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DISSIMILATORY NITRATE UPTAKE IN *PARACOCCUS DENITRIFICANS* VIA A $\Delta\tilde{\mu}_H$-DEPENDENT SYSTEM AND A NITRATE-NITRITE ANTIPORT SYSTEM

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Respiration-driven proton translocation has been studied with the oxidant pulse method for cells of denitrifying *Paracoccus denitrificans* oxidizing H_2 during reduction of O_2, NO_3^-, NO_2^- or N_2O. A simplified scheme of anaerobic electron transport and associated proton translocation is shown that is consistent with the measured $\vec{H}^+/$oxidant ratios. Furthermore, the kinetics and energetics of NO_3^- uptake in whole cells of *P. denitrificans* were studied. For this purpose, we measured H_2 consumption or N_2O production after addition of NO_3^- to a cell suspension, which indirectly gave information about uptake (and reduction) of NO_3^-. It was found that a lag phase in H_2 consumption or N_2O production appeared whenever the membrane potential was dissipated by addition of thiocyanate, carbonyl cyanide *m*-chlorophenylhydrazone or triphenylmethylphosphonium bromide. However, these lag phases were not observed when NO_2^- was present at the moment of introduction of NO_3^-. On the basis of these findings we conclude that there are two uptake systems for NO_3^-. One system is dependent on the proton-motive force and is probably used for initiation of NO_3^- uptake. The other is an NO_3^-/NO_2^- antiport and its function is to take over NO_3^- uptake from the first system.

Introduction

Paracoccus denitrificans is able to denitrify, i.e., to reduce NO_3^- via NO_2^- and N_2O to N_2 in a dissimilative way [1–3]. The respiratory nitrate reductase, which is a molybdenum-containing iron-sulphur protein, reduces NO_3^- to NO_2^- [4]. The localization of the active centre of nitrate reductase is believed to be at the cytoplasmic side of the inner membrane [5]. This belief is based on two facts. In the first place there exists a permeability barrier to chlorate, but not to nitrate, in whole cells and spheroplasts, which can be broken down by addition of the detergent Triton X-100 or sonification of the spheroplasts [6–8]. Second, inside-out vesicles are able to reduce oxygen and nitrate or oxygen and chlorate simultaneously [6].

The subsequent reduction of NO_2^- to N_2O is accomplished by a two-haem (*c*- and *d*-type) nitrite reductase [9,10]. It has been shown that the active centre of nitrite reductase is located at the periplasmic side of the cytoplasmic membrane. First, the protons consumed during reduction of NO_2^- to N_2O by ascorbate/TMPD are taken from the periplasmic space [11,12]. Second, it is possible to prepare intact spheroplasts which have lost their nitrite reductase activity [8].

Reduction of nitrate and nitrite at, respectively, the cytoplasmic and periplasmic side of the cyto-

Abbreviations: $TPMP^+$, triphenylmethylphosphonium cation; $\vec{H}^+/e^-$ ($\vec{q}^+/e^-$), number of protons (charges) translocated across the cytoplasmic membrane during the flow of one electron to an electron acceptor; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

plasm membrane raises the question as to how nitrate is imported into, and nitrite exported from, the cell. Because virtually nothing is known about this process [5], we started a study on the kinetics and energetics of NO_3^- uptake in *P. denitrificans*. The driving force for anion uptake in general and NO_3^- uptake in particular will depend on the mechanism of transport [13]. The ability of the bacterium to discriminate between nitrate and chlorate suggests the presence of a specific carrier system in the cytoplasm membrane. So the uptake is probably not passive, but facilitated. According to Mitchell [14] there are three different systems for facilitated transport: uniport, symport and antiport.

A uniport system for nitrate uptake is unlikely, because in that case the maximum internal nitrate concentration would be restricted to about 0.3% of the external nitrate concentration, due to a steady-state membrane potential of about 150 mV [15], inside negative. Consequently, the cell has to deal with the problem of uptake of NO_3^- against the adverse membrane potential. In the theory, there are several ways to cope with this problem. One way to evade the adverse $\Delta\psi$ is to take up NO_3^- via an H^+-NO_3^- symport system. The membrane electrical potential has no influence on the uptake of NO_3^- in this way, since it is an electroneutral process.

