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Passive penetration of nitrate through the plasma membrane of *Paracoccus denitrificans* and its potentiation by the lipophilic tetraphenylphosphonium cation

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Abstract

Previously, it has been shown that treatment of *Paracoccus denitrificans* cells with phenylglyoxal inhibits the methyl-viologen-linked nitrate reductase activity by blocking the nitrate transporter. This inhibition disappears if tetraphenylphosphonium cation (TPP^+) is added to the assay medium. In the present paper, the following evidence suggests that the effect of TPP^+ results from an increased transmembrane anion permeability and not from transporter reactivation or cell lysis. (1) Beside nitrate, TPP^+ also mediated the utilisation of chlorate, which normally lacks access to the cytoplasm. (2) The TPP^+ pathway had about hundred-times higher K_m values for nitrate and chlorate than nitrate reductase in Triton X-100 permeabilised cells. (3) Although the uncoupler CCCP alone failed to overcome the PG block, it stimulated the operation of the TPP^+ pathway. (4) The method of continuous variations allowed the transport stoichiometry TPP^+/NO_3^- to be determined as 3, indicating charge compensation for nitrate movement and the subsequent transmembrane two-electron redox reaction. Anion uptake was also measured independently from passive swelling of uncoupled spheroplasts in iso-osmotic solutions of ammonium salts. The permeability to nitrate lay in the permeability sequence $Cl^- < NO_3^- < ClO_4^- < SCN^-$ and was further enhanced by TPP^+.

Keywords: Nitrate transport; Nitrate reductase; Permeant ion; Ion pair; Uncoupler; Bacterium

1. Introduction

The utilisation of nitrate as a terminal electron acceptor instead of oxygen in denitrifying bacteria like *Paracoccus denitrificans* is initiated by its active uptake from the environment and subsequent reduction to nitrite in the cytoplasm. Nitrite is then reduced to molecular nitrogen in the periplasmic space of the cells [1]. Major advances have been made over the last decade towards our understanding of enzymatic reactions associated with denitrification whereas little attention has so far been paid to the mechanisms responsible for nitrate and nitrite transport [2]. In our laboratory, we have demonstrated that the use of reduced methyl viologen as a nonphysiological electron donor for nitrate reduction in whole cells provides a convenient experimental tool for analysing transport mechanisms and for screening selective transport inhibitors [3]. Recently, I have shown that treatment of cells with millimolar concentrations of phenylglyoxal (PG), a dicarbonyl modifier of arginyl residues, blocks nitrate transport in *P. denitrificans* without affecting the nitrate reductase itself and that the tetraphenylphosphonium cation (TPP^+) reverses this inhibition [4]. The present investigation is an attempt to elucidate the mode of action of TPP^+. The sum of observations reported here suggests a potentiation of nitrate permeability by TPP^+, apparently resulting from easier diffusion of the lipophilic $TPP^+.NO_3^-$ ion pairs through the lipid part of the membrane.

2. Materials and methods

2.1. Cell growth

P. denitrificans CCM 982 (NCIB 8944) was obtained from the Czech Collection of Microorganisms and cultivated in an anaerobic medium, containing 50 mM sodium succinate as the electron donor and 10 mM nitrate as the

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electron acceptor [3]. Cells were grown in 1-l filled bottles at 30 °C for 22 h, harvested by centrifugation (7000 × *g*, 20 min), washed in 0.1 M sodium phosphate, pH 7.3, suspended in 1 ml of the above buffer per 35 mg of dry cells, and stored on ice.

2.2. Preparation of spheroplasts

Cells from a 1-l culture (~ 0.3 g) were resuspended in 45 ml of 0.5 M sucrose, 20 mM Tris–HCl, pH 7.3, and incubated at 30 °C for 10 min. After the addition of 90 mg lysozyme (Fluka, Product Number: 62971) followed by additional incubation for another 30 min, the spheroplasts were collected by 20 min of centrifugation at 7000 × *g*, washed twice and resuspended in 9 ml of the sucrose medium. The suspension was stored on ice for no more than 3 h until assayed.

