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Nitric oxide formed by nitrite reductase of *Paracoccus denitrificans* is sufficiently stable to inhibit cytochrome oxidase activity and is reduced by its reductase under aerobic conditions

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Nitric oxide, generated by the action of purified nitrite reductase, inhibited the oxidase activity of both membrane vesicles from anaerobically grown *Paracoccus denitrificans* and bovine heart submitochondrial particles. In the former case, the inhibition was relatively short-lived and its duration was reduced either by decreasing the concentration of nitrite or raising the ratio of vesicles to nitrite reductase enzyme. These observations indicate that nitric oxide, at least at low concentrations, was sufficiently stable in the presence of oxygen to allow diffusion between proteins in aqueous solution. The shorter inhibition period with *P. denitrificans* membrane vesicles implies that the nitric oxide reductase of the vesicles is active in the presence of oxygen and has a sufficiently high affinity for nitric oxide to remove it from oxidase enzymes by competition. These observations are related to previous reports of potent inhibition under certain conditions of oxidase activity of *P. denitrificans* cells by a molecular species produced from nitrite. The implications of the deduced stability of nitric oxide in aerobic solutions are considered with respect to both the phenomenon of aerobic denitrification and the synthesis of nitric oxide in mammalian cells.

Introduction

After growth under anaerobic, denitrifying conditions, the electron transport chain of the bacterium *Paracoccus denitrificans* possesses reductases for nitrate, nitrite, nitric oxide and nitrous oxide [1,2]. After some years of debate, recent experiments with this organism have strongly indicated that a discrete reductase exists for nitric oxide, which is a free intermediate in denitrification [3,4]. In addition to the four reductases of the denitrifying pathway, cells grown anaerobically in batch culture also possess cytochrome oxidase activity. There is evidence that a least some cytochrome aa_3 is present in such cells [5], but incomplete inhibition of oxidase activity by antimycin or myxothiazol, potent inhibitors of the cytochrome bc_1 complex in this organism [5,6], indicates the presence of an alternative oxidase that branches at the level of ubiquinol from the pathway to cytochrome aa_3 [6]. The activities of the four reductases of the denitrification pathway are normally inhibited in the presence of oxygen when the reducing substrates are physiological [1–7]. However, some of the reductases will function in the presence of oxygen if the permeability to protons or other ions of the cytoplasmic membrane is perturbed [8,9]. For example, permeabilisation of the cytoplasmic membrane with low titres of Triton X-100 results in the simultaneous reduction of nitrate and oxygen [8]. Reduction of nitrite under aerobic conditions was also observed when electrons were fed into the electron transport chain from isoascorbate plus TMPD [7].

In concert with the absence of reduction of nitrate or nitrite under unperturbed aerobic conditions, neither of these ions at millimolar concentration or less has any inhibitory effect upon the reduction of oxygen. But after permeabilisation, the introduction of nitrate caused a partial inhibition of the oxidase rate [7], consistent with a partition by competition of electron transport to oxygen and nitrate. Kučera and Dadák [9] found that addition of nitrite to cells treated with a protonophore caused potent inhibition of oxidase activity. This was suggested to reflect the binding of nitrite to a site or sites on oxidase(s) that became accessible to this anion only after collapse of the proton electrochemical gradient across the cytoplasmic membrane. Subsequently,

Abbreviations: DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine.

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Parsonage et al. [10], together with Kučera et al. [11], found that the inhibition occurred at such low concentrations of nitrite that the anion itself could not be the inhibitory agent, which therefore appeared to be a diffusible species derived from nitrite. Parsonage et al. [10] considered that this species could be nitric oxide or nitroxyl ion. At the time, the latter, rather than the former, was being considered as an intermediate species between nitrite and nitrous oxide in reactions catalysed by *P. denitrificans* [10], and thus it was appropriate to equate nitroxyl tentatively with the inhibitor. Furthermore, if the inhibitory species were nitric oxide it was difficult to explain features of the inhibition. These were that the inhibition reversed shortly after the disappearance of detectable nitrite, but that the period of inhibition was accompanied by essentially zero oxygen consumption [10]. Nitric oxide reacts readily with oxygen and thus it was not apparent why oxygen did not remove the inhibitory nitric oxide. The lack of oxygen consumption was, however, recognised to be consistent with a hypothesis that the cells possessed a nitric oxide reductase enzyme that was active in the presence of oxygen and maintained the steady-state dissolved nitric oxide concentration sufficiently low that reaction with oxygen was insignificant [10]. The present paper presents evidence in favour of the latter hypothesis. The results also have some bearing on the recent finding that nitric oxide can act as a ligand and messenger in mammalian cells under aerobic conditions [12].

