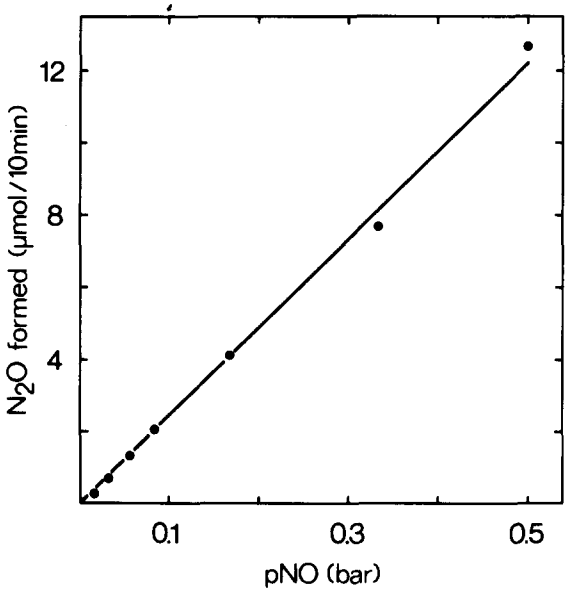

Fig. 2. Reduction of NO to N_2O by iron(II) ascorbate as a function of the partial pressure of NO. The reaction mixture contained 100 µmol sodium ascorbate, 3 µmol $FeSO_4$ and 400 µmol Tris-HCl, pH 7.2, in a final volume of 3.0 ml. The reaction was started by addition of NO (8–500 mbar).

tions above pH 7.0 by ascorbate in the presence of catalytic amounts of Fe^{2+}. The presence of buffering components such as Tris, imidazole, or phthalate was without effect on the reaction. A weak suppression of N_2O formation was observed with Good's buffers at 100 mM when compared to the control with Tris-HCl: Hepes, 20%; Mes, 15%; Mops, 18%; and Tes, 17%. However, Tricine at 100 mM (pH 7.0) inhibited the reaction to 75%. Phosphate was strongly inhibitory and was usually not used as buffering sytem due to the precipitation of secondary iron phosphate unless the inhibitory action was of advantage in lowering nonenzymatic reduction. The inhibitory effect of the buffering components presumably is a consequence of their metal-chelating properties [11].

The pH profile of NO formation from nitrite and N_2O formation from NO by iron(II) ascorbate is shown in Fig. 1. Nitrite was reduced only below pH 6, whereas reduction of NO to N_2O increased sharply between pH 6 and 7, and was constant up to pH 10. Changes of pH in the reaction mixture up to a total consumption of 30 µmol NO were smaller than 0.3 units.

Effect of reactant concentrations and influence of metal ligands

The reduction of NO as a function of its partial pressure is shown in Fig. 2. Within the range 8–500 mbar the reaction was first order with respect to NO and could not be saturated with this substrate. Therefore, it was not possible to determine the reduction of NO as a function of the Fe^{2+} concentration only. The effect of Fe^{2+} was determined thus at three arbitrary concentrations of NO (Fig. 3). The small rate observed when no iron was added could be due to adventitious iron in chemicals or buffer solutions, since no particular precautions were taken to remove it from individual components. The dependency on Fe^{2+} was found to be biphasic with a break at about 0.1 mM iron. At this concentration the gas-liquid partitioning presumably becomes rate limiting because of the low solubility of NO in an aqueous system. Based on the data from Fig. 3 at low iron concentration, the molar activity of Fe^{2+} was about 0.1 s^{-1} (NO reduction). Of other metals

