Oscillations of nitric oxide concentration in the perturbed denitrification pathway of *Paracoccus denitrificans*

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The metabolism of nitric oxide in *Paracoccus denitrificans* has been studied using a Clark-type electrode. The uncoupler carbonyl cyanide *m*-chlorophenylhydrazine (CCCP) and the SH reagent *N*-ethylmaleimide, both of which released nitric oxide from cells respiring nitrite, were found to be efficient inhibitors of nitric oxide reductase activity. Control experiments with another uncoupler, pentachlorophenol, showed that the inhibitory effect of CCCP was not the result of a decrease in membrane potential. The denitrification pathway in cells with partly inhibited nitric oxide reductase, or in a reconstituted system containing purified nitrite reductase and membrane vesicles, exhibited marked sustained oscillations of nitric oxide concentration. The occurrence of the oscillations was strictly dependent on the initial concentration of nitrite. The observed oscillatory kinetics is considered to reflect two regulatory signals destabilizing the denitrification pathway, namely the inhibition of nitric oxide reductase by nitric oxide and/or by nitrite.

INTRODUCTION

Denitrification is by definition the special case of bacterial dissimilatory nitrate reduction where the end products are gaseous. Considerable evidence has appeared over the past few years that the conversion of nitrate into nitrogen in cells of *Paracoccus denitrificans* involves four separate enzymes (membrane-bound nitrate and nitric oxide reductases and soluble periplasmic nitrite and nitrous oxide reductases). Consequently, the whole process can be described as a linear sequence of four enzymic reactions (Scheme 1) [see Stouthamer (1991) for a review]. In spite of the large number of studies devoted to the structure of denitrifying enzymes, details on how the electron flow in the pathway is regulated have not yet been fully understood. A mathematical model of denitrification based on simple Michaelis–Menten kinetics for each reductive step in Scheme 1 was assumed to be sufficient to account for the observed patterns of the transient NO_2^- , NO and N_2O accumulation in resting-cell suspensions of three denitrifiers (Betlach & Tiedje, 1981). However, there are some observations suggesting this assumption may not be completely accurate in *P. denitrificans*. For example, it has been demonstrated that denitrifying enzymes in this bacterium do not work independently but compete for a nearly constant limited flow of redox equivalents supplied from dehydrogenases (Alefounder *et al.*, 1983; Kučera *et al.*, 1983a,b, 1986b, 1987b) and that nitrite reductase and nitrous oxide reductase are inhibited by NO (Dhesi & Timkovich, 1984; Carr *et al.*, 1989) similarly to terminal oxidases (Kučera *et al.*, 1986a, 1987a; Carr & Ferguson, 1990a; Kučera & Skládal, 1990a).

A recent investigation in this laboratory established that the uncoupler carbonyl cyanide 3-chlorophenylhydrazine (CCCP) added in micromolar concentrations to the cells of *P. denitrificans* respiring nitrite brought about an intensive release of NO which could be trapped by horse heart cytochrome *c*. Under certain experimental conditions damped oscillations in the concentration of the cytochrome *c*–NO complex took place and reduced cytochrome *c* accumulated in the reaction mixture (Kučera, 1989). These observations raised two problems that are worth considering. The first question is whether the uncoupler affects the denitrification process solely by lowering the membrane

potential or by a more specific interaction with the denitrifying enzymes. The second question concerns the role of exogenous cytochrome *c* and the relevance of its redox reaction with NO (yielding NO_2^- and reduced cytochrome) to the oscillatory behaviour of the system.

With the aim to answer the above questions, experiments were carried out in the absence of mammalian cytochrome *c* using a Clark-type electrode for monitoring the concentration of NO. The data reported here: (i) identify the NO-reducing activity as an important target site for a variety of inhibitory agents (lipophilic uncouplers/SH reagents and small molecules such as NO, NO_2^- and NH_2OH) and (ii) provide evidence for the oscillations of NO concentration in the denitrification pathway with decreased activity of nitric oxide reductase.

