ELSEVIER

Biochimica et Biophysica Acta 1276 (1996) 203–209

BBA Biochimica et Biophysica Acta

A study on the transport and dissimilatory reduction of nitrate in *Paracoccus denitrificans* using viologen dyes as electron donors

Igor Kučera [a,*], Petr Kaplan [b]

[a] *Department of Biochemistry, Faculty of Science, Masaryk University, Kotlářská 2, 61137 Brno, Czech Republic*
[b] *Department of Biochemistry, Faculty of Medicine, Masaryk University, Komenského nám. 2, 66243 Brno, Czech Republic*

Received 21 February 1996; revised 31 May 1996; accepted 7 June 1996

Abstract

The controversial subject of nitrate transport into the denitrifying cells of *Paracoccus denitrificans* was studied employing methyl or benzyl viologen cation radicals as electron donors for the respiratory nitrate reductase. In intact cells, the oxidation of methyl viologen by nitrate was sensitive to low concentrations of the uncoupler carbonyl cyanide m-chlorophenylhydrazone ($I_{50} = 0.8$ μM). Treatment of cells with 0.1% Triton X-100 (1) completely relieved the inhibition by the uncoupler, (2) raised the values of $K_m(NO_3^-)$ and V_m from 23 ± 5 μM and 6.4 ± 0.3 nmol MV s^{-1} mg dw^{-1} to 120 ± 10 μM and 13.5 ± 0.5 nmol MV s^{-1} mg dw^{-1}, respectively, and (3) changed the type of inhibition by azide from noncompetitive ($K_i = 0.18 \pm 0.01$ μM) to competitive ($K_i = 0.18 \pm 0.02$ μM) with respect to nitrate. On the other hand, permeabilisation had little effects on the activation energy (≈ 57 kJ mol^{-1}) and on the maximal degree of inhibition by 2-heptyl-4-hydroxyquinoline-*N*-oxide (≈ 50%). Observations (1) and (2) suggest an active transport mechanism for nitrate, whereas (3) can be best explained by a simultaneous leakage of the accumulated nitrate from the cells. Results from a HPLC analysis of cellular nitrate levels and their changes elicited by azide (an increase) or uncoupler (a decrease) are compatible with the occurrence of a cytoplasmic nitrate pool linking up nitrate transporter and nitrate reductase.

Keywords: Nitrate reductase; Nitrate transport; Viologen; (*Paracoccus denitrificans*)

1. Introduction

The first enzymic step of the dissimilatory reduction of nitrate in *Paracoccus denitrificans* and many other bacteria is the reduction of nitrate to nitrite catalysed by the membrane-bound respiratory nitrate reductase [1]. Since the nitrate binding site of the enzyme resides on the inner aspect of the cytoplasmic membrane [2], a transmembrane movement of nitrate must take place. However, although this translocation has repeatedly been proposed to have a prominent role in the regulation of anaerobic respiration [3–6], there is currently no agreement concerning the underlying molecular mechanisms. Indirect evidence in favour of an electroneutral nitrate/nitrite antiport initiated by a potential-dependent uptake of nitrate in symport with protons was provided by Boogerd et al. [7] who found that the dissipation of membrane potential by permeant ions or by an uncoupler resulted in a lag period between the introduction of nitrate and the onset of its reduction; the lags disappeared when nitrite was present at the moment of nitrate addition. On the other hand, measurements of the osmotic swelling of spheroplasts or the decay of K^+-diffusion potentials in membrane vesicles failed to detect either symport or antiport system [8]. Consequently, consideration was given to the possible existence of a nitrate-specific pore that would allow access of nitrate directly to the active site of its reductase from the periplasm. Further argument for this mechanism of nitrate entry derived from the fact that the apparent K_m value for nitrate observed with intact cells approached that of purified nitrate reductase when duroquinol was the reductant [9].

The experiments described in the present study were designed to obtain additional quantitative data on nitrate utilisation by denitrifying cells of *P. denitrificans*. Nitrate-dependent oxidation of viologen dyes (methyl or benzyl viologen cation radicals) is employed as a sensitive means of monitoring nitrate reduction. This kinetic approach is combined with an examination of intracellular

* Corresponding author. Fax: +42 5 41211214; e-mail: ikucera@chemi.muni.cz.

PII S0005-2728(96)00079-5

nitrate levels by ion-exchange high performance liquid chromatography.