2.3. Incubation with phenylglyoxal

In order to study the influence of phenylglyoxalation, either intact cells or spheroplasts (~ 7 mg ml^{-1}) were treated at 30 °C for 30 min with 15 mM phenylglyoxal in 0.1 M sodium phosphate, pH 7.3, or 0.5 M sucrose, 20 mM Tris–HCl, pH 7.3, respectively. After chilling on ice, the bacteria were spun down (9500 × *g*, 5 min) and washed once with a modifier-free medium.

2.4. Assay for methyl viologen oxidation

Methyl viologen:acceptor oxidoreductase activity was measured under anaerobic conditions in an assay mixture (2.5 ml) containing N_2-saturated 0.1 M sodium phosphate (pH 7.5), 1 mM methyl viologen, and approximately 3 mg dw of cells [3]. Before the reaction was initiated by injection of either nitrate or chlorate, the reaction mixture was reduced chemically to give an absorbance at 600 nm of 1.3 by adding a small quantity of a freshly prepared solution of $Na_2S_2O_4$ in 10 mM NaOH. Oxidation of methyl viologen was monitored at 600 nm, using a millimolar absorption coefficient of 11.4.

2.5. Measurement of spheroplasts swelling

The osmotic swelling of spheroplasts was measured as a decrease in optical absorption at 500 nm in a cuvette of 1 cm light-path using a Cary 118C spectrophotometer. A triggering agent was added to spheroplasts suspended in 2.5 ml of isotonic medium (30 °C) consisting of 0.25 M ammonium salt (NH_4Cl, NH_4NO_3, NH_4ClO_4, or NH_4SCN) and 0.2 mM sodium azide, buffered with 20 mM Tris to pH 7.3. The final concentration of spheroplasts, evaluated in terms of dry mass, was about 0.3 mg ml^{-1}. Swelling rate was quantified as $-\Delta A_{500}/\Delta t$ (s^{-1}), calculated, in all cases, from a tangent to the initial portion of the trace A_{500} versus time.

2.6. Data analysis

Kinetic data represent the mean values from at least three replicates ± S.E.M. Nonlinear regression analysis was performed with the kinetic software package EZ-FIT developed by Perrella [5].

3. Results

Fig. 1 shows the results of an experiment in which the rate of MV^+ oxidation in PG-treated cells was measured as a

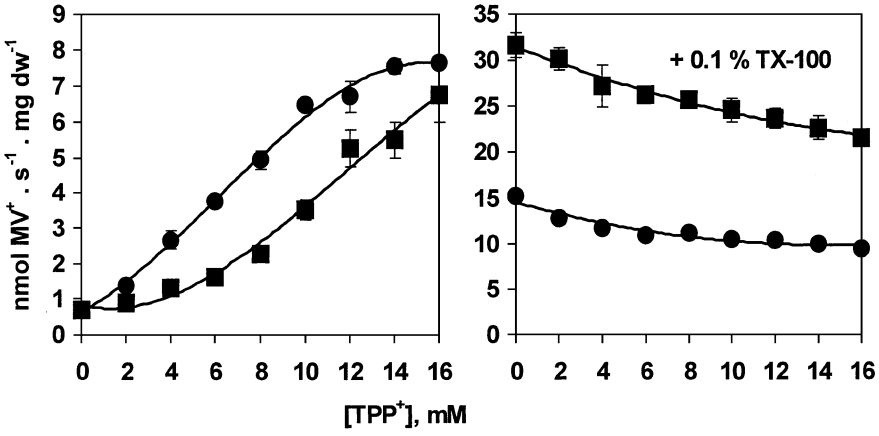

Fig. 1. Concentration dependence of the effect of TPP^+ on the reduction of nitrate (●) or chlorate (■) by methyl viologen in phenylglyoxalated cells. Bacteria were preincubated with phenylglyoxal in 0.1 M sodium phosphate, pH 7.3, as detailed in the Section 2.3. The enzyme assays were carried out under standard conditions (Section 2.4) at the TPP^+ concentrations indicated on the abscissa. The reaction was initiated with either 10 mM nitrate or 10 mM chlorate. The right panel represents the assays done in the presence of 0.1% Triton X-100. An intact cell samples not exposed to phenylglyoxal and TPP^+ had the activities of 6.53 ± 0.09 and 1.08 ± 0.06 nmol MV s^{-1} mg dw^{-1} with nitrate and chlorate, respectively.