MATERIALS AND METHODS

P. denitrificans (NCIB 8944), obtained from the Czechoslovak Collection of Micro-organisms as CCM 982, was grown anaerobically at 30 °C for 22 h in 1-litre bath cultures on minimal medium containing 50 mM-succinate and 10 mM-nitrate (Kučera *et al.*, 1983a). Stock cell suspensions were kept on ice and used within 12 h after harvest. The preparation of periplasmic and membrane fractions and the isolation of nitrite reductase were as described (Kučera & Skládal, 1990a,b).

NO was generated from a solution of KNO_3 (9 g/100 ml) in saturated $\text{K}_4\text{Fe}(\text{CN})_6$ by addition of dilute acetic acid under N_2 atmosphere (Brauer, 1954). The gas was passed through a KOH trap to remove trace amounts of NO_2 and collected by bubbling through 10 ml of oxygen-free 0.1 M-sodium phosphate, pH 7.3, containing 10 mM-glucose, 20 units of glucose oxidase (*Aspergillus niger*) and 200 units of catalase (bovine liver) to ensure anaerobiosis (Carr *et al.*, 1989). NO concentration in saturated solutions was calculated with the use of published values of Bunsen's coefficient (Gmelins Handbuch, 1936).

The production and uptake of NO was monitored by an amperometric technique based on that of Zimmer *et al.* (1985). A Clark-type probe consisting of a gold cathode 0.7 cm in diameter, a conical silver anode electrolytically coated with AgCl, a saturated KCl electrolyte and a polypropylene (Mikroten) membrane permeable to gases was used. The NO

Abbreviations used: CCCP, carbonyl cyanide 3-chlorophenylhydrazine; FCCP, carbonyl cyanide 4-trifluoromethoxyphenylhydrazine; NEM, *N*-ethylmaleimide; NBS, *N*-bromosuccinimide; PCP, pentachlorophenol; TMPD, *NNN'*-tetramethyl-*p*-phenylenediamine.

Scheme 1.

probe was fitted to the top of a closed electromagnetically stirred chamber of 2.1 ml volume equilibrated at 30 °C, to which additions could be made with a Hamilton syringe through a narrow entry port. A polarizing voltage of -0.80 V was applied between the cathode and anode, and the signal from the cathode was amplified and recorded by a universal polarograph OH-105 (Radelkis, Budapest, Hungary). The relation between the output current and NO concentration was linear at concentrations up to 0.3 mM with the slope equal to 0.012 ± 0.003 A/M (the mean $\pm$ S.E.M. of eight separate experiments); the selectivity factor for NO over N_2O was typically about 40. NO_2^- concentration in the withdrawn samples was determined colorimetrically (Snell & Snell, 1949) and corrected for NO_2^- formed via the oxygen-mediated decomposition of NO during the assay (1 mol of NO_2^- /2 mol of NO oxidized).

13-Ethylberberine fluorescence reflecting the membrane energization (Mikeš & Kovář, 1981; Mikeš & Dadák, 1983) was measured in a 1 cm cuvette using an Aminco-Bowman spectrofluorimeter at excitation wavelength 420 nm (2 mm slit) and emission wavelength 520 nm (1 mm slit). Output from the fluorimeter detection system was transmitted to a strip-chart recorder to provide a permanent experimental record. Oxidoreduction of intracellular cytochrome *c* was followed with a Shimadzu U.V.-3000 spectrophotometer at the wavelength couple 550–540 nm.

The sources of the chemicals were as follows. CCCP, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP), glucose oxidase and *N*-bromosuccinimide (NBS) were purchased from Sigma Chemical Co.; *NNN'*-tetramethyl-*p*-phenylenediamine (TMPD) was from Fluka; *N*-ethylmaleimide (NEM) was from Koch-Light. 13-Ethylberberine and pentachlorophenol (PCP) were kindly supplied by Dr. Mikeš from our Department. Other chemicals were reagent grade or of the highest quality available.

