Interference of chlorate and chlorite with nitrate reduction in resting cells of *Paracoccus denitrificans*

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When grown anaerobically on a succinate + nitrate (SN) medium, *Paracoccus denitrificans* forms the membrane-bound, cytoplasmically oriented, chlorate-reducing nitrate reductase Nar, while the periplasmic enzyme Nap is expressed during aerobic growth on butyrate + oxygen (BO) medium. Preincubation of SN cells with chlorate produced a concentration-dependent decrease in nitrate utilization, which could be ascribed to Nar inactivation. Toluene treatment rendered Nar less sensitive to chlorate, but more sensitive to chlorite, suggesting that the latter compound may be the true inactivator. The Nap enzyme of BO cells was inactivated by both chlorate and chlorite at concentrations that were at least two orders of magnitude lower than those shown to affect Nar. Partial purification of Nap resulted in insensitivity to chlorate and diminished sensitivity to chlorite. Azide was specific for SN cells in protecting nitrate reductase against chlorate attack, the protective effect of nitrate being more pronounced in BO cells. The results are discussed in terms of different metabolic activation of chlorine oxoanions in both types of cells, and limited permeation of chlorite across the cell membrane.

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INTRODUCTION

Chlorate enters the environment from industrial sources such as pulp and paper plants, from its use as a non-selective contact herbicide and defoliant, and as a by-product of water disinfection. It exerts toxic effects on plants and bacteria, which are generally supposed to arise as a result of its enzymic conversion to a highly reactive reduction product, probably chlorite. Early studies and their follow-up work have provided strong evidence that the enzyme responsible for this metabolic activation is nitrate reductase (Aberg, 1947; Goksoyr, 1951, 1952; Fahraeus, 1952; Pichinoty *et al.*, 1969; Piéchaud *et al.*, 1969). Nitrate, on the one hand, potentiates chlorate toxicity by enhancing the synthesis of nitrate reductase, whereas, on the other hand, it slows down chlorite formation by competing with chlorate for the active site of the enzyme (Fahraeus, 1952).

A number of bacteria have the ability to perform respiratory or dissimilatory nitrate reduction due to the possession of the nitrate reductases membrane-bound Nar and periplasmic Nap (reviewed by Moreno-Vivian *et al.*, 1999; Philippot & Hojberg, 1999). Nar reduces chlorate, and its activity is inhibited by low concentrations of azide. Nap is generally regarded as azide-insensitive, and does not reduce chlorate, although the enzyme of *Rhodobacter sphaeroides* DSM 158

probably mediates the use of chlorate as an ancillary oxidant during phototrophic growth (Roldan *et al.*, 1994; Castillo *et al.*, 1996).

How does chlorate enter the cell cytoplasm? Early studies on nitrate uptake in plants have identified nitrate transporters as mediators of the influx of chlorate, giving support to the use of $^{36}\text{ClO}_3^-$ as a radiotracer transport analogue for nitrate (Deane-Drummond & Glass, 1982; Ruiz-Cristin & Briskin, 1991). This view has been subsequently modified in the light of results obtained by studying the kinetics of chlorate absorption, and interactions between chlorate and nitrate in intact plants (Kosola & Bloom, 1996), and results from electrophysiological experiments with *Xenopus* oocytes expressing individual nitrate transporter genes (Zhou *et al.*, 1998, 2000). Currently, it appears that the ability to transport chlorate or chlorite is restricted to some members of the proton-dependent oligopeptide transport (POT) family (Galván & Fernández, 2001). The situation with regard to bacteria is even more uncertain. Uptake experiments with intact and disrupted cells of the bacterium *Paracoccus denitrificans* have suggested that its nitrate transporter does not transport chlorate (John, 1977; Alefounder & Ferguson, 1980). The possibility still exists, however, that chlorate may act as an inhibitor of nitrate transport in bacteria. A recent study (Rusmana & Nedwell, 2004) has drawn attention to the possible applicability of chlorate for distinguishing between the contribution of Nar and Nap to nitrate removal in natural microbial communities.

Abbreviations: BO, butyrate + oxygen; MV, methyl viologen; SN, succinate + nitrate.

The authors have shown that there is complete inhibition by chlorate of nitrate conversion to ammonium in the growing culture of *Klebsiella pneumoniae*, which has only the *nar* operon. In contrast, in *Comamonas testosteroni*, which has both *nar* and *nap* operons, only some 50 % inhibition of denitrification is attained. Since the active site of Nar resides on the inner aspect of the cytoplasmic membrane, these observations have been interpreted as reflecting a block of nitrate import into the cell, caused by chlorate.