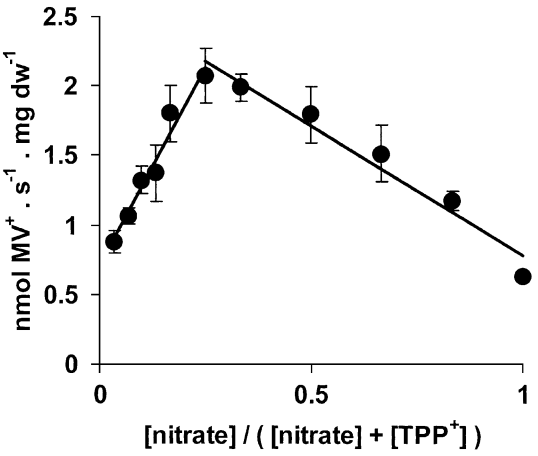

Fig. 2. A continuous variation (Job) plot showing that the optimal combining ratio for the nitrate reductase reaction in phenylglyoxalated cells is ~ $3TPP^+/NO_3^-$. The total concentration was fixed ($[NO_3^-]+[TPP^+]=11.1$ mM), but the mole fraction was allowed to vary as indicated.

Table 1
Estimation of the kinetic constants (K_m and V_{max}) for nitrate and chlorate utilisation by phenylglyoxalated cells via the TPP^+-mediated pathway and in the presence of Triton X-100

	Nitrate		Chlorate	
	K_m (mM)	V_{max} (nmol MV s^{-1} mg dw^{-1})	K_m (mM)	V_{max} (nmol MV s^{-1} mg dw^{-1})
TX-100 absent	10 ± 1	6.8 ± 0.3	14 ± 3	6.0 ± 0.6
TX-100 present	0.12 ± 0.01	12.5 ± 0.3	0.42 ± 0.05	31 ± 1

Kinetics was measured in cells pretreated with phenylglyoxal and washed (Section 2.3). The assay medium (Section 2.4) contained 10 mM TPP^+ to mediate anion transport. When indicated, 0.1% Triton X-100 was also present. The reaction was initiated by nitrate or chlorate at 1 of 10 concentrations over the range 2–48 mM (Triton X-100 absent) or 0.04–3.9 mM (Triton X-100 present). The K_m ± S.E. and V_{max} ± S.E. values shown represent nonlinear fits of data sets to the Michaelis–Menten equation.

function of the TPP^+ concentration. The rate was found to increase over the 0–10 mM concentration range. A second finding shown in Fig. 1 is that not only nitrate but also chlorate could be reduced in the presence of TPP^+. Thus, because in intact cells the active site of nitrate reductase is normally inaccessible to chlorate [6], it appeared that TPP^+ did not remove the PG block as such, but rather promoted a bypass around it.

To further define this bypass, I first sought to determine the kinetic parameters of terminal acceptor utilisation at 10 mM TPP^+ and compare them with those obtained after disrupting the permeability barrier with 0.1% Triton X-100. In all cases, the kinetic data led to hyperbolic dependencies from which the apparent Michaelis constants and limiting velocities could be estimated (Table 1). Since the bypass pathway exhibited a K_m that was about 100-fold higher than the value for the permeabilised cells, a non-specific damage to the membrane caused by TPP^+ could be ruled out.

When present at a high external concentration, TPP^+ is expected to move in the inward direction, dissipating the transmembrane potential gradient. If membrane depolarisation is the sole reason for the observed enhancement of nitrate entry in the presence of TPP^+, exposure of PG-treated cells to an uncoupler should exert a similar bypass effect. This was not the case. In cells treated with 15 mM PG for 30 min (0.1 M sodium phosphate, 30 °C), 10 μM CCCP caused even a modest decrease in the MV^+-nitrate reductase activity (from 0.85 ± 0.02 to 0.69 ± 0.02 nmol MV^+ s^{-1} mg dw^{-1}). Of interest, however, is that CCCP noticeably stimulated the TPP^+-mediated pathway (an increase of the activity from 5.2 ± 0.2 to 9.2 ± 0.4 nmol MV^+ s^{-1} mg dw^{-1} on addition of 10 μM CCCP to the reaction mixture containing 10 mM TPP^+), while it had no effect on control cell samples permeabilised by Triton X-100. Since CCCP is known to inhibit the physiological nitrate transport in *P. denitrificans* [3,4], the observed stimulation by CCCP further supports the notion that the TPP^+-mediated pathway does not involve the nitrate transporter.