RESULTS

Accumulation of NO during the reduction of NO_2^-

In agreement with a previous report (Kučera, 1989), it was observed that cells of *P. denitrificans* respiring low concentrations of NO_2^- in the presence of succinate failed to accumulate amperometrically detectable NO. The same kind of result was obtained with the mixture of 0.2 mM-TMPD and 5 mM-ascorbate, donating electrons directly to the terminal part of the respiratory chain at the level of cytochromes *c*. However, the situation changed if the above mixture contained the uncoupler CCCP as well. Fig. 1 shows that, in the presence of increasing concentrations of CCCP, the accumulation of NO was enhanced accordingly. At low concentrations of the uncoupler, NO was found to oscillate with a gradual increase of both the amplitude and the period, whereas at high (saturating) concentration of CCCP the oscillations vanished and only a rapid accumulation of NO followed by a slow decrease occurred. The maximal concentration of NO obtained in this case was approx. 0.3 mM which corresponded to 6% of the NO_2^- added initially.

From the literature it would be predicted that elevated concentrations of NO should inhibit the nitrite reductase activity of the cells. To assess this possibility, experiments analogous to those in Fig. 1 were carried out in which NO_2^- disappearance from the reaction mixture was followed by taking small samples for NO_2^- analysis. As can be seen in Fig. 2, nitrite reductase was almost completely inhibited at NO concentrations around

Fig. 1. Time-course of NO_2^- reduction by cells of *P. denitrificans*

The electrode chamber contained 2.1 ml of 0.1 M-sodium phosphate, pH 7.3, 0.2 mM-TMPD, 5 mM-ascorbate and 1 mg dry weight of cells. The cells were allowed to respire until the electrode indicated that anaerobiosis had been attained. At this point 1 (---), 2 (— · —), 3 (·····) or 4 (—) nmol of CCCP was added and after 5 min the reaction was started by the addition of 10 μmol of NO_2^- . ·····, No added uncoupler. The vertical bar shows the current change (3×10^{-7} A) corresponding to the addition of 50 nmol of NO (final concentration 24 μM).

Fig. 2. Inhibition of nitrite reductase by NO

Conditions were as described in the legend to Fig. 1, except that 1.37 mg dry weight of cells and 1 mM- NO_2^- were used. Samples were withdrawn and assayed for NO_2^- at the times indicated. ---, no added uncoupler; —, 5 μM -CCCP.

0.3 mM. Under the conditions of the oscillations, the actual concentrations of NO lay well below the maximal value in Fig. 2 and, accordingly, at most a partial transient inhibition of NO_2^- reduction could be observed (results not shown).

The effects of different concentrations of NO_2^- on the rate of the uncoupler-induced NO production are presented in Fig. 3. The most striking feature is that the accumulation of NO was not observable unless the initial concentration of NO_2^- had reached a certain threshold value. As the amount of the uncoupler was raised, this threshold concentration of NO_2^- shifted downwards and the maximal rate of NO production (attainable at high NO_2^- concentrations) increased.

While screening a number of agents, it has been established that the ability to elicit the production of NO from NO_2^- is not restricted to a particular structure of phenylhydrazone-propanedinitrile. For instance, oscillatory phenomena comparable with those seen in Fig. 1 took place after treatment of

Fig. 3. Dependence of the maximal rate of NO accumulation on the concentrations of NO_2^- and CCCP

The reaction mixture contained, in a total volume of 2.1 ml, 0.1 M-sodium phosphate, pH 7.3, and either 0.2 mM-TMPD, 5 mM-ascorbate plus 0.64 mg dry weight of cells (○, ●) or 12.5 mM-succinate plus 1.3 mg dry weight of cells (□). On exhaustion of the dissolved O_2 , 1 (○), 2.5 (□) or 25 (●) μM -CCCP was added and incubated with the cells for 5 min before initiation of the reaction with the given concentration of NO_2^- . The maximal rate was calculated from the slope of the tangent to the inflection point.

bacterial cells with 0.5 mM-NEM (0 °C, 120 min) or 0.5 mM-NBS (0 °C, 30 min) (results not shown). NO also accumulated in the presence of millimolar concentrations of hydroxylamine. In this case, however, reproducible oscillations of NO concentration were not obtained, probably because hydroxylamine can be rapidly converted into ammonia by the dissimilatory nitrite reductase of *P. denitrificans* (Kučera & Skládal, 1990b).