Another system for the entry of NO_3^- is a symport of NO_3^- with more than one H^+. The operation of such a system will not only be promoted by the chemical gradient of NO_3^- and H^+, but also by the membrane electrical potential. A third possibility is the existence of an electroneutral NO_3^-/NO_2^- antiport system.

Studies on the transport systems for NO_3^- uptake were performed with cells grown on succinate and nitrate or H_2, CO_2 and nitrate. We show that there are two transport systems for the entry of NO_3^-. One is believed to function as an electrogenic H^+-NO_3^- symport and the other as an NO_3^-/NO_2^- antiport. Cells grown with H_2, CO_2 and NO_3^- were also used to determine the efficiency of energy conservation with H_2 as substrate and O_2 or nitrogenous oxides as electron acceptors. H^+/oxidant ratios with H_2 as substrate are presented. We conclude on the basis of these stoicheiometries that the efficiency of energy conservation during denitrification is 50% of the efficiency during O_2 respiration with H_2 as substrate. Preliminary accounts of parts of this work have been presented [16,17].

Materials and Methods

Microorganism and growth conditions. P. denitrificans NCIB 8944 was the experimental organism. Anaerobic NO_3^--limited continuous cultures were performed essentially as described by Boogerd et al. [12]. The medium contained 40 mM KNO_3, 50 mM sodium succinate, 0.1% yeast extract (Oxoid, code L21) and 1 ml trace element solution/l. The trace element solution contained: 100 mM $CaCl_2$, 90 mM $FeCl_3$, 50 mM $MnCl_2$, 25 mM $ZnCl_2$, 10 mM $CoCl_2$, 5 mM $CuCl_2$, 5 mM H_3BO_3, 10 mM Na_2MoO_4 and 5 ml concentrated H_2SO_4/l, 3 M HCl. The culture volume was 330 ml, the specific growth rate (μ) was 0.15 h^{-1} and the growth temperature was 35°C. Cells were harvested, subsequently washed twice with 50 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.0) and then resuspended in this buffer at approx. 30 mg dry wt. cells/ml. The suspension was immediately used.

The medium used for growth in continuous culture on H_2, CO_2 and NO_3^- contained 40 mM KNO_3, 0.05 or 0.1% yeast extract, 0.01% titriplex I and 1 ml trace element solution/l. 100% H_2 and 5% CO_2/95% N_2 were supplied separately to the culture, both at a flow rate of 5 l/h. Growth temperature was 30°C, pH was maintained at a value of 7.5 by addition of 1 M KOH and the culture was stirred at 600 rpm. Harvesting and washing procedures were the same as for succinate-grown cells.

Measurement of H_2 consumption. H_2 was determined polarographically using a Clark electrode (Yellow Springs Instrument Co.) The Ag electrode was electrolytically covered with AgCl as described by Janz [18] to improve performance. The electrode was polarized at −0.4 V [19,20]. The initial rate of H_2 uptake was determined from the tangent of the H_2 trace in time. The extent of H_2 uptake was taken to be the sum of the amount of H_2 consumed according to the H_2 trace and the amount of H_2 diffused into the suspension during the reaction. The mean H_2 diffusion was measured from the H_2 trace after each reaction had finished.

The signal of the electrode was calibrated with 100% H_2 and recorded on a Kipp recorder. Depending on the sensitivity of the H_2 electrode this signal varied from 0.2 to 1.0 mV for 100% H_2-saturated buffer at 25°C (H_2 concentration 0.789 mM). A continuous stream of H_2 was directed at the liquid surface. Temperature was controlled with a thermocouple and a sensor at 25°C. The suspension was continuously stirred and the working volume was 2.0 ml. The reaction was started by the injection of electron acceptor. 100 mM standard solutions of KNO_3 or KNO_2 in water and 100% N_2O-saturated water of 25°C (N_2O concentration 24.5 mM) were used.