2. Materials and methods

2.1. Bacterial strain and growth conditions

Paracoccus denitrificans NCIB 8944 was obtained from the Czech Collection of Microorganisms as CCM 982, and grown anaerobically at 30°C for 22 h in 1-litre batch cultures on the minimal medium composed of (in mM): sodium succinate (50), KNO_3 (10), KH_2PO_4 (30), Na_2HPO_4 (15), NH_4Cl (30), $MgSO_4$ (1), Na_2MoO_4 (0.5) and ferric citrate (0.03) (pH 7.3). Cells were harvested by centrifugation (6200 × *g*, 20 min) and washed with 0.1 M sodium phosphate (pH 7.3), before being resuspended in the same buffer. Stock cell suspensions were kept on ice and used within 8 h after harvest.

2.2. Assay for viologen dyes

The oxidation of viologen cation radicals was followed at 600 nm on a Cary 118C spectrophotometer with cuvette holders thermostatted at 30°C. A 1-cm cuvette fitted with a rubber stopper, contained in 2.5 ml, 0.1 M sodium phosphate (pH 7.3), 1 mM methyl or benzyl viologen and bacterial cells. Triton X-100 (0.1%), CCCP or nitrate reductase inhibitors were also present when indicated. After the mixture was bubbled with nitrogen for 2 min, a freshly prepared solution of 0.25 M $Na_2S_2O_4$ in 0.25 M $NaHCO_3$ was added to give an absorbance of 1.3 to 1.5 and the reaction started by addition of the appropriate electron acceptor ($NaNO_3$ or $NaNO_2$). Initial rates were calculated from the slope of the reaction curve at zero time by using millimolar absorption coefficients of 11.4 and 10.4 for MV^+ and BV^+ respectively [10].

2.3. Analysis of cellular nitrate

Nitrate levels inside the cells were determined by the method described by Romero et al. [11] with several modifications. A 0.5 ml-portion of the reaction mixture (0.1 M sodium phosphate, pH 7.3, 0.25 M sucrose, 0.1 mM methyl viologen, bacterial cells) was placed in a stoppered 1.5-ml Eppendorf tube containing 0.27 ml of the Dow Corning 550 silicone oil (Fluka Chemie AG, Buchs, Switzerland), beneath which was 0.1 ml of a solution of 20 mM sulfamic acid in 2 M H_3PO_4. Then 5 mM $Na_2S_2O_4$ was added, and after incubation for 3 min at 30°C the reaction was initiated by 2 mM $NaNO_3$. At 20 s, the tube was spun for 2 min at 15 000 rpm in a MPW-310 bench-top centrifuge (Mechanika precyzyjna, Warsaw, Poland). The cells sedimented into the phosphoric acid solution at the bottom of the tube, whereas the supernatant remained above the oil layer. A critical factor in this procedure was the inclusion of sucrose in the medium for cells to pass rapidly through the water-oil interface. Samples of 10 μl of the acid cell lysate were subjected to ion-exchange HPLC. The liquid chromatograph consisted of an LCP 4000 high pressure pump (Ecom, Prague, Czech Republic), an LCI 20 injector (Laboratory Equipments, Prague, Czech Republic) and an LC 55B detector (Perkin-Elmer GmbH, Vienna, Austria) set at 215 nm and coupled to a 1040/2 MTA strip-chart recorder (Kutesz, Budapest, Hungary). Separations were obtained using a column (0.3 × 15 cm) of HEMA-S 1000, 10 μm (Tessek Ltd., Prague, Czech Republic) with a guard column (0.3 × 3 cm) containing the same material as the main column. The solvent, 10 mM sodium phosphate (pH 6.8), was delivered at 0.7 ml min^{-1}. External standard was used to identify and quantify the peak corresponding to nitrate. Assays were reproducible for nitrate concentrations at least as low as 10 μM. A correction for extracellular nitrate trapped during centrifugation was based on determination of the volume occupied by [^{14}C]sucrose (Kučera et al., unpublished results). The internal volume of cells was taken to be 2 μl mg dw^{-1} [12].

2.4. Data analysis

The numerical values reported are means of at least four replicates (deviation from mean < 10%). Kinetic parameters and their standard errors are given as determined by nonlinear regression, using the program EZ-FIT developed by F. Perrella [13].