Correlation between the release of NO from NO_2^- -reducing cells and the inhibition of NO consumption

Hoglen & Hollocher (1989) have shown that NBS strongly inhibits the nitric oxide reductase of *P. denitrificans*. The accumulation of NO described above therefore could originate in a lowered efficiency of the NO-reducing step in the presence of the agents tested. To check this hypothesis, presumed inhibitory effects on the reduction of NO were examined. Actually, it was found that both CCCP and NEM inhibited nitric oxide reductase activity of *P. denitrificans* cells in a time-dependent manner, 50% inhibition being achieved at concentrations equal to 1.0 μM (5 min preincubation with cells) or 0.37 mM (1 h preincubation) respectively (Fig. 4). The inhibitory pattern of FCCP, another uncoupler commonly used, was comparable with that of CCCP (not shown). On the other hand, hydroxylamine (2.5 mM) acted immediately with 50% inhibition. These observations indicate that all the compounds tested can specifically disturb the step $\text{NO} \rightarrow \text{N}_2\text{O}$ of the denitrification pathway without significantly affecting the reduction of NO_2^- .

Interaction of CCCP with the denitrification pathway

The use of 13-ethylberberine as a potential-dependent fluorescent probe made it possible to show that the concentration of CCCP required for the dissipation of membrane potential in cells of *P. denitrificans* respiring oxygen was similar to the concentration inhibiting the reduction of NO (approx. 1 μM). This might mean that the utilization of NO would depend on

Fig. 4. Sensitivity of NO respiration by cells of *P. denitrificans* to uncouplers and a lipophilic thiol-blocking reagent

Nitric oxide reductase activities were determined by following the initial rate of NO consumption in the reaction mixture (2.1 ml) comprising 0.1 M-sodium phosphate, 0.2 mM-TMPD, 5 mM-ascorbate and 1 mg dry weight of cells. CCCP (○) or PCP (●) was introduced 5 min before addition of NO (210 nmol). NEM (□) treatment of cells was performed separately in a phosphate buffer with NEM at the concentrations shown in the figure. After 1 h incubation at 30 °C, the cells were spun down and resuspended in a buffer without inhibitor for nitric oxide reductase measurement. An activity of 100% corresponds to a value of 4.9 nmol of NO/s per mg dry wt. The arrows indicate the conditions under which the energization of cytoplasmic membrane was examined (see Fig. 5).

Fig. 5. Generation of a membrane potential by electron transport to oxygen in cells of *P. denitrificans*

The cuvette was filled with 2 ml of 0.1 M-sodium phosphate, pH 7.3, containing 0.2 mM-TMPD, 5 mM-ascorbate, 30 μM -13-ethylberberine, 2000 units of catalase (bovine liver) and 1.2 mg dry weight of cells. The temperature was 20 °C. At the time indicated by the arrow, 200 nmol of H_2O_2 was added. a, untreated cells; b, cells preincubated for 5 min with 5 μM -PCP; c, cells preincubated for 1 h with 1.25 mM-NEM as specified in the legend to Fig. 4. In a separate experiment it was shown that neither PCP nor NEM produced any discernible quenching of the fluorescence at the concentrations used.

energization of the bacterial membrane. In analogous experiments, however, membrane potential was almost completely collapsed by a structurally different uncoupler, PCP, at the concentration that did not influence the activity of nitric oxide reductase. This clearly follows from Figs. 4 and 5 along with the fact that NEM (another inhibitor of nitric oxide

Fig. 6. Effect of CCCP on the reoxidation of cytochromes *c* by NO in cells of *P. denitrificans*

NO (400 nmol) was added to the N_2 -sparged medium containing 0.1 M-sodium phosphate, pH 7.3, 0.2 mM-TMPD, 5 mM-ascorbate and 0.9 mg dry weight of cells. The test volume was 3 ml, the temperature 30 °C, the wavelength couple 550–540 nm, and the optical pathway 1 cm. a, Control without treatment; b, in the presence of 16 μ M-CCCP.