Considering the existing uncertainties, I thought it worthwhile to characterize interactions of chlorate with nitrate transport and metabolism in more detail. *P. denitrificans* was chosen as a model organism because its Nar and Nap content can be easily manipulated by the choice of specific carbon and energy sources for cultivation (Sears *et al.*, 1993). Resting cells were used throughout the incubation experiments, and nitrate reductase activities, instead of nitrate concentrations, were followed to identify any possible inactivation of the enzymes by the chlorine oxoanions tested.

METHODS

Organism and growth conditions. *P. denitrificans* strain CCM 982 used in these studies was obtained from the Czech Collection of Microorganisms. It was grown at 30 °C to early stationary phase, either anaerobically in a minimal medium (Kučera & Kaplan, 1996) supplemented with 50 mM succinate and 10 mM nitrate, or aerobically in the presence of 20 mM butyrate. Cells were collected by centrifugation, and washed with and resuspended in 0.1 M sodium phosphate buffer, pH 7.3. Bacterial cells obtained in these ways were designated SN (succinate + nitrate) and BO (butyrate + oxygen), respectively.

Tolueneization. A cell suspension of about 35 mg dry weight ml⁻¹ was warmed to 37 °C, mixed with 0.05 % (v/v) toluene, and left at 37 °C for 30 min. Centrifuged and washed permeabilized cells were kept in ice-water for up to 1 h until used.

Subcellular fractionation. Individual fractions of cytoplasmic membrane and periplasm were isolated as described by Alefounder & Ferguson (1980). Nap present in the crude periplasmic fraction was further purified by ion-exchange chromatography on an FPLC anion-exchange column (Mono Q HR10/10; Pharmacia) with a linear gradient of 0–800 mM NaCl in 20 mM Tris chloride, pH 8.0.

Chlorate/chlorite treatment. Cells suspended at a concentration of 15 to 20 mg dry weight ml⁻¹ in 0.1 M sodium phosphate buffer, pH 7.3, were supplemented with chlorate or chlorite, and incubated for 30 min at 30 °C. After washing, cells were resuspended in fresh buffer, and used immediately for enzyme activity determination.

Assay of methyl viologen cation radical (MV^{•+})-linked enzyme activities. The reaction mixture was maintained at 30 °C and contained, in a total volume of 2.6 ml, 0.1 M sodium phosphate, pH 7.3, 1 mM methyl viologen (MV), and an adequate number of cells. When indicated, 0.1 % Triton X-100 was also included. The mixture was rendered anoxic by bubbling with nitrogen, and titrated with a Na₂S₂O₄ solution in 10 mM NaOH until *A*₆₀₀ reached 1.5. After adding an electron acceptor (usually 10 mM nitrate or chlorate) to start the reaction, the oxidation of MV^{•+} was monitored at 600 nm ($\Delta\epsilon_{600}$ 11.4 mM⁻¹ cm⁻¹).

Data analysis. Maximal velocity *V*_{max}, Michaelis constant *K*_m, inhibition constant *K*_i for competitive inhibition and their SEs were

determined from non-linear regression analysis, using the kinetic software EZ-FIT (Perrella, 1988). Approximate 95 % confidence intervals for *V*_{max} and *K*_m were computed as the best-fit value ± SE multiplied by the appropriate *t* statistic with *n*–2 degrees of freedom, where *n* is the number of data points. Symbols in figures represent means ± SEM from at least four replicates. Error bars are omitted if they are smaller than the heights of the symbols.

Chemicals. Most chemicals used in this study were obtained from Sigma–Aldrich, and were of the highest grade available. The purity of commercial chlorite was checked by iodometric titration in 1 M acetic acid (Goksoyr, 1952).