The increased penetration of anions through the lipid bilayer in the presence of TPP^+ may be related to the formation of a lipid-soluble ion pair complex. Therefore, I attempted to obtain information about a possible transport stoichiometry in a continuous variation experiment in which the sum of $[NO_3^-]$ and $[TPP^+]$ was kept constant but their mole ratios varied (Fig. 2). The rationale behind this experiment is as follows. Assuming that an association equilibrium involving one nitrate anion and n TPP^+ cations takes place on the outer phase boundary (water solution/lipid membrane), the concentration of the associate within the membrane is expected to be proportional to the product

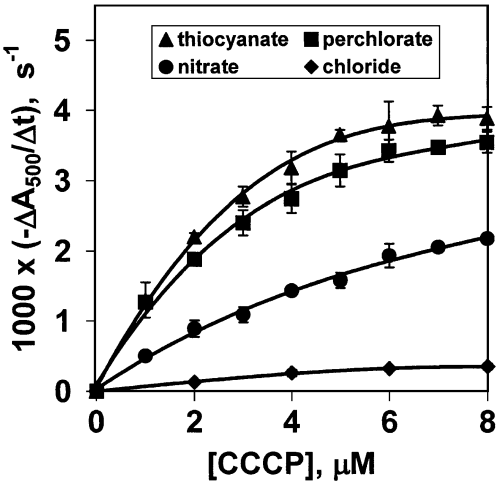

Fig. 3. Swelling of spheroplasts induced by CCCP in isoosmotic solutions of ammonium salts. The initial rates of swelling following CCCP addition are plotted versus CCCP concentration. See Section 2.5 for composition of the swelling assay media.

$[NO_3^-][TPP^+]^n$, where the square brackets denote the concentrations in the solution. At external nitrate concentrations lower than the apparent Michaelis constant for nitrate (~ 10 mM at 10 mM TPP^+, see Table 1), the pool of cytoplasmic nitrate is small and nitrate reductase works effectively as an indicator enzyme, measuring the rate of nitrate transport. Since this rate is determined by the membrane concentration of the conducting species, a plot of the velocity versus the mole ratio should have a maximum corresponding to the unknown stoichiometry. From Fig. 2, we have $[NO_3^-]/([NO_3^-]+[TPP^+]) = 0.25$ and $n=[TPP^+]/[NO_3^-] = 3$, indicating that three TPP^+ cations seemingly participated in the transport of one nitrate anion. Similar results were obtained with chlorate instead of nitrate (not shown).

The observed 3:1 stoichiometry, which markedly differs from the expected 1:1 ratio, raised the concern that the result holds only for the given system where the transmembrane nitrate reductase reaction takes place. To evaluate this possibility, an independent biochemical approach was taken, based on the passive swelling of spheroplasts in an isoosmotic solution of ammonium salts. Swelling requires net uptake of both cation and anion; hence, import pathways must be provided for both the anion and H^+ (electroneutral NH_3 diffuses freely). In Fig. 3, a comparison is offered of the swelling rates with four ammonium salts, when the proton conductance was raised by addition of increasing amounts of CCCP. A flattening of the titration profiles at high titres of uncoupler gives an indication that anion flux across the membrane becomes rate limiting for net salt transport and swelling. The observed swelling rates at high proton leakage enables to rank the permeabilities of the plasma membrane to the anion tested in the following increasing order: Cl^-, NO_3^-, ClO_4^-, SCN^-.