Fig. 7. Oscillations of NO concentration in the reconstituted system as a function of the nitrite reductase/membrane vesicles ratio

Sodium phosphate buffer, pH 7.3 (2.1 ml; 0.1 M) plus 0.2 mM-TMPD, 5 mM-ascorbate, 0.12 mg of purified nitrite reductase and membrane vesicles in an amount equivalent to 1.35 (---), 2.7 (---) or 5.4 (---) mg of protein was placed into the electrode chamber and, once dissolved O_2 had been consumed, the reaction was started by adding 10 μ mol of NO_2^- . —, no added membranes.

reductase) does not uncouple the cells. Thus the effect of CCCP on NO consumption does not seem to be associated with the changes in membrane potential.

As illustrated in Fig. 6, the addition of NO to the anaerobically

Fig. 8. Oscillations of NO concentration in the reconstituted system at a fixed nitrite reductase/membrane vesicles ratio

The conditions were as described in Fig. 7 except that the following amounts of nitrite reductase/membrane vesicles were used (mg of protein): a, 0.06/1.35; b, 0.12/2.7; c, 0.24/5.4.

Fig. 9. Inhibition of the NO reduction of *P. denitrificans* membrane vesicles by excess substrate (a) and/or by NO_2^- (b)

The rates were measured with 0.27 mg of protein in 2.1 ml of 0.1 M-sodium phosphate (pH 7.3)/0.2 mM-TMPD/5 mM-ascorbate and corrected for non-enzymic reduction of NO in the absence of membranes. In (b), the initial concentration of NO was 20 μ M.

grown cells caused a transient reoxidation of cellular cytochromes *c*. Since these redox changes could be prevented by the simultaneous presence of CCCP, it appears likely that a terminal reaction in the electron-transport chain to NO is blocked under these conditions.

Oscillations of NO concentration in the reconstituted system nitrite reductase/membrane vesicles

A more direct approach used for assessing the role of single respiratory components in the oscillations of NO concentration was the reconstitution of the involved redox chain from its soluble and membrane-bound parts. As can be seen in Fig. 7, almost undamped oscillations of NO concentration appeared after the ratio of nitric oxide reductase to nitrite reductase had been raised by adding membrane vesicles to the reaction mixture containing NO_2^- , TMPD plus ascorbate and a fixed amount of purified nitrite reductase. When the amounts of both NO-producing and NO-consuming enzymes were increased (at their constant ratio), the oscillations became markedly higher and narrower, whereas the period shortened only insignificantly (Fig. 8). The maximal rates of NO accumulation, determined as slope

Fig. 10. Interference of NO_2^- with NO metabolism in intact cells of *P. denitrificans*

Cells (1 mg dry wt.) were added to 2.1 ml of a solution containing 0.1 M-sodium phosphate, pH 7.3, 0.2 mM-TMPD and 5 mM-ascorbate in the reaction chamber of a NO electrode. Further additions were: NO, 0.2 μmol in both cases; NO_2^- , 10 μmol and 90 mmol.

values in inflex points, were proportional to the amounts of nitrite reductase present.

Kinetic signals destabilizing the denitrification pathway

In a preceding paper (Kučera, 1989) dealing with the oscillatory phenomena in denitrification, attention has been drawn to possible modifications of the catalytic properties of the enzymes involved by the concentrations of denitrification intermediates. This possibility is now substantiated through the results summarized in Figs. 9 and 10. Fig. 9(a) shows that the plot of rate against substrate concentration for the reduction of NO by membrane vesicles does not obey the hyperbolic law, but that the rate passes through a maximum at approx. 10 μM -NO and then falls again. The same activity was inhibited by millimolar concentrations of NO_2^- (Fig. 9b).