RESULTS

To evaluate whether chlorate is capable of inhibiting nitrate import in *P. denitrificans*, the rate of MV^{•+} oxidation in whole SN cells was measured over a range of nitrate concentrations from 0 to 100 µM. Since SN cells contain predominantly the Nar enzyme, the active site of which is oriented toward the cytoplasm, it was expected that any alterations in nitrate import caused by chlorate would affect the kinetics of the reaction, as has previously been shown for inhibition of the nitrate transporter by phenylglyoxal (Kučera, 2003a). Nevertheless, addition of 5 mM chlorate to the assay medium did not significantly influence the shape of the saturation curve and the calculated best-fit kinetic parameters (Fig. 1). This negative result indicates that the inhibition, if it exists at all, cannot be instantaneous, but must take an amount of time to develop. Thus, the next experiment included a 30 min exposure of SN cells to 1–6 mM chlorate. Decreases in the activity were clearly seen,

Fig. 1. Utilization of low concentrations of nitrate by SN cells in the absence (○) or presence (●) of 5 mM chlorate. Initial velocity (*v*) of MV^{•+} oxidation was measured at the starting nitrate concentration indicated. Direct fits of respective datasets to the Michaelis–Menten equation yielded estimates ± SE (95 % confidence intervals) for *K*_m of 25 ± 8 (5.4–44.6) and 18 ± 6 (3.3–32.7) µM, and for *V*_{max} of 5.6 ± 0.7 (3.9–7.3) and 4.9 ± 0.6 (3.4–6.4) nmol MV^{•+} s⁻¹ (mg dry weight)⁻¹. Considerable overlaps of confidence intervals indicate that neither of the two kinetic parameters was significantly affected by 5 mM chlorate (*P* < 0.05).

although not only at the whole-cell level: the effect of chlorate pre-treatment persisted when the cell membrane was lysed by the addition of Triton X-100, with either nitrate or chlorate as an electron acceptor (Fig. 2). The nearly parallel course of all three curves in Fig. 2 suggests that the Nar itself, rather than the nitrate transporter, was the major molecular target for chlorate action.

Since chlorate enters the cells of *P. denitrificans* via diffusion through the lipid bilayer of the plasma membrane (Kučera, 2003b), experiments were undertaken to elucidate the role of membrane integrity. Fig. 3 compares the chlorate-concentration dependency of SN cells previously subjected to toluenization with that of the intact cells. Toluenization caused chlorate to lose its inactivator properties to a large extent. It therefore appears that chlorate must have undergone a metabolic conversion before becoming an inactivator of Nar, and that this conversion was slowed down after toluenization, probably because of a lack of endogenous substrates. The true inactivating species for Nar may be chlorite, the product of enzymic chlorate reduction, since chlorite sensitivity of Nar in toluenized cells did not much differ from chlorate sensitivity of the same enzyme in intact cells.

The results presented so far seem to be consistent with the view that chlorite formed from chlorate, by virtue of Nar, might be responsible for subsequent enzyme inactivation. Consequently, similar incubation experiments were performed with BO cells, which catalyse nitrate respiration mainly via the periplasmic Nap enzyme, previously reported

Fig. 2. Effect of prolonged exposure of SN cells to different concentrations of chlorate on the MV^+ -linked enzyme activities. SN cells were incubated for 30 min with the indicated concentrations of chlorate and assayed for the activity of MV^+ -nitrate reductase with 0.1 % Triton X-100 absent (○), and for MV^+ -chlorate reductase in the presence of 0.1 % Triton X-100 (▲). The results are plotted as a percentage of the maximum activity obtained with a control sample incubated without chlorate. These 100 % activities (means $\pm$ SEM, $n=4$) correspond to 5.1 ± 0.2 , 18 ± 1 and 54 ± 1 nmol MV^+ s^{-1} (mg dry weight) $^{-1}$, respectively.

Fig. 3. Susceptibility of the MV^+ -chlorate reductase activity of intact (open symbols) and permeable (solid symbols) SN cells to inactivation by chlorate (circles) or chlorite (squares). A suspension of freshly harvested SN cells was divided into two parts, one of which was subjected to toluenization. Both non-toluenized and tolueenized cells were then exposed for 30 min to various concentrations of chlorine oxoanions, and assayed for MV^+ -chlorate reductase in the presence of 0.1 % Triton X-100. Activities of 100 % (mean $\pm$ SEM, $n=4$) correspond to 46 ± 2 and 41.7 ± 0.3 nmol MV^+ s^{-1} (mg dry weight) $^{-1}$ for non-toluenized and tolueenized cells, respectively.

not to reduce chlorate. Somewhat surprisingly, the nitrate reductase activity of these cells turned out to be far more prone to inactivation by chlorate than that of SN cells (Fig. 4). The concentrations of chlorate necessary to achieve 50 % removal of the total activity (Nap plus Nar, measured in Triton-lysed samples) over a 30 min incubation period