3. Results

Fig. 1 illustrates the absorbance changes when methyl or benzyl viologen radicals were oxidised by nitrate in the presence of anaerobically grown cells. Inspection of the records reveals significant kinetic differences. While with MV^+ as the electron donor a virtually linear decay curve was obtained, the decay curve of BV^+ has a more complex profile, characterized by a slow initial phase followed by a progressive acceleration. Inclusion of 0.1% Triton X-100, which is thought to render the cytoplasmic membrane permeable toward small ions, caused a substantial increase in the reaction rates and abolished the lag in the onset of BV^+ oxidation.

Fig. 1 also shows experiments performed by substituting an appropriate amount of nitrite for nitrate. Considerably lower rates were observed under these conditions demonstrating that conversion to nitrite was predominantly responsible for oxidising viologen radicals by nitrate.

The pronounced enhancement of the MV^+ and BV^+ by treatment of cells with Triton X-100 (Fig. 1) might have resulted either from increased penetration of exogenous substances into the cells or from removing a competition between the non-physiological reductant and cytoplasmic

electron donors. The latter explanation seemed unlikely, since the addition of 5 mM succinate did not affect the rate of MV^+ oxidation within the accuracy of the measurements, irrespective of the absence or presence of Triton. To explore the possibility that the stimulation may be due to an activation of nitrate reductase by sodium ions, as had been reported for the dissimilatory nitrate reductases in other bacteria [14], we used a Tris chloride buffer instead of sodium phosphate for washing and incubating cells and replaced $NaNO_3$ and $Na_2S_2O_4$ by Tris nitrate and aminoiminomethansulfonic acid, respectively. The resultant pattern of behaviour essentially similar to that in Fig. 1 corroborated that the low activity in intact cells was not caused by the lack of sodium ions to activate nitrate reductase.

For a detailed investigation of nitrate metabolism, MV^+ was chosen in preference to BV^+ to avoid the complex response and to allow a better evaluation of initial rates. As can be seen in Fig. 2, both intact and Triton-treated cells displayed a classical saturation kinetics with respect to external nitrate concentration. However, there appeared to be marked differences in the values of kinetic parameters, the most notable being a 5-fold increase in the apparent K_m value for NO_3^- upon permeabilisation. On the other hand, the data pertaining to the effect of temperature were mutually comparable. From the linear parts of the Arrhenius plots shown in Fig. 3, an activation energy could be estimated at 57 kJ mol^{-1} in both cases, which corresponds to a Q_{10} value (20–30°C) of 2.16.

Next, experiments were undertaken with the aim of comparing the sensitivity of intact and permeabilised cells to the typical agents known to affect transmembrane proton gradient or electron flow to nitrate. The data from

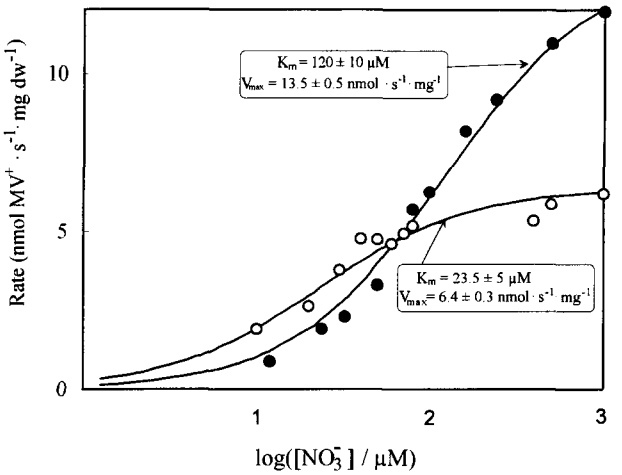

Fig. 2. Dependence of the observed initial rates for the oxidation of MV^+ in intact (○) and permeabilised (●) *P. denitrificans* cells as a function of nitrate concentration. The lines and the numerical values represent the fits of data sets to the Michaelis-Menten equation. Substrate concentration is displayed on a logarithmic scale for the sake of convenience.

titrations by the protonophorous uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP) are given in Fig. 4. It is apparent that CCCP caused a marked depression of the initial rate of MV^+ oxidation in intact cells. This inhibition was concentration-dependent (half-maximal effect at 0.8 μM), decreasing in size with time and could be completely relieved by permeabilisation. In agreement with Boogerd et al. [15], we could find no inhibition by CCCP in inside-out vesicles (results not shown).