Kučera et al. [13] have recently presented evidence of a different kind leading to their conclusion that nitric oxide is probably the potent inhibitor of oxidase activity that is formed from nitrite by cells of *P. denitrificans* in the presence of a protonophore.

Materials and Methods

Anaerobic growth of *P. denitrificans* NCIB 8944, preparation of membrane vesicles therefrom, synthesis of nitric oxide and purification of nitrite reductase from *P. denitrificans* as well as measurement of dissolved oxygen and nitrite concentrations were as described in a previous paper [4] and in the figure legends. Submitochondrial particles were prepared from bovine heart mitochondria as described by Ferguson et al. [14].

Results

As explained in the Introduction, nitric oxide produced from nitrite by the action of nitrite reductase could be responsible for the inhibition of oxidase activity observed with cells of *P. denitrificans* under certain conditions. To test this explanation, the effects of nitrite on the oxidase activity of bovine heart submitochondrial particles were examined in the presence or absence of purified nitrite reductase. Since these mitochondrial membranes do not possess enzymes for reduction of oxy-species of nitrogen, use of this experimental system eliminated any possibility that the inhibitor of oxidase activity could be formed from added nitrite by an enzyme activity of *P. denitrificans* other than that of the nitrite reductase.

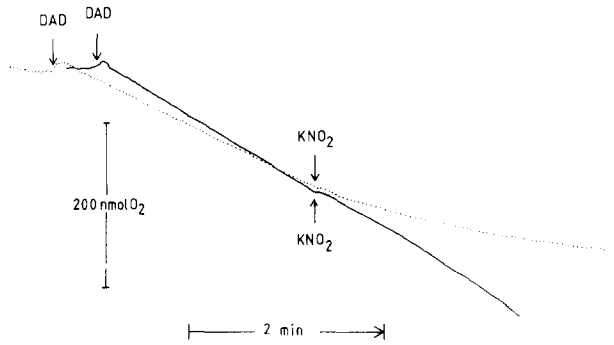

Fig. 1. The oxidase activity of bovine heart submitochondrial particles is inhibited by a product formed by the action of nitrite reductase on nitrite. A reaction chamber fitted with an oxygen electrode (platinum cathode, polarised at −0.6 V) contained in a total volume of 2 ml at 30 °C the following: 10 mM Tris-acetate (pH 7.3), 1 mM magnesium acetate, 5 mM isoascorbate and 0.94 mg protein bovine heart submitochondrial particles. 0.3 mM DAD was added to initiate respiration followed by KNO_2 to a final concentration of 0.1 mM as shown. The difference between the record marked (———) and that marked (· · · · · ·) is that 45.7 μg cytochrome cd_1 plus 120 μg cytochrome c_{550} were also present in the latter.

Fig. 1 shows that addition of nitrite in the micromolar concentration range to bovine heart submitochondrial particles supplied with isoascorbate plus DAD as substrate had no effect on the rate of electron flow to oxygen. Introduction of nitrite in the presence of its reductase rapidly caused an almost complete inhibition of oxygen reduction (Fig. 1). This inhibition persisted for at least 20 min. The interpretation of this experiment is that the nitric oxide generated by nitrite reductase was sufficiently stable to diffuse, even in the presence of oxygen, to a very tight binding site of inhibition on the submitochondrial particles.

In a second series of experiments nitrite was added to vesicles prepared from anaerobically grown *P. denitrificans* in order to test for an inhibitory effect, in the presence of nitrite reductase, upon oxidase activity of membranes. Strong inhibition by low concentrations of nitrite (less than 100 μM) was observed under these conditions (Fig. 2), whereas up to 200 μM nitrite was without effect in the absence of the nitrite reductase (not shown). The duration of the inhibition was approximately proportional to the concentration of nitrite added (Fig. 2) and persisted for a short period after the concentration of nitrite had fallen below detectable limits (not shown). It should be noted that in Fig. 2 the oxygen consumption arose from both the vesicles and the nitrite reductase, which itself has oxidase activity

