The inhibitory effect of nitrite on the oxidase activity of cells of *Paracoccus denitrificans*

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It was observed with anaerobically grown cells of *Paracoccus denitrificans* which possess dissimilatory nitrite reductase that on increasing the flux of electrons into the terminal part of the respiratory chain by means of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) plus ascorbate the sensitivity of the oxidase activity towards the inhibitory action of nitrite markedly increased. It was shown that under these conditions the reduction of nitrite gave rise to the formation of an agent strongly inhibiting the terminal oxidases, the presence of which was detected using aerobically grown cells or membrane vesicles derived from them. The strong inhibition of TMPD-oxidase activity of membranes was transient in time and could also be brought about by addition of nitric oxide (NO) but not by nitrous oxide (N_2O).

Denitrification *Electron acceptor competition* *Nitric oxide* *Enzyme inhibition* (Paracoccus denitrificans)

1. INTRODUCTION

The exploration of mechanisms participating in the inhibitory action of nitrite on bacterial metabolic pathways has drawn attention in recent years in connection with the use of this substance as an antibacterial conservation agent. With the aerobic bacterium *Pseudomonas aeruginosa* the inhibition of active transport, respiration and oxidative phosphorylation by nitrite was observed [1], the attack on the terminal oxidase being considered as the primary effect [2]. A more complex situation sets in with anaerobically grown denitrifying bacteria which, besides terminal oxidase, possess the dereprimed dissimilatory nitrite reductase. In anaerobically grown cells of *Paracoccus denitrificans* oxygen as terminal acceptor is preferentially utilized over nitrite [3]. On cytoplasmic membrane permeabilization or in the presence of an uncoupler nitrite is progressively reduced and concomitantly the oxidase activity of cells is almost completely inhibited [4–6]. Anaerobic nitrite reduction can also be initiated by increasing the degree of reduction in the terminal part of the respiratory chain (*c*-type cytochromes) by means of a mixture of the artificial electron donor ascorbate and the mediator *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) [7]. Here, we show that the inhibition of oxidase activity of cells caused by nitrite in the presence of TMPD plus ascorbate is strongly enhanced by the course of the nitrite reductase reaction passing in parallel. A plausible mechanism of this effect and some of its physiological consequences are discussed.

2. MATERIALS AND METHODS

P. denitrificans NCIB 8944 obtained as CCM 982 from the Czechoslovak Collection of Microorganisms was grown anaerobically at 30°C in 2 l flasks completely filled with a medium [8] containing 50 mM succinate and 10 mM nitrate. Aerobic cultivations were performed in 2 l flasks vigorously shaken with 500 ml of the medium without nitrate. The cells were harvested in an ear-

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ly stationary stage of growth (after 22 and/or 14 h) and washed with 0.1 M phosphate buffer. Membrane vesicles were derived from the cells after osmotic lysis of spheroplasts obtained by lysozyme treatment followed by differential centrifugation [9]. Protein content was estimated by the biuret method [10]. Rates of oxygen consumption were measured by means of a Clark electrode at 25°C fitted in an electromagnetically stirred vessel of 2 ml volume. Wurster's blue formation was monitored as the increase in absorbance at 563 nm [11] on a Cary 118 C spectrophotometer. Nitrogen oxides NO and N_2O were applied as aqueous solutions saturated at 0°C (at 3.1 and 58 mM, respectively) [12].

3. RESULTS

The cytochrome(s) of the *c* type which in *P. denitrificans* serve as physiological donor for nitrite reductase (cytochrome cd_1) and terminal oxidase [13] can be effectively reduced by TMPD [14]. The influx rate of redox equivalents into the terminal part of the respiratory chain can be conveniently adjusted by varying the TMPD concentration in the mixture with ascorbate. If a simple competition for the electron donor between nitrite reductase and oxidase were valid, it could be expected that in parallel to increasing the TMPD concentration the rate of reduction of the two terminal acceptors NO_2^- and O_2 would be enhanced similarly to what was observed with the NO_2^- and N_2O couple [15]. However, this is at variance with our experiments. The results given in fig.1 show that in the absence of nitrite the dependence of oxygen consumption on TMPD concentration has the expected hyperbolic profile. On the other hand, if nitrite is present in the medium the oxidase activity measured at TMPD concentrations above 0.15 mM slows down. The decrease in oxidase activity at higher TMPD concentrations could not be detected using aerobically grown cells lacking nitrite reductase activity. Hence, it can be concluded that the inhibitory effect is not due to the mere presence of nitrite in the medium but that there exists a junction of this effect with the course of nitrite reduction in the passing reaction of nitrite reductase.