2-n-Heptyl-4-hydroxyquinoline-*N*-oxide (HQNO), which is believed to interact with nitrate reductases at the ubiquinol binding site, was only partially effective at in-

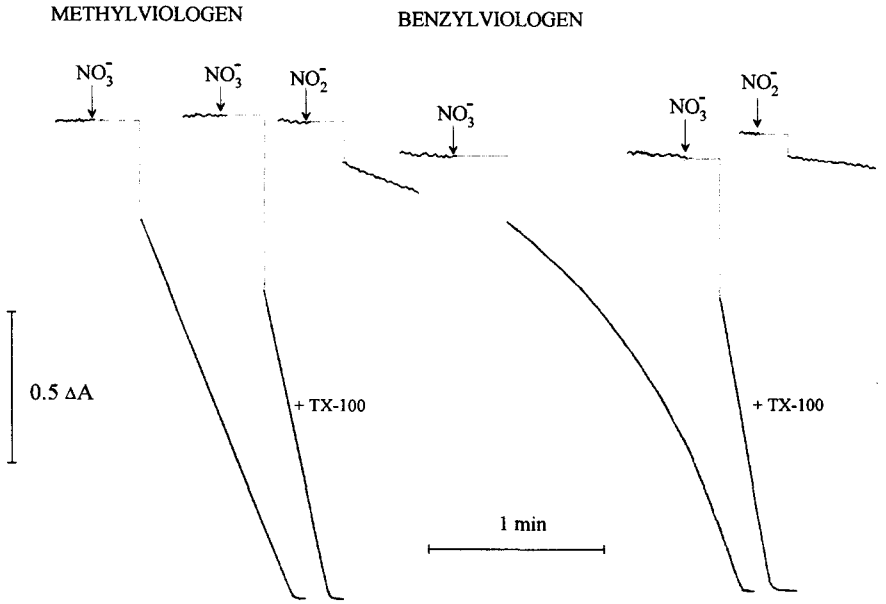

Fig. 1. Typical traces of electron acceptor-induced reoxidation of viologen cation radicals by intact or Triton-permeabilised *P. denitrificans* cells. Bacteria (0.78 mg dry wt.) were suspended in 2.5 ml of the assay mixture described in Section 2. On partial reduction of viologen by dithionite, the reaction was started by the addition of 1 mM-nitrate or nitrite. The vertical bar indicate the change of absorbance at 600 nm.

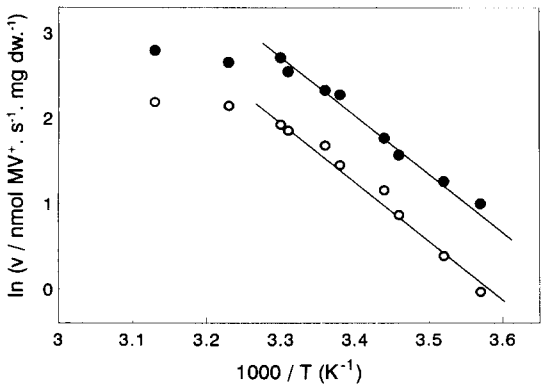

Fig. 3. Arrhenius plot for the temperature dependence of MV^+-nitrate reductase activity of intact (○) and permeabilised (●) *P. denitrificans* cells. Assays were performed under substrate saturating conditions (1 mM NO_3^-).

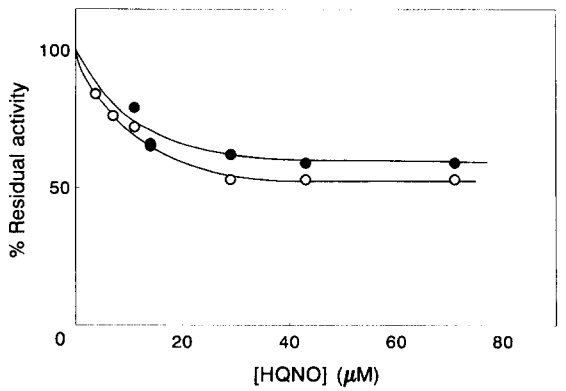

Fig. 5. The effect of HQNO on the oxidation of MV^+ by intact (○) or permeabilised (●) cells in the presence of 1 mM nitrate. The uninhibited (100%) initial rates were 4.2 and 9.9 nmol MV^+ oxidised s^{-1} mg dw^{-1}, respectively.

hibiting MV^+ oxidation. About a half of activity was retained in both intact and permeabilised cells (Fig. 5).