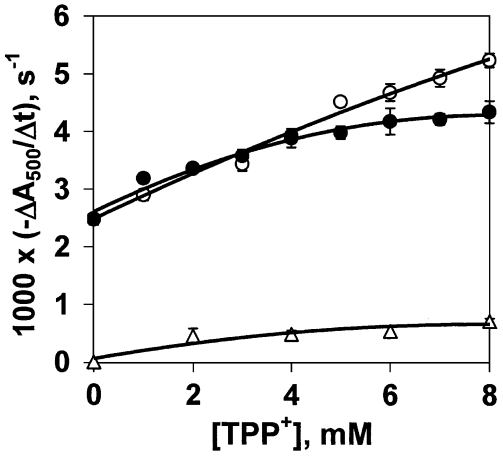

Fig. 4. Effect of TPP^+ on spheroplasts swelling in ammonium nitrate. The initial rates of swelling following TPP^+ addition are plotted versus TPP^+ concentration. Conditions of the assay are the same as described in Section 2.5. △, no CCCP added to the assay medium. ○, ●, 10 μM CCCP added together with TPP^+. ●, spheroplasts were pretreated with phenylglyoxal as described in Section 2.3.

In the next experiments, I looked at the effect of adding TPP^+ to the ammonium nitrate assay medium (Fig. 4). Whereas TPP^+ (≤ 8 mM) alone exhibited only a very low efficiency in producing swelling, it significantly potentiated swelling in response to 10 μM CCCP. For the data in Fig. 4, the synergistic enhancement factor $v_{TPP+CCCP}/(v_{TPP}+v_{CCCP})$ covered a range from 1 to 1.6. Pretreatment of the spheroplasts with 15 mM PG for 30 min at 30 °C (0.5 M sucrose with 20 mM Tris–HCl, pH 7.3) did not affect the swelling rate induced by CCCP at all and reduced the potentiation by TPP^+ of the uncoupler-provoked swelling only marginally (Fig. 4). These results confirm that most of nitrate anion enters the swelling spheroplasts mainly through the lipid bilayer and not via the nitrate transporter.

4. Discussion

Nitrate is often considered to be a lipophilic anion able to cross the membrane without the aid of specialised proteins or ionophores. However, this view was questioned for mitochondria by finding that effective nitrate uniport required opening of the inner membrane anion channel [7]. In the case of the cytoplasmic membrane of *P. denitrificans*, the comparative kinetic analysis of osmotic swelling (Fig. 3) indicates that nitrate has an intermediate permeability between thiocyanate and chloride. The same relation was obtained with *P. denitrificans* membrane vesicles when the decay of potassium diffusion potentials was taken as an estimate of the anion influx [8].

Accepting that the plasma membrane of *P. denitrificans* is to some extent permeable to nitrate, the question inevitably arises as to the physiological significance. Since PG inhibits the major portion (>90%) of nitrate respiration in the whole cells, the operation of an active nitrate transporter likely realises most of nitrate import under in vivo conditions. The negligible contribution of passive nitrate entry possibly bears on the orientation of transmembrane potential difference, which tends to expel the negatively charged nitrate anion from the cytoplasm. This continuous excretion of the accumulated nitrate then may lead to nitrate recycling across the membrane [3].

The results presented in this paper point to a significant inward transmembrane movement of nitrate or chlorate in the presence of TPP^+. Earlier extraction experiments using chloroform or dichloromethane have shown the ability of TPP^+ nitrate and TPP^+ chlorate salts to pass from the aqueous to the organic phase [9,10]. A plausible explanation thus may be that TPP^+ serves as a carrier and transfers the anion in the form of an ion pair complex. The two most striking findings reported here, namely the 3:1 stoichiometry and the TPP^+ + CCCP synergism, can best be understood considering the electroneutrality-based schemes depicted in Fig. 5. According to Fig. 5A, nitrate anion undergoes transport with one TPP^+ and two other TPP^+ cations are taken up