Measurement of N_2O production. A normal Clark oxygen electrode responded to high dissolved concentrations of N_2O, but the signal was never stable. The H_2 electrode, treated as described above, responded very well to N_2O, even to low dissolved N_2O concentrations. A stable signal was obtained in a 5% N_2O-saturated buffer, provided that the polarizing voltage was set at a value of +1.15 V and 1 M KOH plus 100 mM KCl was used as the electrolyte to avoid electrolyte breakdown [21]. The signal of the electrode was calibrated with 5% N_2O/95% N_2 at 25°C (N_2O concentration 1.23 mM) and recorded on a Kipp recorder. 5% N_2O yielded a recorder response of about 1.0–1.5 mV. The volume of the reaction chamber was 2.0 ml. Temperature was maintained at 25°C with a circulating water jacket of 25°C. Because of a small gas/liquid interface diffusion of gas into or out of the suspension was negligible.

Measurement of respiration-driven proton translocation. Cells from H_2, CO_2 and NO_3^- chemostat cultures were harvested, subsequently washed twice with 1.5 mM glycylglycine (pH 7.5), 150 mM KCl and resuspended in this buffer (all steps were performed at 0–4°C). The reaction cell, electrode system and actual measurement procedure were essentially as described before [12].

Chemicals. CCCP, valinomycin, rotenone and antimycin A were added as ethanolic solutions. Potassium thiocyanate and TPMP (bromide salt) were added as aqueous solutions. Acetylene was added as a 100% acetylene-saturated buffer solution of 25°C (acetylene concentration 41.5 mM).

Results

Growth with H_2 as substrate

Growth of *P. denitrificans* under denitrifying, strictly autotrophic conditions appeared to be impossible. Addition of yeast extract to the growth medium was essential for growth with H_2, CO_2 and NO_3^-, as was concluded earlier by several authors [22–24]. In Table I a comparison between growth under mixotrophic and heterotrophic condition is given for illustration. It can be seen that a higher cell density is reached with $H_2 + CO_2$ than with N_2 alone. These former cells also possessed hydrogenase activity, consumed much more NO_3^-

TABLE I

GROWTH OF *P. DENITRIFICANS* UNDER HETEROTROPHIC AND MIXOTROPHIC CONDITION

The basal medium plus 0.01% titriplex I was used with the supplements given in the table. 100% N_2, 5% CO_2/95% N_2 and 100% H_2 were sparged through the culture at a flow rate of 5 l/h for each gas (mixture). Dry weight of cells was calculated from the organic carbon content of the culture. NO_3^- was determined by a colorimetric assay. The growth yield ($Y_{NO_3^-}$) can be calculated from the two preceding columns. Hydrogenase activity, expressed as rate of H_2 uptake during of NO_3^- to NO_2^-, was measured as described in Materials and Methods. The dilution rate of the chemostat cultures was set at a value of 0.029 h^{-1}. For other culture conditions see also Materials and Methods.

Medium supplied	Gases supplied	dry wt. cells (g/l)	NO_3^- used (mM)	$Y_{NO_3^-}$ (g/mol)	$H_2 \rightarrow NO_3^- \nearrow NO_2^-$ activity (nmol H_2/min per mg dry wt.)
40 mM KNO_3 0.1% yeast extract	100% N_2	0.185	9.8	18.9	0
40 mM KNO_3 0.1% yeast extract	5% CO_2 + 95% N_2 100% H_2	0.300	35.7	8.4	101

and showed lower $Y_{NO_3^-}$ values. The hydrogenase activity appeared to be variable, varying from 50 to 250 nmol H_2/min per mg dry wt. However, the density of the culture was proportional to the hydrogenase activity.

Respiration-driven proton translocation with H_2 as substrate

When cells of *P. denitrificans* are supplied with NO_3^-, NO_2^- or N_2O under an H_2 atmosphere an immediate consumption of H_2 takes place (Fig. 1). Addition of NO_3^- results in a biphasic uptake of H_2, while this is not the case with NO_2^- or N_2O. Since the rate of H_2 consumption with NO_2^- is much lower than with NO_3^- or N_2O, as indicated by the numbers along the traces, the second phase in H_2 uptake with NO_3^- is due to reduction of NO_2^- accumulated during the first phase. An increase in H_2 concentration in the suspension is always seen after reduction of the nitrogenous oxides has been completed, due to diffusion of H_2 from the gas phase into the suspension. Calculation of the amount of H_2 consumed during reduction of the different nitrogenous oxides, with a correction for diffusion of H_2 from the gas phase into the suspension, gives H_2/oxidant ratios of 2.40, 1.35 and 0.67 for, respectively, reduction of NO_3^-, NO_2^- and N_2O by H_2. These values correspond reasonably well with the theoretical values of 2.50, 1.50 and 1.00, respectively, which are based on complete reduction of nitrogenous oxides