[15], with each making approximately equal contributions before the addition of nitrite. Clearly, both activities are extensively suppressed following the addition of nitrite (Fig. 2), and in the case of the nitrite reductase this may be a combination of the effect of nitrite competing with oxygen as substrate as well as an inhibition by nitric oxide. In separate control experiments it was shown that addition of 25 μM nitrite to nitrite reductase alone caused substantial inhibition of its oxidase activity, particularly as the oxygen concentration decreased. The observations in Fig. 2, and the lack of inhibition if nitrite reductase is omitted, imply that the inhibition of oxidase activity of vesicles is due to nitric oxide rather than nitrite, and the reversal of the inhibition can be attributed to the conversion of nitric oxide to nitrous oxide by the previously demonstrated nitric oxide reductase activity of the vesicles [4]. If the foregoing explanation of Fig. 2 is valid, increasing the amount of vesicles whilst keeping the quantity of both nitrite and its reductase constant should shorten the period of inhibition of oxidase activity as the total added nitric oxide reductase activity increases. The requirement was satisfied by the experiment shown in Fig. 3. It is also evident that the inhibition of oxidase activity that followed addition of nitrite was more pronounced at lower concentrations of oxygen (Fig. 3). As mentioned above, this can be attributed, at least in part, to the consequence of competition between nitrite and oxygen for the catalytic site of nitrite reductase.

These experiments clearly indicate that it is nitric oxide generated by the action of nitrite reductase that is responsible for the previously observed [9,10] inhibition of oxidase activity following addition of nitrite to cells of *P. denitrificans*. This means that the nitric oxide so generated must be sufficiently stable in the presence of oxygen to permit its diffusion to oxidases. If this is the case it might be predicted that addition of nitric oxide

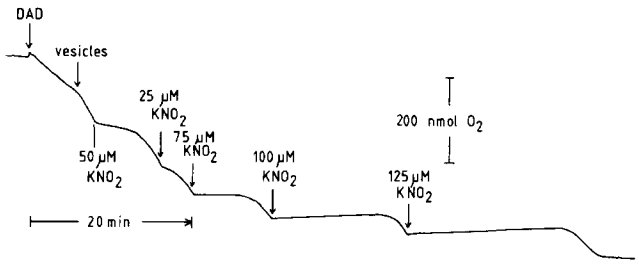

Fig. 2. Inhibitory effect of nitric oxide, formed in situ by action of cytochrome cd_1 on nitrite, upon the oxidase activity of membrane vesicles from *P. denitrificans*. A reaction chamber fitted with an oxygen electrode (gold cathode polarised at −0.8 V) contained in a total volume of 2 ml at 30 °C the following: 10 mM Tris-acetate (pH 7.3); 1 mM magnesium acetate, 5 mM sodium isoascorbate, 0.3 mM DAD and 120 μg cytochrome *c*-550. Respiration was initiated by the addition of 47.5 μg cytochrome cd_1. Subsequently membrane vesicles (0.13 mg protein) and KNO_2 to the final concentrations indicated were added as shown.

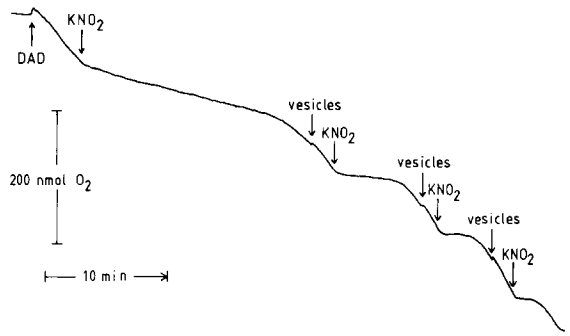

Fig. 3. Effect of varying the ratio of membrane vesicles to cytochrome cd_1 on the duration of the inhibition of oxidase activity following addition of nitrite. A reaction chamber fitted with an oxygen electrode (gold cathode polarised at −0.8 V) contained in a total volume of 2 ml at 30 °C the following: 10 mM Tris-acetate (pH 7.3); 1 mM magnesium acetate, 5 mM sodium isoascorbate, 120 μg cytochrome c_{550}, 47.5 μg cytochrome cd_1 and *P. denitrificans* membrane vesicles (0.03 mg protein). Respiration was initiated by the addition of 0.3 mM DAD. Subsequently 0.07 mg protein of membrane vesicles and KNO_2 to a final concentration of 25 μM were added on three occasions as shown.