One of the possible explanations can be sought in the assumption according to which a product

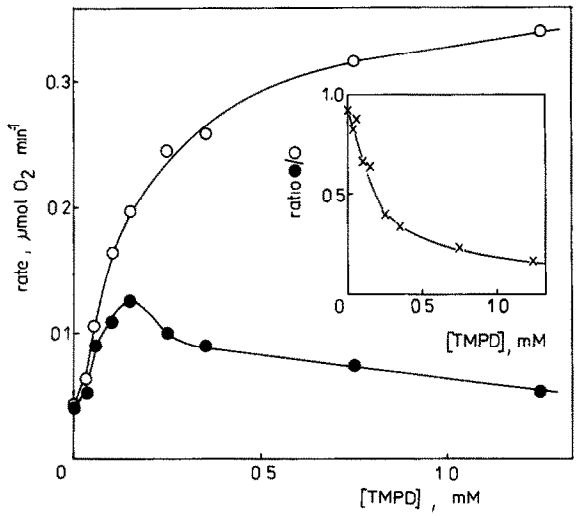

Fig.1. Dependence of oxidase activity of anaerobically grown cells of *P. denitrificans* on concentration of TMPD. The reaction mixture (2 ml) contained 0.1 M sodium phosphate, pH 7.3, anaerobically grown cells (0.75 mg dry wt), 10 mM ascorbate, and the given concentration of TMPD (○). In some cases 0.5 mM sodium nitrite was added (●). (Inset) Profile of the concentration dependence vs the ratio of the two activities (in the presence and absence of nitrite) (×).

strongly inhibiting terminal oxidase is formed in the nitrite reductase reaction. To test the existence of such a mechanism the profiles of dependences of the oxidase activity on TMPD concentration were measured using: (i) anaerobically grown cells, (ii) membrane vesicles derived from cells grown aerobically, and (iii) a mixture which consisted of these two experimental objects, while nitrite was present in all these experiments (fig.2). Under such an experimental arrangement the membrane vesicles serve as a testing system registering the presence of a diffusible inhibitory agent acting on terminal oxidases which is generated during the reduction of nitrite by cells cultivated anaerobically. The analysis of the dependences shown in fig.2 provides evidence that while increasing the mediator concentration the activity of the testing system slows down to almost zero activity at 0.25 mM TMPD; this is in support of the mechanism suggested. Similar results to those in fig.2 were also obtained by using intact cells grown aerobically (not shown).

Further experiments were directed towards

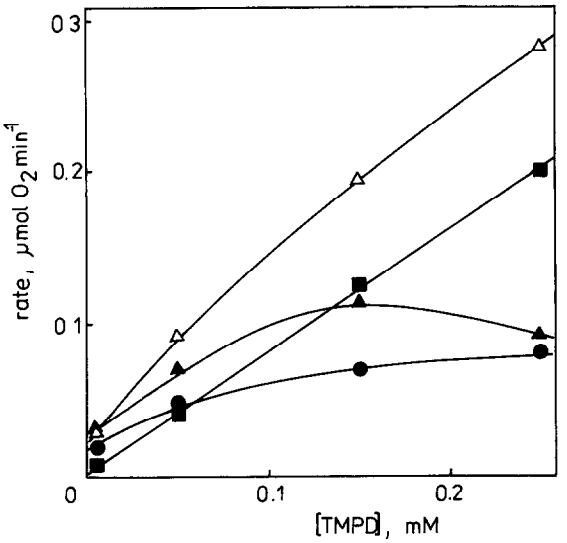

Fig.2. Inhibition of oxidase activity in membrane vesicles by an intermediate generated through the action of anaerobically grown cells of *P. denitrificans*. The reaction mixture (2 ml) consisted of 0.1 mM sodium phosphate, pH 7.3, 5 mM ascorbate, 0.5 mM sodium nitrite, the given concentration of TMPD, 0.3 mg dry wt of anaerobically grown cells (●, ▲) and 1.1 mg protein of vesicles derived from cells grown aerobically (■, ▲). The found estimates of the oxidase activities in the mixture of cells and vesicles (▲) can be compared with values calculated under the assumption that both systems are not mutually affected (△).