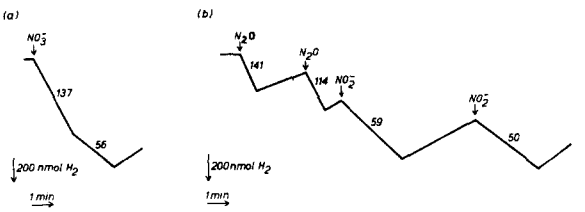

Fig. 1. Reduction of NO_3^-, NO_2^- and N_2O to N_2 by H_2 in *P. denitrificans* cells. Cells from a chemostat culture with H_2, CO_2 and NO_3^- were used. The reaction chamber contained 2.8 mg dry wt. cells in a volume of 2.0 ml. The H_2 trace is shown after the suspension has been saturated with 100% H_2. The numbers along the traces indicate the rate of H_2 uptake (nmol H_2/min per mg dry wt.). The following amounts of nitrogenous oxides were injected: (a) 500 nmol KNO_3; (b) 490 nmol N_2O and 500 nmol KNO_2. Time of addition is denoted by arrows.

by H_2. From the results of Fig. 1 it is clear that in these cells measurements on respiration-driven proton translocation with H_2 as substrate and NO_3^-, NO_2^- or N_2O as electron acceptor can be done. In Table II the observed $\vec{H}^+$/oxidant stoicheiometries are given for reduction of the different acceptor couples by H_2. However, these observed ratios have to be corrected, as argued in our previous article [12], for acceptor scalar proton consumption in the case of reduction of NO_2^- and N_2O, since their reductases are both located at the periplasmic side of the cytoplasmic membrane. No such correction has to be made for NO_3^- or O_2 reduction, because the proton-consuming sites of

TABLE II

RESPIRATION-DRIVEN PROTON TRANSLOCATION WITH H_2 AS SUBSTRATE AND O_2 OR NITROGENOUS OXIDES AS ELECTRON ACCEPTOR

Cells from three different H_2, CO_2 and NO_3^- chemostat cultures were used (4.2 mg dry wt. cells). Proton translocation was measured in 3.0 ml of 1.5 mM glycylglycine (pH 7.5), containing 150 mM KCl, 5 mM $TPMP^+$, 12 μg valinomycin/ml. The suspension was allowed to equilibrate for 1 h at 25°C under a 100% H_2 atmosphere. The $\vec{H}^+$/oxidant ratios are given as averages ± S.D. with the number of pulses in parentheses. Corrections were made as explained in the text.

Acceptor couple	Observed $\vec{H}^+$/oxidant	Corrected $\vec{H}^+$/oxidant	Corrected $\vec{H}^+/e^-$	Predicted $\vec{H}^+/e^-$
NO_3^-/N_2	4.03 ± 0.40 (14)	8.03	1.61	1.60
NO_2^-/N_2	2.87 ± 0.42 (16)	6.87	2.29	2.00
NO_2^-/N_2O	1.68 ± 0.59 (15) [a]	4.68	2.34	2.00
N_2O/N_2	2.06 ± 0.20 (13)	4.06	2.03	2.00
O/H_2O	3.55 ± 0.36 (10)	3.55	1.78	2.00
O/H_2O	3.92 ± 0.41 (10) [a]	3.92	1.96	2.00

[a] Determined in the presence of 100 mM KSCN.