to cells of anaerobically grown *P. denitrificans* would cause a similar inhibition. When this experiment was attempted, the oxygen electrode record showed that there was an immediate consumption of oxygen, presumably owing to the direct reaction of the latter with nitric oxide, and only a very limited phase of inhibition respiration (not shown). When, however, nitric oxide was added to an aerobic suspension of cells in the presence of Triton X-100 a more prolonged period of inhibition was observed following a phase of non-enzymic reaction of nitric oxide with oxygen (Fig. 4). Only brief and partial inhibition was observed if the nitric oxide was exposed to atmospheric oxygen before being added to the cells (Fig. 4). Reaction of nitric oxide with oxygen yields dinitrogen tetroxide, which hydrates to give nitrate and nitrite. Thus, neither nitrite nor nitrate, nor diversion of electrons for their reduction, was responsible for the inhibition observed following addition of nitric oxide. Triton X-100 is known to inhibit nitric oxide reductase of *P. denitrificans* [16] and therefore low concentrations of nitric oxide may persist in the suspension of cells with this detergent and have the inhibitory effect upon oxidase activity seen in Fig. 4A.

Nitric oxide could not, for technical reasons, be directly detected in experiments of the kind described in Figs. 1–4. The Clark-type electrode that detects nitric oxide responds also to oxygen and is somewhat more sensitive to the latter gas. Hence even in experiments (Figs. 2 and 3) in which an electrode responsive to nitric oxide was used, the steady-state concentration of nitric oxide could not be measured against the background concentration of oxygen. The concentration of nitric oxide would have to be in the range of tens of micromolar before it could be reliably estimated in the pres-

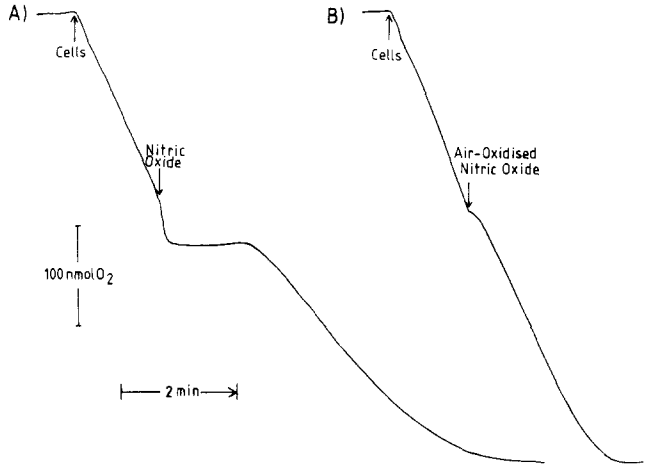

Fig. 4. Effect of nitric oxide upon the rate of oxygen reduction by Triton X-100 treated cells of *P. denitrificans*. A reaction chamber fitted with an oxygen electrode (platinum cathode polarised at −0.6 V) contained in a final volume of 2 ml at 30 °C the following: 10 mM Tris-acetate (pH 7.3); 1 mM magnesium acetate, 10 mM sodium succinate and 0.03% (v/v) Triton X-100. Respiration was initiated by adding cells. In record A an aqueous solution of nitric oxide was added to give a final concentration of 100 μM whilst in record B an equivalent amount of nitric oxide solution that had previously been shaken in air was added.

ence of oxygen. The absence of a substantial upward response of the electrode in Figs. 2 and 3, taken together with arguments advanced in the Discussion, strongly implies that the steady-state concentration of nitric oxide was at most in the very low micromolar range.

Discussion

Data presented in this paper provide clear evidence that purified *P. denitrificans* nitrite reductase acting alone can generate from nitrite a molecular species that is a potent inhibitor of the oxidase activity of both the cytoplasmic membrane of *P. denitrificans* and inner mitochondrial membranes. Since there is clear evidence that this nitrite reductase catalyses formation of nitric oxide [4], it is reasonable to conclude that the inhibition is exerted by nitric oxide, which is well known to have a high affinity for haem centres. This interpretation is also consistent with the release of the inhibition that was observed upon introducing increasing amounts of membrane vesicles into the test system. The nitric oxide reductase activity of such vesicles would have removed nitric oxide, provided this enzyme was active under aerobic conditions and had a high affinity for its substrate. Such high affinity would be expected if nitric oxide is an intermediate in denitrification because this gas would, at modest concentrations, inhibit other cell functions e.g., succinate dehydrogenase [4]. Thus, as is generally observed [1], nitric oxide should be found at barely detectable levels during denitrification. Its toxicity probably accounts for the general failure to obtain growth of denitrifiers with nitric oxide as terminal electron acceptor.