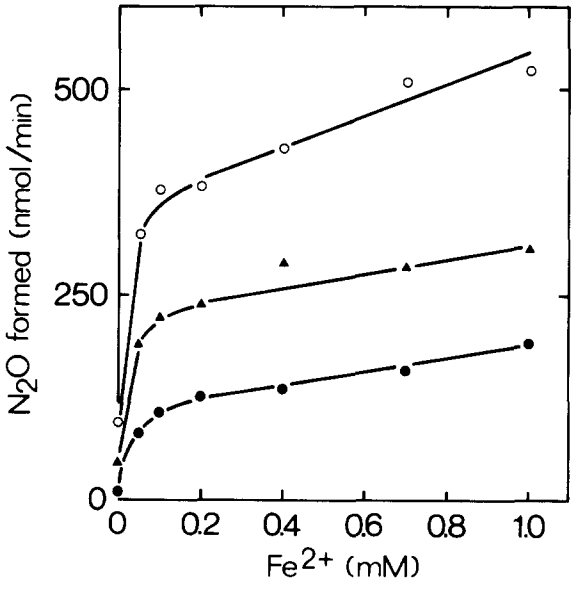

Fig. 3. Rate of N_2O formation form NO by iron(II) ascorbate as a function of the Fe^{2+} concentration. The reaction mixtures contained 400 µmol Tris-HCl, pH 7.5, and 100 µmol sodium ascorbate. $FeSO_4$ was varied from 0 to 1 mM. Three reaction series were started with different partial pressures of NO: (●——●) 42 mbar, (▲——▲) 83 mbar, and (○——○) 167 mbar.

TABLE II

EFFECT OF METAL LIGANDS ON THE REDUCTION OF NO TO N_2O BY IRON(II) ASCORBATE

All ligands were added at a concentration of 10 mM. The reaction mixture contained the standard components in 133 mM Tris-HCl, pH 7.1.

Ligand	N_2O (nmol/min)	N_2O (%of control)
None	268	100
Sodium diethyldithiocarbaminate	0	0
o-Phenanthroline	172	64
Trisodium citrate	88	33
Disodium EDTA	0	0
2,2'-Bipyridyl	0	0
KF	257	96
KSCN	265	99
KCN	72	27
Cysteine	40	15
Lysine	306	114
Histidine	233	87
Asparagine	324	121
Glycine	276	103

tested, only copper gave about 5–10% of the activity compared to an identical iron-containing system.

Several metal-complexing agents were tested for their capability of interacting with the iron(II) ascorbate system in an inhibitory or stimulatory manner. Table II shows that strong iron ligands interfered with NO reduction. At a concentration of 10 mM the most effective inhibitors were EDTA, diethyldithiocarbaminate and 2,2'-bipyridyl. Citrate and cyanide decreased the reaction rate to 33 and 27%, respectively. Thiocyanate and *o*-phenanthroline which are strong ligands for Fe^{3+}, showed no effect or only a moderate inhibitory one. Whereas histidine was slightly, and cysteine strongly inhibitory, the amino acids lysine and asparagine enhanced NO reduction somewhat (Table II).

Stoichiometries of reduction of NO by iron(II) ascorbate

Table III shows the nearly quantitative conversion of NO to N_2O according to the equation:

$$2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O \quad (1)$$

TABLE III

STOICHIOMETRY OF NO REDUCTION BY IRON(II) ASCORBATE

Conditions: 133 mM Tris-HCl, pH 7.1; 1 mM $FeSO_4$; 33.3 mM ascorbate.

NO (μmol)	Reaction time 3 h		Reaction time 15 h	
	N_2O (μmol)	% of theoretical value	N_2O (μmol	% of theoretical value
2.08	0.89	85.6	0.9	86.5
4.17	1.88	90.2	1.87	89.7
8.33	4.04	97.0	3.99	95.8
16.7	7.54	90.3	7.69	92.1

By gas chromatography no reaction products besides N_2O could be detected; in particular no N_2 was found. In addition to the chromatographic evidence, N_2O was identified by its vibration frequency at $\upsilon = 2224\ cm^{-1}$. Disproportionation of NO to NO_2 and N_2O, as well as the stoichiometric formation of a stable nitrosyl complex are not compatible with the data from Table III.

The stoichiometric electron transfer from ascorbate and Fe^{2+} to NO is shown in Table IV. According to Eqn. 1, NO reduction requires two electrons per molecule of N_2O produced. The experimental data for a reaction time of 12 h were found to be in agreement with this value and show that the electrons of Fe^{2+} are also accounted for.