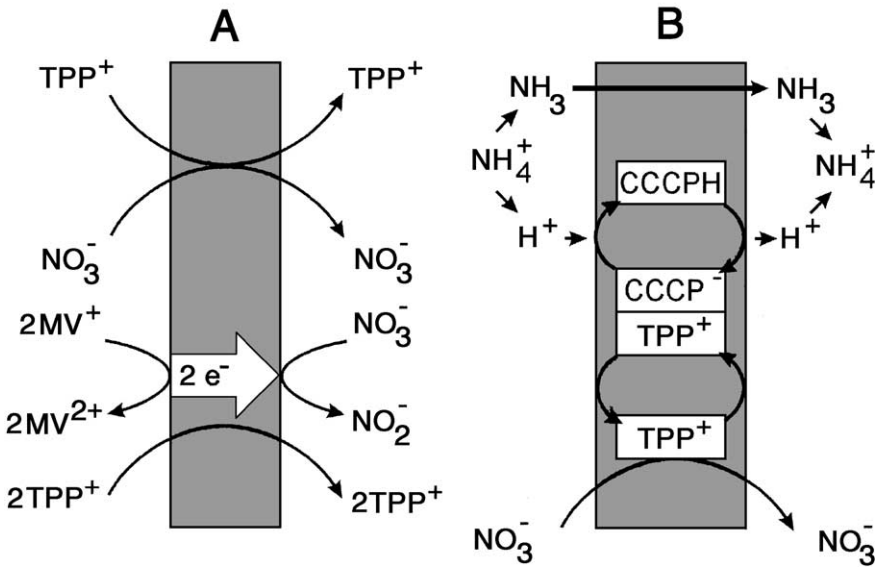

Fig. 5. Models of the TPP^+-mediated nitrate permeation across the cytoplasmic membrane of *P. denitrificans*. Diagram A indicates how the observed stoichiometry $\sim 3TPP^+/NO_3^-$ can be accounted for by coupling the entry of TPP^+ to the translocation of one nitrate anion and two electrons needed for the nitrate reductase reaction. Diagram B represents a possible mechanism whereby the mixture of TPP^+ and CCCP accelerates net influx of ammonium nitrate through formation of more diffusible electroneutral cation:anion pairs.

to compensate the electric field generated by the transmembrane MV^+-nitrate reductase reaction ($2e^-/NO_3^-$). A model pertinent to spheroplasts swelling (Fig. 5B) assumes formation of an ion-pair not only between TPP^+ and nitrate but also between TPP^+ and the ionised form of uncoupler. The evidence for the first interaction comes from the stimulatory effect of TPP^+ on swelling in ammonium nitrate medium containing sufficient CCCP to make the membrane permeable to H^+ (Fig. 4). The second above-mentioned possible interaction was advocated some time ago to explain the stimulating effect of TPP^+ on the rate of protonophore-mediated decay of pH gradient across lipid vesicular membranes [11]. Collectively, diffusion of uncharged single species (CCCP-H, NH_3) and ion pairs ($TPP^+.NO_3^-$, $TPP^+.CCCP^-$) would provide a dual cycle capable of explaining a highly efficient net influx of ammonium nitrate brought about by the mixture of CCCP and TPP^+.

Carrier properties of TPP^+ characterised here offer new possibilities of studying various aspects of nitrate transport in bacteria. For example, the use of TPP^+ should be instrumental to ascertain whether oxygen does indeed inhibit denitrification at the level of nitrate transporter, as was suggested by Alefounder and Ferguson [12]. Another concern relates to the interpretation of existing applications of phosphonium ions. In a pivotal work on nitrate transport in *P. denitrificans*, dissipation of transmembrane potential by high concentrations (5–35 mM) of triphenylmethylphosphonium ($TPMP^+$) bromide served as one of the means of discriminating between the putative energy-dependent and energy-independent transport systems for nitrate uptake of into the cell [13]. Nitrate transport observed in cells depolarised by $TPMP^+$ was originally ascribed to a nitrate–nitrite electroneutral exchange. It is now apparent, however, that nitrate might at least in part move via an unphysiological $TPMP^+.NO_3^-$ ion pairing mechanism. Formation of lipophilic ion pairs may also adversely influence measurements of membrane potential based on phosphonium probes distribution because of an increased binding of the probe to the membrane. An appreciation of the importance of this effect would require careful examination of probe binding as a function of ionic composition of the solution.

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