Activity of nitric oxide reductase under aerobic conditions is strongly indicated by the observations reported in Figs. 2 and 3 and from Fig. 3 an approximate rate of nitric oxide reduction of 100 nmol · min^{-1} · (mg protein)$^{-1}$ can be calculated. This value, which assumes that the rate of reduction of nitrite to nitric oxide was not rate-limiting, is comparable to the rate with the same reductant under anaerobic conditions [4]. The functioning of nitric oxide reductase in the presence of oxygen would distinguish this enzyme from the other reductases of denitrification in *P. denitrificans* [1,7], but is not surprising because the reductase contains haem centres [26] which generally have a higher affinity for nitric oxide than for oxygen. The evidence that the nitric oxide reductase can function under aerobic conditions makes it difficult to understand why Kučera et al. [11] found that the period of an inhibitory effect of added nitric oxide upon the oxidation of TMPD by membrane vesicles from *P. denitrificans* was not altered in proportion to the quantity of vesicles present.

Activity of the nitric oxide reductase under aerobic conditions is also required to account for certain features of the previously observed inhibition in the presence of nitrite of aerobic respiration by cells of *P. denitrificans* treated with either Triton X-100 or a protonophoric uncoupler [9,10]. These are: (i) the disappearance of the added nitrite from the reaction mixture was followed, after a short lag, by relief of the inhibition; (ii) during the inhibited phase essentially zero consumption of oxygen was observed [9,10]. The latter finding means that nitric oxide was not removed by reaction with oxygen, which at first sight is puzzling given the known high reactivity of nitric oxide towards oxygen. A plausible basis for understanding why nitric oxide is not removed through reaction with oxygen is as follows. If the reaction of nitric oxide with oxygen in aqueous media follows the same third order kinetics as the reaction in the gas phase and in carbon tetrachloride solution [17] then at low nitric oxide concentrations (which appear in the rate equation as a squared term) the rate of nitric oxide reaction with oxygen would be far from instantaneous [18]. If the nitric oxide reductase activity of cells has very high affinity, then this condition of very low nitric oxide concentration could be satisfied with the result that the potent inhibition of oxidase activity reported previously [9,10] could be observed.

In the case of the experiments with submitochondrial particles and nitrite reductase reported in this paper it appears that nitric oxide was able to build up to a sufficient concentration so as to inhibit strongly the oxidase activity of the particles. The slow consumption of oxygen in this experiment (Fig. 1) may well reflect

the uncatalysed reaction of nitric oxide with oxygen. The rate of oxygen reduction following introduction of nitric oxide, either via nitrite reductase or as a gas (Figs. 2–4), was less when membranes possessing nitric oxide reductase activity were present, suggesting that the steady-state concentration of nitric oxide was kept sufficiently low that reaction with oxygen was insignificant.

Record A in Fig. 4 shows that addition of nitric oxide caused an immediate decrease in the oxygen concentration. The change in oxygen concentration was however, significantly less than expected if the reaction $2NO + O_2 \rightarrow 2NO_2$ were to approach completion. The anticipated stoichiometric consumption of oxygen was observed if the same amount of nitric oxide was introduced to a reaction mixture that contained solely the buffer. The following is an interpretation of the events subsequent to addition of nitric oxide in experiment A of Fig. 4. The nitric oxide is proposed to have three immediate fates: (i) reaction with dissolved O_2; (ii) reduction by the nitric oxide reductase which is presumed only partially inhibited by Triton X-100 under these conditions; (iii) binding to cellular components, e.g., oxidases, which causes inhibition. Process (i) is suggested, for the reasons indicated above, to slow to a negligible rate once the concentration of nitric oxide has fallen to a certain level. The concomitant operation of process (ii) prevents stoichiometric reaction of added nitric oxide with oxygen. During the inhibited phase of oxygen respiration nitric oxide is also formed by reduction of the nitrate and nitrite generated by hydration of the initial product of the reaction of nitric oxide with oxygen. Once replenishment of nitric oxide is complete its reductase is sufficiently effective at scavenging for low concentrations of nitric oxide that the inhibition of oxidase activity is substantially reversed. In view of these considerations the precise stoichiometry of reaction of nitric oxide with oxygen may be complex but some reaction should always be expected. Thus it is difficult to understand how Krul [19] could have observed the inhibition by nitric oxide of oxidase activity of denitrifying bacteria without the significant removal of dissolved oxygen through uncatalysed reaction with the added nitric oxide.