the nitrate reductase and the oxidase are situated at the cytoplasmic side of the inner membrane. There is also no need to correct for scalar proton production associated with membrane-bound H_2 oxidation [25], since it has been shown that these protons are liberated at the cytoplasmic side of the inner membrane [26]. When the corrected $\vec{H}^+/e^-$ values are divided by the number of electrons involved in reduction, this yields the corrected $\vec{H}^+/e^-$ values. On the basis of the previously published scheme of proton translocation and electron transport to NO_2^-, N_2O and O_2 (Ref. 12; see also Fig. 7), extended with the knowledge that reduction equivalents from H_2 enter the respiratory chain at the level of ubiquinone [27,28] (see next section), we can predict the $\vec{H}^+/e^-$ ratios for reduction of these electron acceptors by H_2. Comparison of the corrected $\vec{H}^+/e^-$ values with the predicted $\vec{H}^+/e^-$ ratios demonstrates that the measured $\vec{H}^+/e^-$ values are in full agreement with the predicted ratios. A corrected $\vec{H}^+/e^-$ of 1.61 for reduction of NO_3^- to N_2 by H_2 is expected, since electrons destined for nitrate reductase pass only site IIa and not site IIb (see next section).

Reduction of NO_3^- to NO_2^- by H_2

Washed cell suspension of *P. denitrificans* do not always possess an NO_2^-- or N_2O-dependent H_2 uptake. Moreover, when these activities are present in a washed suspension they are only measurable at the beginning and diminish to zero after some time, at least in potassium phosphate buffer. The activities present at the beginning can be completely blocked by antimycin A (10 μg/ml). So with these cells it is possible to terminate reduction of NO_3^- at the level of NO_2^-, whenever it is required. This reduction of NO_3^- to NO_2^- by H_2 is shown in Fig. 2a. There is an immediate consumption of H_2 after introduction of NO_3^- into a suspension of *P. denitrificans* cells and this H_2 uptake proceeds at a constant rate without the biphasic character of the H_2 trace in Fig. 1. Addition of NO_2^-, however, does not result in uptake of H_2, whereas H_2 consumption again starts immediately after a second addition of NO_3^-. Hence, NO_3^- is not reduced beyond NO_2^-. Calculation of the amount of H_2 consumed during reduction of NO_3^- to NO_2^- yields an H_2/NO_3^- stoicheiometry of 1.0, which means that the following reaction has