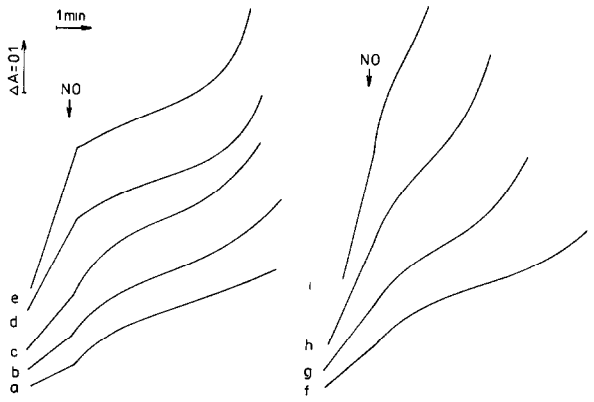

Fig.3. The inhibitory effect of nitric oxide on the oxidation of TMPD by membrane vesicles derived from anaerobically grown cells of *P. denitrificans*. The reaction mixture (3 ml of 0.1 M sodium phosphate, pH 7.3; temperature, 20°C) contained 0.17 mM TMPD and various amounts of membranes (curves a–e): 0, 0.02, 0.04, 0.1, 0.2 mg protein, respectively; 0.04 mg protein and various concentrations of TMPD were present in the cuvette in case f–i: 0.08, 0.17, 0.33, 0.83 mM. In all experiments the reaction was started by TMPD addition. At the time indicated by the arrow, NO was introduced as 20 μl of aqueous solution saturated at 0°C (62 nmol NO).

assessing at least preliminarily the character of the postulated substance which is inhibitory to terminal oxidases. The measurements were undertaken where the effect of nitrogenous oxides, i.e. NO and N_2O, as possible intermediates in the denitrification process was tested on the oxidase activity of membrane vesicles. The activity was monitored spectrophotometrically as the rate of Wurster's blue generation (in the absence of ascorbate). The applied procedure has an advantage over the commonly used measurement of oxygen consumption in a greater sensitivity and especially in the fact that the nonenzymatic interaction of oxygen with added NO does not interfere. It was found that N_2O even at 1 mM was without effect on the TMPD oxidase activity of the membrane. In contrast, NO was strongly inhibitory even when applied at very low (of the order of 0.01 mM) concentrations (cf. fig.3). The same effect could be observed on addition of a solution of nitrosyl sulphate. The observed inhibition was only of transient character in both cases, this being manifested by a delay observed on the record indicating the time course of Wurster's blue formation. The duration of the delay was proportional to the amount of NO introduced into the mixture (not shown).

In an attempt to clarify the cause of the transient character of the inhibitory action of NO the concentrations of membranes and of TMPD were successively changed; the results are depicted in fig.3. It is evident that the concentrations of membrane fragments used were without effect on the period of duration of the inhibitory phase (see traces a–e). This probably means that the disappearance of the inhibitor from the medium is not an enzymatic event catalyzed by membranes. The variation of the initial concentrations of TMPD brought about a different effect. The corresponding records (see fig.3, curves f–i) designate the superposition of enzymatic and nonenzymatic oxidation of TMPD; the relative portion of the

nonenzymatic process is raised at higher initial concentrations of TMPD. The rate of Wurster's blue generation decreases upon NO addition, being diminished to the value which corresponds to the nonenzymatic oxidation of TMPD. The duration of the inhibitory phase is decreased with increased TMPD concentrations, indicating that its oxidation can contribute to inhibitor degradation.

4. DISCUSSION

The present findings provide evidence that anaerobically grown cells of denitrifying bacteria possess the ability to enhance markedly the inhibitory action of nitrite on terminal oxidases (TMPD plus ascorbate as electron donor). The results obtained are consistent with the mechanism according to which a potent inhibitor of terminal oxidases is generated during the course of enzymatic reduction of nitrite by nitrite reductase (cytochrome cd_1). Since a similar inhibitory effect was achieved by addition of NO it may be assumed that this substance plays the role of an inhibitor. Under the experimental conditions, however, NO is exposed to further alteration, thus an intermediate rise in another inhibitory product in situ originating from NO cannot be excluded as well. Finally, it should be mentioned that NO may be generated as an intermediate during the denitrification process in *P. denitrificans* although it was indirectly proven that it does not accumulate at marked concentration in the reaction medium [15]. The interaction of NO with bacterial redox systems has also been reported (for example the formation of nitrosyl complexes with bacterial terminal oxidases [16,17], Fe-S protein centres [18], and the inhibition of photosynthetic electron transport [19]). All these findings attest to the fact that bacterial respiration can be inhibited even by a nitrogenous substance whose degree of oxidation of nitrogen is lower than +3.

The appearance of a respiratory inhibitor during the nitrite reductase reaction in *P. denitrificans* obviously plays a role in switching the electron flow from oxygen towards nitrite. This effect can be attained besides others through the increased intensity of electron flow into the terminal part of the respiratory chain [7] but also as a consequence of the slight inhibition of terminal oxidases by hydroxylamine [20]. The common feature of both cases can be seen in the enhancement of the electron flux towards nitrite reductase, this probably resulting in the generation of a nitrogenous intermediate in a concentration sufficient for the inhibition of terminal oxidases. A similar mechanism may be valid in effects observed upon cytoplasmic membrane permeabilization and in the effects brought about by an uncoupler [4–6].

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