TABLE IV

STOICHIOMETRY OF ELECTRON TRANSFER

Conditions: 133 mM Tris-HCl, pH 7.1; the reactions were started by addition of 41.7 μmol NO.

Ascorbate (μmol)	Fe^{2+} (μmol)	Total e^- (μmol)	N_2O found (μmol)		e^-/N_2O (12 h)
			2 h	12 h [a]	
2.5	5	10	3.66	5.05	1.98
5.0	5	15	5.41	8.15	1.84
10.0	5	25	8.06	12.5	2.0

[a] The reaction was completed at 12 h and no further N_2O formation was observed on longer reaction times.

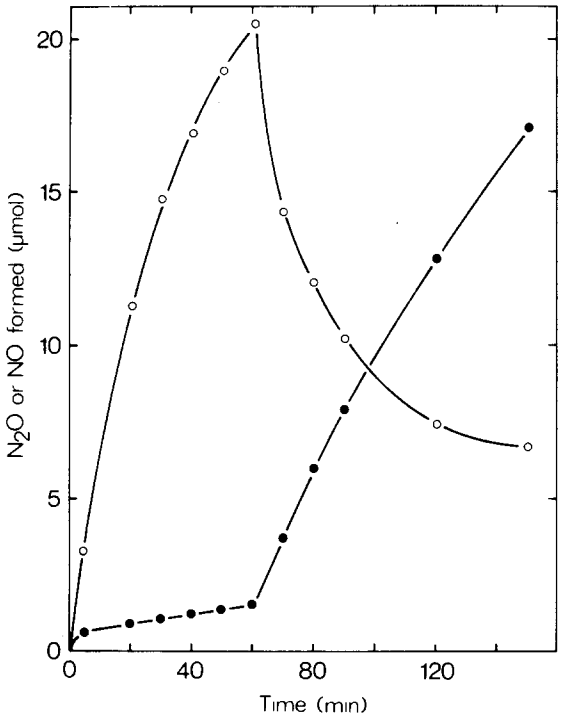

Fig. 4. Formation of N_2O by cytochrome *cd* in the presence of Fe^{2+}. The reaction mixture contained in 133 mM Tris-HCl, pH 7.5, 100 μmol sodium ascorbate, 0.5 μmol phenazine methosulfate and 140 μg purified cytochrome *cd* from *Pseudomonas perfectomarinus*. The reaction was started by the addition of 100 μmol $NaNO_2$. At 60 min, 3 μmol $FeSO_4$ were added. (○——○) NO, (●——●) N_2O.

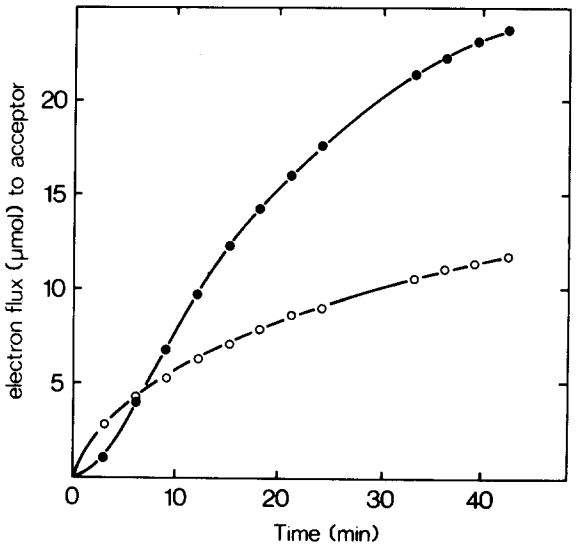

Fig. 5. N_2O as apparent product of nitrite reduction by cytochrome *cd* in the presence of Fe^{2+}. The assay mixture contained in a final volume of 3.0 ml, 300 μmol Tris-HCl, pH 7.5, 3 μmol $FeSO_4$, 300 μg purified cytochrome *cd*, 100 μmol sodium ascorbate, and 0.5 μmol phenazine methosulfate. The reaction was started by the addition of 50 μmol $NaNO_2$. (○——○) NO, (●——●) N_2O.