The effect of NO_2^- of retarding the reduction of exogenous NO was also apparent when intact cells were used (Fig. 10). Unlike with membrane vesicles, however, the extent of inhibition could not be evaluated because of the interfering simultaneous production of NO via nitrite reductase. Nevertheless, the accumulation of NO in the presence of a high concentration of NO_2^- (Fig. 10) accords with the view that the inhibition at the level of nitric oxide reductase is operative. The cells were less susceptible to substrate inhibition by NO than were membrane vesicles. Typically, only about 30 % reduction of the maximal rate was achieved at 100 μM -NO.

DISCUSSION

Progress towards realization of the role of NO as a free intermediate in the denitrification pathway of *P. denitrificans* has partly come from the studies concerned with the differential inhibition of NO_2^- and NO uptake by various agents such as Triton X-100 (Shapleigh *et al.*, 1987), CCCP (Kučera, 1989; this study) or azide (Goretski & Hollocher, 1990). The inhibitors of nitric oxide reductase activity known so far (including those characterized in the present study) can now be classified into three distinct categories: inorganic nitrogen-containing compounds (N_3^- , NH_2OH , NO_2^- , NO), lipophilic organic agents (CCCP, NEM, NBS) and detergents (Triton X-100). Although the exact mechanism for the inhibition cannot be defined at this time, it is probable that the inorganic compounds bind to the haem centres which were identified in the nitric oxide reductase of *P. denitrificans* (Carr & Ferguson, 1990b; Dermastia *et al.*, 1991). This possibility draws some support from the fact that the titres necessary for inhibiting reduction of NO blocked also the oxidase activity of cytochrome *aa*₃ (Kučera *et al.*, 1986a; Kučera

& Skládal, 1990a). The sensitivity to NEM, a well-known thiol-blocking reagent, may indicate the involvement of a buried nucleophilic (possibly thiol) group. The same group may be covalently modified by CCCP, which is able to react with a variety of thiols (Drobnica & Šturdík, 1979; Antalík *et al.*, 1984) and was shown to diminish the reactivity of *Escherichia coli* membrane proteins towards NEM (Kaback *et al.*, 1974). Future studies using the purified enzyme should elucidate whether a thiol group is involved in the reduction of NO. In this connection it is interesting to note that the redox reaction of NO with thiols was considered to be a general technique for the preparation of symmetrical disulphides (Longhi *et al.*, 1962). Alternatively, a common denominator for the inhibitory action of the hydrophobic compounds studied may lie in their solubility in lipid phase which would enable them to affect the activity of nitric oxide reductase in a non-specific way, e.g. by changing the local environment of the active site or by modulating the interactions between the membrane-anchored subunits of the enzyme.

After the completion of this work, Carr & Ferguson (1990a) surprisingly characterized the nitric oxide reductase activity of *P. denitrificans* cells as being insensitive to the uncoupler FCCP. Unfortunately, the lack of essential description about the experimental conditions makes any comparison between the latter and the present papers hardly possible. It seems likely that different initial concentrations and incubation periods may account, at least in part, for this discrepancy.

To my knowledge, the data presented in this paper provide the first example of metabolic oscillations in an anaerobic respiratory pathway. Three aspects deserve consideration.

First, the catalytic concentrations of nitrite reductase and nitric oxide reductase obviously play a critical kinetic governing role in the dynamic behaviour of the denitrification pathway. When these parameters are varied (e.g. by inhibiting the nitric oxide reductase), the steady state can become unstable and, as a consequence, the system jumps from a monotonous time pattern to a periodic regime. Whether the conditions necessary for the oscillations to occur can be met *in vivo* remains unknown. A rise in nitrite reductase/nitric oxide reductase ratio might take place in parallel with variations in environmental factors such as pH and concentrations of naturally occurring compounds that inhibit the reduction of NO.