In an early study using particulate and purified nitrate reductase from *P. denitrificans*, Forget [16] observed a strong inhibition by azide, acting in competition with nitrate. A Dixon-type analysis presented in Fig. 6 demonstrates that this was also the case with the permeabilised cells. Intact cells differed considerably regarding the type of inhibition which was noncompetitive rather than competitive at low concentrations of azide (up to approx. 0.3 μM). However, despite this difference, the inhibition constants (got either from the horizontal coordinates of the points of the straight lines or as a result of non-linear regression) were identical ($K_i = 0.18$ μM).

The above findings left some uncertainty as to the sites of inhibition in intact cells. In an attempt to clarify further this issue, the amount of nitrate present in cells was determined by the procedures described in Section 2. Fig. 7 shows the results of an experiment in which cells were incubated with nitrate for 20 s. When added at concentrations inhibiting the reduction of NO_3^- by MV^+, CCCP

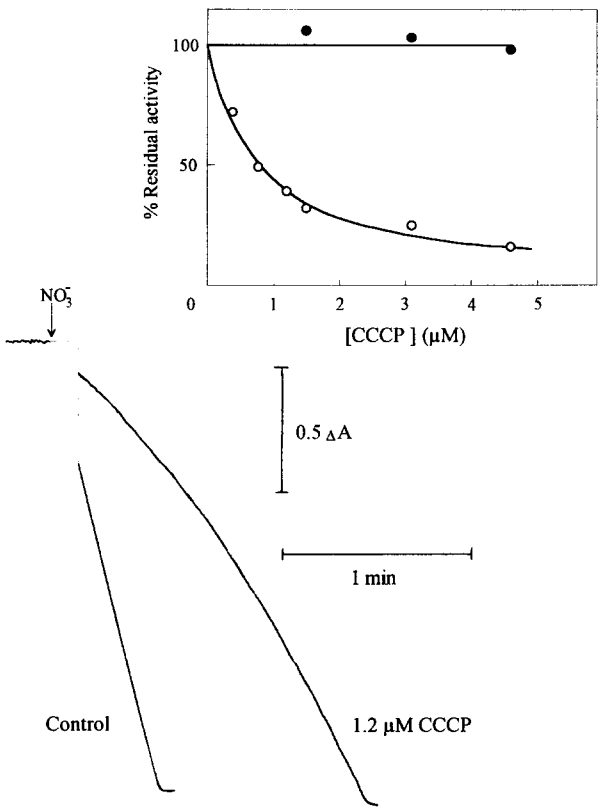

Fig. 4. Inhibition in *P. denitrificans* cells (3.5 mg dry wt.) of electron transport from MV^+ to NO_3^- by CCCP. The inset shows the inhibition titration of intact (○) and permeabilised (●) cells. The uninhibited (100%) initial rates were 3.9 and 13.9 nmol MV^+ oxidised s^{-1} mg dw^{-1}, respectively.

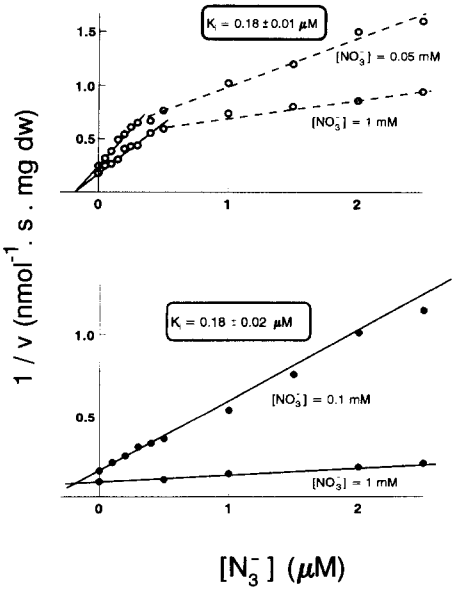

Fig. 6. Dixon plots showing the inhibition of MV^+-dependent nitrate reduction by azide in intact (○) and permeabilised (●) *P. denitrificans* cells. The abscissa of the intersection point of the straight lines is equal to $-K_i$.