Coupling of nonenzymatic reduction of NO to an enzymatic NO generator

With ascorbate/phenazine methosulfate as electron donor and nitrite as substrate, we observed the formation of NO and N_2O in various fractions during the purification of nitrite reductase (cytochrome *cd*) (Ref. 9, and unpublished results). The amount of N_2O relative to NO decreased from about 25% in the 235800 × *g* supernatant to less than 10% with the homogeneous enzyme. However, on addition of iron to the assay mixture containing purified enzyme, the formation of N_2O increased dramatically, whereas the initially formed NO decreased to a lower steady-state level (Fig. 4). When iron was added to the reaction mixture together with cytochrome *cd* a simultaneous production of NO and N_2O was observed, the latter with a short lag phase (Fig. 5). The total electron flux in this system was considerably greater to N_2O than that to NO. The same sample showed no gas production from nitrite when heat-denatured cytochrome *cd* was used.

Properties of the membrane-bound nitric oxide reductase activity

Attempts to substitute ascorbate/phenazine methosulfate or NADH-FMN by electron donors which would not cause chemical NO reduction but allow at the same time high enzymatic activities were unsuccessful, as were previous attempts by others with cell-free extracts from *Alcaligenes* [12,13]. The apparent K_m for phenazine methosulfate with the membrane system was 2 μM. The pH profile of the enzymatic reduction of NO by membranes from *Ps. perfectomarinus* was similar to that of the particulate NO-reducing fraction from *Alcaligenes faecalis* [14]. We observed an activity maximum at about pH 5.2. Towards alkaline pH the activity decreased sharply, but increased again slightly above pH 7.5, presumably because of a higher contribution of nonenzymatic reduction of NO (Fig. 1).

TABLE V

DISTRIBUTION OF ENZYMATIC REDUCTION OF NO WITH ASCORBATE PHENAZINE METHOSULFATE AS ELECTRON DONORS IN *PSEUDOMONAS PERFECTOMARINUS*

Conditions as under Materials and Methods except 133 mM acetate buffer, pH 5.5; no addition of $FeSO_4$.

Fraction	Protein (mg)	Activity (nkat)	Spec. act. (nkat/mg)	Yield (%)
Crude extract	1415	452.8	0.320	100
Pellet (29000× *g*)	108	85.7	0.793	19
Supernatant (29000× *g*)	1302	507.8	0.390	112
Membranes (235800× *g*)	580	976.3	1.683	216
Supernatant (235800× *g*)	721	27.2	0.038	6

The effect of the partial pressure of NO on the enzymatic reaction was tested in the range 11–222 mbar (Fig. 6). At a low membrane concentration we observed a saturation-type response, whereas at higher membrane concentrations an optimal partial pressure of NO was observed at about 0.1 bar. This finding is similar to the NO-reducing activity of an *Alcaligenes* sp. which had an optimum at 10% NO in the gas phase [12].

The ligands, which at a 10-fold molar excess over iron inhibited completely the nonenzymatic reaction (cf. Table II), were also examined for their effect on the membrane-bound nitric oxide reductase. Neither 10 mM EDTA nor cysteine, which at this concentration were strong inhibitors of the nonenzymatic reaction, affected the rate of the enzymatic process, whereas 10 mM diethyldi-

TABLE VI

SUPRESSION BY EDTA OF NONENZYMATIC REDUCTION OF NO IN A MEMBRANE FRACTION FROM *PSEUDOMONAS PERFECTOMARINUS*

Conditions: 133 mM Tris-HCl, pH 7.1; 1 mM $FeSO_4$; 10 mM disodium EDTA; 5 mg membrane protein; 0.17 mM phenazine methosulfate (PMS); NO 110 mbar. Asc., ascorbate (33.3 mM).