In record B, Fig. 4, the added nitrate and nitrite (formed by the pre-oxidation and hydration of the nitric oxide) might have been expected from previous work [10] to have been converted to an inhibitor, now recognised to be nitric oxide, of oxidase activity. The relatively brief and partial inhibition observed in Fig. 4B indicates that in that particular experiment the accumulation of nitric oxide formed from nitrate and nitrite was insufficient to cause prolonged inhibition. The important feature of Fig. 4B is that it acts as a control for Fig. 4A, thus establishing that nitric oxide itself was responsible for the inhibition depicted in the latter figure.

Careful inspection of Fig. 2 and Fig. 4(A) shows that during the inhibited phase of oxygen reaction that follows addition of nitrite there is an upward drift of the electrode response. Clark-type electrodes can detect nitrous oxide [20] and, although the operating conditions used in the experiments of Figs. 2 and 4 were not optimal for measurement of nitrous oxide, we attribute the upward deflections, which were repeatedly observed, to the detection, at low sensitivity, of the nitrous oxide synthesised by nitric oxide reductase. The presence of oxygen inhibits reduction of nitrous oxide by cells of *P. denitrificans* [20], which accounts for the formation of nitrous oxide in the experiment with cells (Fig. 4). In retrospect, the same upward electrode drift can be discerned in Fig. 9 of Ref. 10.

The foregoing discussion provides an explanation for some of the aspects of the inhibition of the aerobic respiratory chain in *P. denitrificans* by a product derived from nitrite that were not obviously or immediately consistent with the inhibitor being nitric oxide [10]. The observations and arguments in this paper show that nitric oxide is the inhibitor. However, the considerations discussed here concerning the synthesis of nitric oxide and its stability in the presence of oxygen do not in themselves explain why nitric oxide should be formed from nitrite under aerobic conditions only after permeabilisation of cells or treatment with a protonophore [9,10]. Kučera et al. [13] have argued that under these conditions the oxidases of the respiratory chain become more sensitive to inhibition by nitrite. Such inhibition, even to the rather slight degree that will be achieved by very low (tens of micromolar) concentrations of nitrite, would then permit some electron flux to be diverted to nitrite reductase. Synthesis of nitric oxide and strong inhibition of oxidase would then ensue. Alternatively, as observed by Alefounder et al. [7], the donation of electrons at a high rate to nitrite reductase with isoascorbate plus TMPD would have the same effect in permitting reduction of nitrite in the presence of oxygen. An additional factor might be that the agent used to permeabilise the cells, Triton X-100, is now known to inhibit nitric oxide reductase [16]. Thus in the presence of this detergent even a very minor amount of aerobic nitrite reduction could generate sufficient steady-state concentration of nitric oxide to have an inhibitory effect. Whether or not this effect occurs, there is no evidence that a similar effect could operate in the presence of the protonophore FCCP. In our hands this reagent, unlike Triton X-100, did not inhibit the nitric oxide reductase activity of cells of *P. denitrificans*.

The inhibition of aerobic respiration by *P. denitrificans* oxidase systems due to nitric oxide formation following addition of nitrite leads to consideration of some wider implications. First, are similar effects observed with other species of bacteria? There are reports

that very low concentrations of added nitrite can inhibit electron transport to oxygen in other organisms, for example in *Rhizobium 'hedysari'* strain HCNT1 [21]. As the inhibition is not observed in the presence of an inhibitor of nitrite reductase [22] it is probable that nitric oxide is the agent responsible in this organism as well. There is also an implication for the concept of aerobic denitrification [23]. If an organism can reduce nitrite to nitric oxide under aerobic conditions, without the perturbations that are necessary with *P. denitrificans*, then one might predict that nitric oxide-dependent inhibition of oxidase activity ought to prevent concomitant reduction of both oxygen to water and nitrate to the gaseous products that have been reported for *Thiosphaera pantotropha* [23]. This requires further investigation. The reduction of nitrite to nitric oxide discussed here may also mean that in some instances the preservative effect of nitrite might be due to a suicide inhibition of cell growth through enzymic generation of nitric oxide. Finally, the present work illustrates that, at least at low concentrations, nitric oxide in aqueous solution has significant stability in the presence of oxygen, consistent with its proposed role as an endothelium derived relaxant factor [12] and contrary to a suggestion [24] that it may be too unstable for such a role. The synthesis of nitric oxide by macrophages [25] might be directed towards the killing of aerobic bacteria through the inhibition of oxidase activity described and discussed in the present paper.

Acknowledgements

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