Fig. 4. Inactivation of the MV^+ -nitrate reductase activity of BO cells (open symbols) or a periplasmic fraction (solid symbols) by chlorate (circles) or chlorite (squares). Samples were incubated for 30 min in the presence of the oxoanion concentrations shown, and assayed for MV^+ -nitrate reductase activity. Measurements with the cells were made in the presence of 0.1 % Triton X-100. Activities of 100 % (mean $\pm$ SEM, $n=4$) correspond to 13 ± 1 nmol MV^+ s^{-1} (mg dry weight) $^{-1}$ (cells) and 4.4 ± 0.2 nmol MV^+ s^{-1} (mg protein) $^{-1}$ (periplasm).

were found to be $\sim 30 \mu\text{M}$ for BO cells, as opposed to $\sim 3 \text{ mM}$ for SN cells (cf. Fig. 1). Even more dramatic was the difference in responses to external chlorite (IC_{50} $10 \mu\text{M}$ versus 9 mM).

The findings concerning the unexpected Nap inactivation in whole BO cells prompted analogous investigation in cell-free systems. A periplasmic fraction containing the enzyme was prepared and subjected to incubation with various concentrations of chlorate or chlorite. Fig. 4 shows that chlorate was completely ineffective at causing loss of enzyme activity. Chlorite clearly acted as an inactivator, but at concentrations at least 10 times higher than those needed for intact cells. Similar observations were also made with a partially purified Nap. When a time-course of its residual activity was examined for 30 min after adding 0.4 mM chlorite, the data followed pseudo-first-order reaction kinetics with a rate constant of $2.3 \times 10^{-4} \text{ s}^{-1}$ (results not shown). In agreement with Moreno-Vivian *et al.*, (1999), the ratio between MV^+ -chlorate- and MV^+ -nitrate-specific activities of the purified Nap was extremely low, not exceeding 0.05. When both electron acceptors were applied together, chlorate behaved as a competitive inhibitor of the MV^+ -nitrate reductase activity of Nap [$K_i(\text{ClO}_3^-)$ $6 \pm 3 \text{ mM}$, $K_m(\text{NO}_3^-)$ $0.35 \pm 0.08 \text{ mM}$].

Given the limited, or even absent, ability of Nap to reduce chlorate to chlorite, one must ask the reason for rapid inactivation of Nap by chlorine oxoanions in the cellular environment. The possibility that this effect was due to chlorite produced by a simultaneously present Nar was tested, based on selective inhibition of Nar by azide (Fig. 5). In control experiments with SN cells, azide effectively protected their Nar from inactivation by chlorate. The dependence of the extent of this protection upon azide concentration obeyed saturation kinetics, with a half-maximal effect at $\sim 10 \mu\text{M}$, which compares well with the

Fig. 5. Protective effect of azide on the inactivation by chlorate of MV^+ -nitrate reductase in SN (●) and BO (■) cells. Bacteria were incubated for 30 min in the presence of 10 and 0.5 mM chlorate, respectively, and different concentrations of azide, before assaying for MV^+ -nitrate reductase with 0.1% Triton X-100 added. Open symbols refer to control samples incubated without chlorate.

known K_i for azide inhibition of the Nar enzyme in *P. denitrificans* (Kučera & Kaplan, 1996). In contrast, the presence of azide afforded no protection against chlorate in BO cells, supporting the contention that the inactivation of Nap proceeds here independently of Nar.

In their natural environment, bacteria rarely encounter chlorine oxoanions in the total absence of nitrate. It was therefore of interest to repeat some of the above-described inactivation experiments under conditions of nitrate supplementation. To accomplish this, three representative chlorate and chlorite concentrations were chosen to cover the range of sensitivity for each type of cells (SN or BO). Fig. 6 shows that 10 mM nitrate effectively prevented inactivation caused by low concentrations of chlorate or chlorite; this effect was more pronounced in BO cells containing Nap.

Fig. 6. Ability of nitrate to protect the MV^+ -nitrate reductase activity of SN (upper panel) and BO cells (lower panel) from inactivation by chlorate or chlorite. Bacteria were incubated for 30 min with the indicated concentrations of chlorine oxoanions, either in the absence (open bars) or simultaneous presence (solid bars) of 10 mM nitrate. The reaction mixture for activity measurement contained 0.1% Triton X-100. Data were calculated relative to controls (100%), equal to 16.0 ± 0.4 and $4.1 \pm 0.2 \text{ nmol MV}^+ \text{ s}^{-1} (\text{mg dry weight})^{-1}$, respectively.