Components	N_2O formed (%)
Asc. + PMS	0–2
Asc. + PMS + Fe	168
Asc. + PMS + Fe + EDTA	0–2
Membranes + Asc. + PMS	100
Membranes + Asc. + PMS + EDTA	79
Dialyzed membranes [a] + Asc. + PMS	77
Dialyzed membranes [a] + Asc. + PMS + EDTA	75
Membranes + Asc. + PMS + Fe	237
Membranes + Asc. + PMS + Fe + EDTA	82

[a] Membranes were dialyzed for 12 h at 4°C against 133 mM Tris-HCl, pH 7.1.

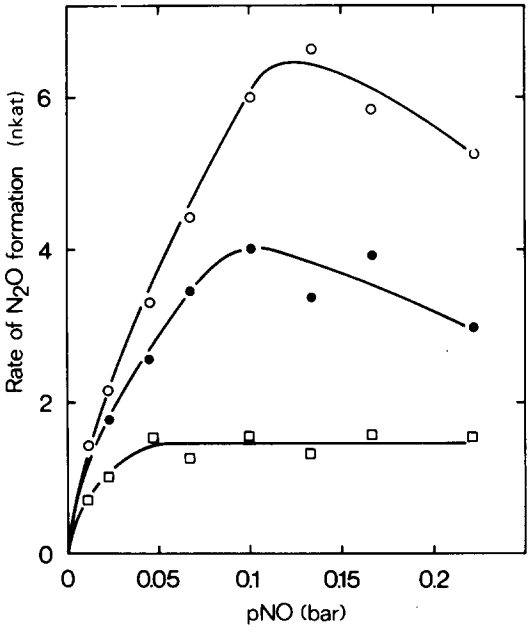

Fig. 6. Rate dependency of NO reduction on the membrane and NO concentration. Standard reaction conditions with 67 mM acetate buffer, pH 5.5. The protein concentration was 0.8 mg/ml (□——□), 2.1 mg/ml (●——●), and 4.2 mg/ml (○——○).

thiocarbaminate and citrate both inhibited the membrane-bound activity about 70%.

The distribution of NO-reducting activity on fractionated centrifugation of a cell-free extract is shown in Table V. The activity assays were run at pH 5.5, where the rate of nonenzymatic N_2O formation was reduced to less than 15%. The ascorbate/phenazine methosulfate-dependent activity was found exclusively in the membrane fraction sedimenting at $235\,800 \times g$. On high-speed centrifugation we found a sharp increase in the NO-reducing activity of the membrane fraction. Preliminary evidence indicates that the soluble cell fraction of *Ps. perfectomarinus* contains an inhibitory agent which suppresses membrane-bound reduction of NO. A similar observation was made by Matsubara and Iwasaki [13] with the particulate NO-reducing fraction from *A. faecalis.*

Table VI demonstrates elimination of the nonenzymatic contribution of NO reduction on addition of EDTA to the membrane fraction. In this case the process was studied at pH 7.1 to have the nonenzymatic reaction operating at its maximal rate. About 20% of the total activity was due to nonenzymatic reduction. When 100 μM iron was added, N_2O formation increased 2.4-fold over the basal activity; however, it still could be fully suppressed by 10 mM EDTA.

Discussion

NO is formed by two types of respiratory nitrite reductases [9,15–22] and remains the subject of discussions with respect to its functional position in nitrite respiration [2,23–27]. If NO is the only and final product of the presently known nitrite reductases, an additional nitric oxide reductase is required for its further conversion to N_2O. The physiological reduction of NO to N_2O is usually attributed to a membrane-bound enzyme which has not yet been characterized, but preliminary evidence shows it to be different from nitrite reductase. Phenazine methosulfate-ascorbate-dependent particulate enzymes were found in *Alicaligenes* sp. (formerly known as *Pseudomonas denitrificans*) [12,28] and in *A. faecalis* [13] where this activity was indicated not to be due to cytochrome *cd*, even though the latter, when purified from the soluble cell fraction, had weak NO-reducing activity [14]. We have found in *Ps. perfectomarinus* an NO-reducing activity which is associated with the cytoplasmic membrane [9] and have shown here that it accounts for the total NO-reducing ability of a cell-free system when assayed with ascorbate/phenazine methosulfate (nonenzymatic reduction was largely suppressed by low pH). Payne and co-workers [7,8] described a soluble nitric oxide reductase in the same bacterium which was active with a poorly defined reductant system of malate, endogenous dehyddrogenase, NAD^+, $NADP^+$, FMN and FAD. The authors also indicated that the minimal particle weight of their enzyme was 800000, and since it eluted in the void volume of Sephadex G-200 it could as well have been larger. Previous attempts to isolate nitric oxide reductase focussed on *Ps. aeruginosa* [29] and *Ps. stutzeri.* For the latter, reduction of NO to N_2 was claimed [30]. Gases in this case were measured by a manometric technique which has led in a similar situation to an erroneous assignment (cf. Ref. 31); alternatively, a complex of nitric oxide and nitrous oxide reductase would have to be studied inadvertently. This, however, is unlikely considering the lability of nitrous oxide reductase.