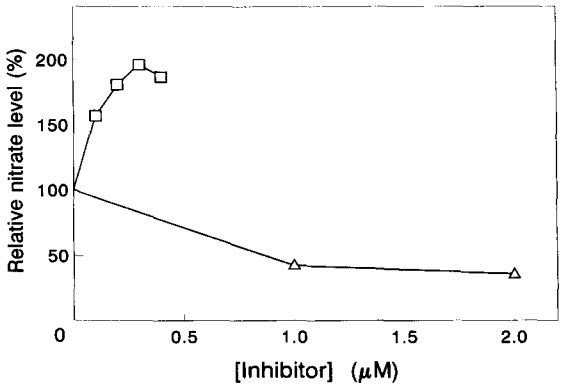

Fig. 7. Dependence of the relative nitrate level in cell lysates on CCCP (△) or azide (□) concentrations. Each Eppendorf tube contained 4.0 mg dry weight of cells and the indicated concentration of the inhibitor. Further treatment of the samples and determination of nitrate followed the protocol explained in Section 2. The 100% value corresponds to an intracellular concentration of ≈ 90 μM, as estimated from experiments in which no inhibitors were added and [^{14}C]sucrose was included in the incubation mixture.

decreased the attained intracellular level of nitrate. This is consistent with the view that CCCP specifically disturbs nitrate uptake into cells. The reason why azide had an opposite effect on intracellular nitrate (a 2-fold increase) could be a preferential inhibition of nitrate reductase.

4. Discussion

Jones and Garland [17] carried out an extensive study testing the ability of several bipyridylium cation radicals to cross the cytoplasmic membrane of *Escherichia coli* and to react with the membrane-bound nitrate reductase. These authors clearly distinguished two pathways involved in the reduction of the enzyme. One of them, termed indirect, was highly specific for Diquat radical, sensitive to HQNO and received electrons at the periplasmic aspect of the cytoplasmic membrane. The other pathway, termed direct, could utilize all the radicals tested and faced to the cytoplasm. It was also possible to show that BV^+ could easily traverse the membrane and donate electrons to the direct pathway, whereas the impermeable MV^+ acted as an effective electron donor only in broken cells.

The above conclusions drawn about *E. coli* seem not to be fully applicable to *P. denitrificans*, as evidenced by a relatively high MV^+-dependent nitrate reductase activity associated with intact cells (Fig. 1) and its partial inhibition by HQNO (Fig. 5). In two studies with purified nitrate reductase from *E. coli* [18] and *P. denitrificans* [9], HQNO was found to inhibit the ubiquinol-dependent enzyme activity but not the reduction of nitrate by MV^+. Conceivably in cells of *P. denitrificans* a significant part of MV^+ oxidation is occurring via ubiquinone pool within the membrane. An important aspect of the results in Fig. 5 is that detergent treatment did not influence partitioning of electrons from MV^+ between the HQNO-sensitive and HQNO-insensitive pathways. The changes in kinetic behaviour upon permeabilisation may therefore be due not to a diversion of electron influx from one reducing pathway to another but rather to an increased permeability for nitrate.

Recently, the occurrence of a periplasmic respiratory nitrate reductase has been recognised in the denitrifiers *Thiosphaera pantotropha* [19] and *P. denitrificans* [20]. As the periplasm is freely accessible to MV^+, the nitrate dependent oxidation of MV^+ by intact cells (Fig. 1) could have been accomplished by this reductase. In principle, the presence of the periplasmic nitrate reductase would pose serious difficulties in interpreting our experimental data, but can be definitely ruled out on the basis of the observed 85% inhibition at 2.5 μM azide (Fig. 6) taken together with the fact that even 20 μM azide failed to affect the activity of the authentic periplasmic enzyme [20].

An independent determination of nitrate transport activity in whole cells is certain to be difficult due to metabolic impacts (rapid conversion to nitrite) as well as the absence of a readily available radiotracer. Our experience with bipyridylium salts suggests that the enzyme catalysed reaction of the accumulated nitrate with MV^+ could be a convenient albeit indirect means of monitoring the transport. In this way the transport was shown here to be saturable (and thus facilitated) (Fig. 2). The apparent K_T value found (23 ± 5 μM) is somewhat higher than that estimated previously from nitrate decay curves (< 5 μM) [8]; nevertheless, it lies within the range for pure cultures of other denitrifying bacteria [21,22].