Second, the oscillations of NO concentration occur even in a reconstituted cell-free system, i.e. in the absence of any soluble cytochrome *c* except for nitrite reductase (cytochrome *cd*₁). Thus the previously described oxidation of NO to NO_2^- by cytochrome *c* (Ehrenberg & Szczepkowski, 1960; Kučera, 1989, 1990) does not appear to be essential for the oscillations, although it may modify the detailed kinetics of the system. Both the demonstration of the oscillations in the systems depleted of nitrous oxide reductase and a high selectivity factor of the NO probe (see the Materials and methods section) argue against the possibility that the registered periodic current response contains a significant contribution from build up (and subsequent reduction) of N_2O .

Third, the experiments of Figs. 9 and 10 suggest that step 3 of the denitrification pathway (see Scheme 1) can be negatively affected by the preceding denitrification intermediates NO_2^- and NO. Such types of feed-forward kinetic signals are known to destabilize linear metabolic pathways and have been recognized as possible sources of multistability and metabolic oscillations (Reich & Sel'kov, 1981). Substrate inhibition by NO offers a plausible explanation for the S-shaped progress curves obtained during the assay of nitric oxide reductase (Goretski & Hollocher, 1990; Dermastia *et al.*, 1991; but cf. Carr *et al.*, 1989). Further investigation will have to take into account a marked dependence of the inhibitory pattern on the type of enzyme preparation and the electron donor system used. The inhibition of nitric oxide

Fig. 11. Two stable stationary states in the denitrification pathway

The variable v_1 is given by the difference between the rates of NO formation via nitrite reductase (which undergoes product inhibition by this metabolite) and of NO consumption via non-enzymic processes. v_2 and v_2'' represent the rates of NO reductase reaction at various $V_{\max}$ values. The stationary states (S) are defined by intersections of the v_1 curve with the v_2 curve. Note that if the system approaches a stationary state of the first type (S_1), the derivative $d[\text{NO}]/dt = v_1 - v_2$ decreases monotonically with increasing $[\text{NO}]$, whereas in the case of the second type (S_2), a transient maximum appears.

reductase by NO_2^- may be related to the observations of Vosswinkel *et al.* (1991) who found that the formation of NO by a denitrifying *Pseudomonas aeruginosa* strain increased with increasing concentrations of NO_2^- in the growth medium. This was suggested to reflect a modification of the nitrite reductase (or its complex with nitric oxide reductase) to a form that easily released NO and did not convert it into N_2O . The observed accumulation of NO can now be accounted for by a direct interaction of NO_2^- with a separate NO-reducing enzyme without postulating any changes in the nitrite reductase reaction mechanism.

In order to facilitate further discussion let us consider a kinetic scheme depicted in Fig. 11. The intersection of the curves v_1 and v_2 indicates a steady state in which NO production from NO_2^- is counterbalanced by its consumption via nitric oxide reductase and via other (non-enzymic) processes. It is easy to see that two different types of stable stationary states can exist, corresponding to the intersection points situated on either the increasing or decreasing part of the v_2 curve. Very low concentrations of NO observed during denitrification (Goretski *et al.*, 1990) and a high nitric oxide reductase activity of denitrifying cells indicate that the stationary state attained under physiological conditions belong to the first type. The second-type stationary state might become accessible when the flux through the nitric oxide reductase is sufficiently lowered by a specific inhibitor (compare curves v_2' and v_2'' in Fig. 11). As far as the kinetics of approaching this new state is considered, our model predicts that the time-dependence of NO concentration should at first be concave upward as in autocatalytic reactions, which agrees fairly well with the experimental findings [Fig. 1 and Fig. 1 in Kučera (1989)]. However, to explain the oscillatory behaviour, it seems reasonable to admit that the existence of stable stationary states may not be obligatory. This would be the case provided that at least one of the

curves does not have a fixed position due to the dependence of v_1 or v_2 on a hitherto unknown variable. In fact, preliminary calculations have demonstrated the possibility of sustained oscillations of NO concentration in the system where a redox reaction of pseudo-first order serves as the source of a hypothetical electron donor for nitric oxide reductase (I. Kučera, unpublished work). Evidently, additional and more refined data must be obtained to reconcile all experimental information with either the above or, if necessary, a completely different model.

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