Ascorbate/phenazine methosulfate and NADH-FMN have been used nearly exclusively in studies on the formation and the reduction of NO, since they were the only electron donors that sustained high in vitro activities. It is extremely difficult, if not impossible, to assess where in previous work a nonenzymatic reduction of NO might have contributed to the observed reactions, because controls might not have been stated expressly. N_2O formation by nitrite reductase as the consecutive process of enzymatic and nonenzymatic reaction is particularly prone to fallacy. The classical procedure to establish an enzyme-catalyzed reaction will fail, unless specific tests for nonenzymatic NO reduction are done separately.

An opposite pH dependency and a differential sensitivity towards metal ligands are two useful properties to discriminate enzymatic and nonenzymatic reduction of NO by iron(II) ascorbate. The membrane-bound activity of *Ps. perfectomarinus* had its optimum at pH 5.2, and was studied in a range where the contribution of nonenzymatic reduction was small. At higher pH the

nonenzymatic reaction increased rapidly but was suppressed effectively by EDTA. This is of importance for nitric oxide reductases with pH optima at neutral or weakly alkaline pH [12,29]. Awareness of the ease of nonenzymatic reduction of NO will be relevant for further attempts to characterize nitric oxide reductase and for product studies with nitrite reductase. A third point deserving attention is the use of NO in bacterial growth studies.

Reduction of NO to N_2O by iron(II) ascorbate mimics nitric oxide reductase and provides a model for possible mechanistic insights. Contrasting with a recent hypothesis by Averill and Tiedje [23] that suggested reaction of a ferrous-nitrosyl complex with nitrite to yield coordinated N_2O_3, and further reduction via oxyhyponitrite to N_2O, the iron(II) ascorbate system, in the absence of nitrite, must operate via an alternative mechanism and represents at the same time a novel type of reaction for the reduction of NO to N_2O. The brown color of the reaction mixture is indicative of the formation of a nitrosyl complex. Since iron(II) without ascorbate did not reduce NO under standard conditions, ascorbate should be an integral part of the iron-nitrosyl complex that allows transfer of its electrons to NO. To the best of our knowledge, the reactivity and structure of an ascorbate-iron-nitrosyl complex has not been studied yet. Visser [32] proposed from theroretical considerations bridging of the O-1 and O-2 atoms of ascorbate by iron and the formation of a ferryl complex as the active species in a powerful hydroxylating system consisting of iron(II) ascorbate and EDTA (Udenfriend system) [33]. Hydroxylation does not necessarily require EDTA, being somewhat slower without it [34]. Thus, we assume a high degree of analogy between Udenfriend's system and ours. Chemical studies have indicated the presence of five- and six-coordinate nitrosyl complexes [35]. Both are EPR active, thus EPR should be a useful probe for further studies of the iron(II) ascorbate system. Preliminary evidence shows the system to be paramagnetic under turnover conditions (unpublished results). Since stepwise addition of NO to Fe^{2+} would give only a diamagnetic $\{Fe(NO)_2\}^8$ species, a more complex reaction is likely to occur to give an EPR-active species, e.g., $\{Fe(NO)_2\}^9$ or $[Fe(NO)_2]^+$.

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