It has been reported several times that the addition of CCCP exerts little effect on the rates of succinate, glucose, or viologen radicals-supported nitrate uptake, as measured by the use of a nitrate electrode, in *P. denitrificans* [23,24] or *Pseudomonas aeruginosa* [25]. On the contrary, in *P. denitrificans* cells grown on H_2, CO_2 and NO_3^-, the uptake of H_2 during reduction of NO_3^- was subject to almost complete inhibition by micromolar concentrations of CCCP [7]. Likewise, $^{13}NO_3^-$ uptake by *E. coli* was drastically inhibited by 10 μM CCCP [26] and 0.75 μM CCCP gave 50% inhibition of nitrite production from nitrate in *Flexibacter canadensis* [22]. In view of this apparent discrepancy it was of interest to find a transient inhibition of MV^+-dependent nitrate reductase in intact, but not permeabilised cells (Fig. 4), complemented by a considerable reduction in the intracellular level of NO_3^- elicited by CCCP (Fig. 7). Our data thus extend previous indications that metabolic energy is required to initiate nitrate delivery to the cytoplasm [7]. Low initial rates with benzyl viologen in comparison with methyl viologen (Fig. 1) may arise from an electrophoretic dissipation of the membrane potential by inward movement of the more lipophilic cation radical of the former compound, leading to inhibition of nitrate transport.

If a unidirectional transport of nitrate precedes its con-

version to nitrite inside the cell then a competitive inhibitor of nitrate reductase might be predicted to have no effect on the measured overall rate. This is because in the presence of inhibitor, the intracellular nitrate should readily set the level which is just sufficient to prevent the reaction rate from changing. However, in sharp contrast to the above expectation, azide was shown to be almost equally effective at inhibiting MV^+-dependent NO_3^- reduction in both intact and permeabilised cells (Fig. 6).

An explanation, consistent with the well documented permeability of *Paracoccus* cytoplasmic membrane for nitrate anion [8,27], would anticipate that the presence of a transmembrane potential difference negative inside promotes a continuous excretion of the accumulated nitrate out of the cell leading to nitrate cycling across the membrane. A precedent for such a 'pump and leak' mechanism occurs in the nonvacuolated alga, *Cyanidium caldarium*, where a short-term regulation of the net nitrate uptake through changes in nitrate efflux was evidenced [28]. This kept internal nitrate at relatively low concentration, even when nitrate reductase was severely inhibited by cyanate. In a somewhat related study, nitrogen isotopic discrimination was used to measure the ratio of nitrate efflux to nitrate influx for the cyanobacterium *Synechococcus* sp. R2 [29]. Once again, the decreases in NO_3^- reduction brought about by the addition of increasing concentrations of tungstate into the growth medium were found to be accompanied by increases in the relative extent of nitrate efflux.

It may be useful to discuss here, in more details, a simple kinetic model of nitrate cycling where the rates of both nitrate reduction and leakage are proportional to the concentration of nitrate inside the cells. The condition of steady state applied to intracellular nitrate yields:

$$\frac{V_T[NO_3^-]_{out}}{K_T + [NO_3^-]_{out}} = L[NO_3^-]_{in} + \frac{V_{NR}}{K_m^{app}}[NO_3^-]_{in}$$

Here, K_T and $K_m^{app} = K_m(1 + [N_3^-]/K_i)$ denote, respectively, the half-saturating concentration of nitrate for its transport into the cells and the apparent Michaelis constant of nitrate reductase for nitrate in the presence of azide. V_T and V_{NR} represent the maximal rates of nitrate transport and enzymic conversion, respectively, while L specifies the rate of leakage at the internal nitrate concentration equal to 1. Solving for $[NO_3^-]_{in}$, one obtains:

$$[NO_3^-]_{in} = \frac{K_m^{app} V_T [NO_3^-]_{out}}{(LK_m^{app} + V_{NR})(K_T + [NO_3^-]_{out})}$$

Consequently, the rate at which nitrate is consumed by nitrate reductase in intact cells can be expressed in terms of $[NO_3^-]_{out}$ as follows

$$v = \frac{V_T^{app}[NO_3^-]_{out}}{K_T + [NO_3^-]_{out}} \quad (1)$$

with

$$V_T^{app} = V_T \frac{V_{NR}}{LK_M^{app} + V_{NR}} < V_T$$

Since only the V_T^{app} term contains K_m^{app} and is therefore affected by azide, the rate expression (Eq. (1)) predicts a noncompetitive inhibition by N_3^- relative to external nitrate. Moreover, if $LK_m^{app} \gg V_{NR}$, it can be easily proven that the resulting inhibition constant should approach that for the competitive inhibition of nitrate reductase in the permeabilised cells (K_i). Both these predictions are in line with the experimental observations (Fig. 6).