DISCUSSION

Rapid conversion of chlorate to toxic chlorite by the membrane-bound respiratory Nar of denitrifying bacteria is already well established, and has previously been exploited for the isolation of Nar mutants of *P. denitrificans* (Calder *et al.*, 1980) and of its close relative *Paracoccus pantotrophus* (formerly *Thiosphaera pantotropha*) (Bell *et al.*, 1993). As documented here (Fig. 2), Nar itself becomes a target for subsequent inactivation. In this respect, it behaves similarly to the algal assimilatory NADH-dependent Nar, which undergoes inactivation by both endogenously formed and externally added chlorite (Solomonson & Vennesland, 1972). The present study advances our knowledge of chlorate toxicity, in that it demonstrates that chlorate is extremely active against Nap. Incubation of cells with just 10 μ M chlorate sufficed for marked depression of enzyme activity (Fig. 4). This finding poses a serious dilemma because Nap binds chlorate only with low affinity (as indicated by a relatively high K_i for chlorate), and its chlorate reductase activity is very low, if not virtually non-existent. Moreover, at least 10-fold higher concentrations of chlorite had to be applied to comparably inactivate Nap in a separate periplasmic fraction (Fig. 4). Therefore, a possibility to be considered is that some kind of metabolic activation of chlorine oxoanions occurred in the BO cells. In contrast to the reduction of chlorate by Nar, this process appeared to be insensitive to micromolar concentrations of azide (Fig. 5). Identification of the true inhibitory species and the underlying reaction mechanisms must, of course, await further experimentation. Hypothetically, one may speculate about chlorine dioxide, known to result from peroxidase-catalysed dismutation of chlorite (Hewson & Hager, 1979), or about hypochlorite, a postulated intermediate of haem-dependent chlorite reduction (Kelly *et al.*, 1981). A functional group of nitrate reductases susceptible to non-catalysed chemical oxidation may be the dithiolene linkage in the molybdenum cofactor (Rajagopalan & Johnson, 1992). If the hypothesis of specific chlorate activation in BO cells holds true, one could predict the existence of mutants that would be insensitive to chlorate, and would show normal activity of nitrate reductase. Obtaining such mutants would assist in identification of the responsible genes and their protein products.

An aspect not to be overlooked is an apparently much lower permeability for chlorite than for chlorate, which can be inferred from comparison of the concentration dependencies in Fig. 3. A consequence may be that chlorite, once formed by Nar from the more diffusible chlorate, tends to remain entrapped in the cytoplasm, and does not influence the neighbouring cells. This type of reasoning has been applied earlier regarding the possibility of isolating chlorate-resistant colonies after growth in media supplemented with chlorate (Pichinoty *et al.*, 1969). Since Nar resides within the membrane, while Nap is a soluble protein, uneven accessibility could account, at least partly, for the observed difference in sensitivity of the two enzymes to chlorite (compare Figs. 3 and 4).

The nitrate transporter of *P. denitrificans* is highly homologous to that of *P. pantotrophus* (Wood *et al.*, 2001), and belongs to the nitrate/nitrite porter (NNP) family, which also includes the nitrate/nitrite transporters of *Escherichia coli*. For the latter organism, it has been reported that chlorate concentrations up to 30 mM do not affect the rate of nitrite production from nitrate catalysed by intact cells, in the presence of MV as an electron donor (Noji & Taniguchi, 1987). To ensure that no type of inhibition might have escaped attention, the kinetic measurements made with *P. denitrificans* (Fig. 1) employed a series of nitrate concentrations, including those well below saturation of the transporter. The clear absence of any short-term effect of chlorate on the kinetic parameters questions the suggestion (Rusmana & Nedwell, 2004) that chlorate might directly inhibit nitrate transport. Other possible mechanisms should be taken into account to explain the observed, apparently specific, suppression of cytoplasmic nitrate reduction by chlorate. Of particular concern, it seems that the above authors worked with cultures grown on nitrate. Based on the data in Fig. 6, the presence of nitrate should offer greater protection from chlorate attack to the periplasmic Nap compared to the cytoplasmic Nar, and the reduction of nitrate is then indeed expected to take place predominantly in the periplasm. Further work is needed to establish whether nitrate simply out-competes chlorate and chlorite for binding to their target sites, or whether there is also a contribution from chlorite removal by its reaction with the redox-active nitrogenous compounds involved as intermediates in the periplasmically located denitrification pathway.

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