Although nitrate cycling may provide a satisfactory explanation for the inhibitory pattern of azide in intact cells, alternative possibilities are not completely ruled out. The observation on the similar values for intact and permeabilised cells could also be taken as indicative of some type of interaction between nitrate transporter and nitrate reductase which in turn would control nitrate influx. Indeed, there are several reports describing a direct cooperation between the enzymes and the membrane-bound transport proteins for the enzyme substrates or products in mitochondria [30]. The involvement of such an assembly in the denitrification pathway would, however, be difficult to reconcile with the observed increase in $[NO_3^-]_{in}$ caused by azide (Fig. 7) which can be readily interpreted in terms of a cytoplasmic nitrate pool linking up nitrate transporter with nitrate reductase, without any necessity of assuming a direct nitrate transfer to the enzyme.

Acknowledgements

We thank Mrs. J. Neužilová for expert technical assistance. This work was supported by Grant 204/93/2054 from the Grant Agency of the Czech Republic.

References

[1] Ferguson, S.J. (1994) Antonie van Leeuwenhoek 66, 89–100.
[2] John, P. (1977) J. Gen. Microbiol. 98, 231–238.
[3] Alefounder, P.R. and Ferguson, S.J. (1980) Biochem. J. 192, 231–240.
[4] Noji, S. and Taniguchi, S. (1987) J. Biol. Chem. 262, 9441–9443.
[5] Hernandez, D. and Rowe, J.R. (1987) Appl. Environ. Microbiol. 53, 745–750.
[6] Hernandez, D. and Rowe, J.R. (1988) J. Biol. Chem. 263, 7937–7939.
[7] Boogerd, F.C., Van Verseveld, H.W. and Stouthamer, A.H. (1983) Biochim. Biophys. Acta 723, 415–427.
[8] Parsonage, D., Greenfield, A.J. and Ferguson, S.J. (1985) Biochim. Biophys. Acta 807, 81–95.
[9] Craske, A. and Ferguson, S.J. (1986) Eur. J. Biochem. 158, 429–436.
[10] Kristjansson, J.K. and Hollocher, T.C. (1980) J. Biol. Chem. 255, 705–707.
[11] Romero, J.M., Lara, C. and Guerrero, M.G. (1989) Biochem. J. 259, 545–548.
[12] McCarthy, J.E.G., Ferguson, S.J. and Kell, D.B. (1981) Biochem. J. 196, 311–321.

[13] Perrella, F. (1988) Anal. Biochem. 174, 437–447.
[14] Yamaoka, K., Kato, M. and Kamihara, T. (1988) Biochem. Int. 16, 829–834.
[15] Boogerd, F.C., Appeldoorn, K.J. and Stouthamer, A.H. (1983) FEMS Microbiol. Lett. 20, 455–460.
[16] Forget, P. (1971) Eur. J. Biochem. 18, 442–450.
[17] Jones, R.W. and Garland, P.B. (1977) Biochem. J. 164, 199–211.
[18] Morpeth, F.F. and Boxer, D.H. (1985) Biochemistry 24, 40–46.
[19] Bell, L.C., Richardson, D.J. and Ferguson, S.J. (1990) FEBS Lett. 265, 85–87.
[20] Sears, H.J., Ferguson, S.J., Richardson, D.J. and Spiro, S. (1993) FEMS Microbiol. Lett. 113, 107–112.
[21] Betlach, M.R. and Tiedje, J.M. (1981) Appl. Environ. Microbiol. 42, 1074–1084.
[22] Wu, Q. and Knowles, R. (1994) Can. J. Microbiol. 40, 576–582.
[23] Alefounder, P.R., McCarthy, J.E.G. and Ferguson, S.J. (1981) FEMS Microbiol. Lett. 12, 321–326.
[24] Kim, C.-H., Kristjansson, J.K., White, M.M. and Hollocher, T.C. (1982) Biochem. Biophys. Res. Commun. 108, 1126–1130.
[25] Hernandez, D., Dias, F.M. and Rowe, J.J. (1991) Arch. Biochem. Biophys. 286, 159–163.
[26] Rowe, J.J., Ubbink-Kok, T., Molenaar, D., Konings, W.N. and Driessen, A.J.M. (1994) Mol. Microbiol. 12, 579–586.
[27] Kučera, I., Karlovský, P. and Dadák, V. (1982) Biologia (Bratislava) 37, 809–814.
[28] Fuggi, A. (1989) Biochim. Biophys. Acta 974, 141–148.
[29] Shearer, G., Schneider, J.D. and Kohl, D.H. (1991) J. Gen. Microbiol. 137, 1179–1184.
[30] Sandor, A., Johnson, J.H. and Srere, P.A. (1994) J. Biol. Chem. 269, 29609–29612.