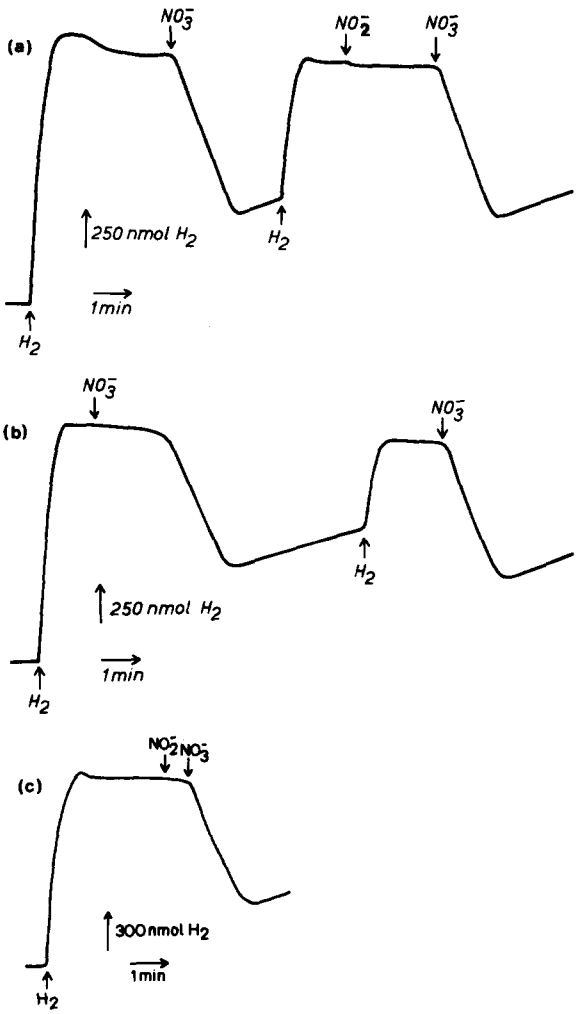

Fig. 2. Effect of SCN^- on reduction of NO_3^- to NO_2^- by H_2 in *P. denitrificans* cells. The reaction chamber contained 2.6 mg dry wt. cells from an H_2, CO_2 and NO_3^- chemostat culture and 20 μg antimycin A in 2.0 ml. Additions were made at the arrows as follows: (a) 1000 nmol KNO_3 or KNO_2; (b) 1000 nmol KNO_3; (c) 2000 nmol KNO_2 and 1000 nmol KNO_3. In b and c also 100 mM KSCN was present. H_2 denotes the moment of saturation of the suspension with 100% H_2.

occurred:

$$H_2 + NO_3^- \rightarrow H_2O + NO_2^- \quad (1)$$

This reaction is not influenced by a concentration of 10 μg antimycin A/ml that completely inhibits the NO_2^-- and N_2O-dependent H_2 uptake. The reaction in Eqn. 1 is insensitive not only to anti-

mycin A but also to rotenone. A concentration as high as 90 μM rotenone does not inhibit the rate of H_2 uptake during reduction of NO_3^- to NO_2^-. These findings indicate that H_2 donates its electrons to the respiratory chain after the rotenone-sensitive and before the antimycin A-sensitive site, presumably at the level of ubiquinone. The same conclusion has been drawn for aerobic, autotrophic growth of *P. denitrificans* with H_2 [27,28].

By following the H_2 consumption in time we can, according to Eqn. 1, indirectly study the kinetics of NO_3^- uptake and reduction. The presence of the permeant anion SCN^-, an inhibitor of nitrate reductase [29], in this system brings about unexpected results. There is no H_2 uptake after introduction of NO_3^- until about 2 min have passed. By that time H_2 is consumed at the same rate as that in the absence of thiocyanate (Fig. 2b). Surprisingly enough, H_2 uptake starts immediately after addition of a second amount of NO_3^- (and also after all following additions; not shown). The duration of the lag phase observed after the first addition of NO_3^- is dependent on the SCN^- concentration, the preincubation time with SCN^- and the condition of the cell preparation. The length of the lag phase is proportional to the concentration of SCN^-, at least in the 20–100 mM range. Thiocyanate concentrations below 20 mM are not always effective in producing a lag phase. With concentrations above 100 mM inhibition of the rate of NO_3^--dependent H_2 uptake is observed, but the lag phase does not disappear. The observed H_2/NO_3^- ratio is always close to the theoretical value of 1.0 when the activity of the route from H_2 to NO_3^- is higher than about 100 nmol H_2/min per mg dry wt. Cell preparations with activities lower than 100 in general show H_2/NO_3^- ratios substantially lower than 1.0. Therefore, in these cases the added NO_3^- is not only reduced by H_2 but also by endogenous substrates. Nevertheless, whatever H_2/NO_3^- ratio or uptake rate of H_2 is measured, under appropriate conditions one can observe a lag phase upon first addition of NO_3^- in the presence of SCN^-.

Concerning the immediate H_2 uptake after addition of a second amount of NO_3^- we noticed that, whenever there was a lag phase in H_2 consumption upon first addition of NO_3^-, it did not show up at the second addition. The only difference in experimental conditions at the first and second supply of NO_3^- is the presence of NO_2^-, as a consequence of the validity of Eqn. 1. Therefore, it could be the presence of NO_2^- at the time of the second addition that prevents the lag phase. This suggestion is confirmed in the experiment shown in Fig. 2c. Addition of NO_2^- prior to NO_3^- results in an instantaneous uptake of H_2 after the first addition of NO_3^- in the presence of the same concentration of SCN^- as in Fig. 2b. All following additions of NO_3^- show also immediate H_2 consumption upon introduction of NO_3^-, as expected (not shown).

Because of the results shown in Fig. 2 we propose that an explanation of these phenomena can be found in the existence of two transport systems for NO_3^- uptake. A $\Delta\psi$-dependent system that is sensitive to the $\Delta\psi$ dissipator SCN^- and an NO_3^-/NO_2^- antiport system that is not sensitive to SCN^-.

When the entry of NO_3^- into the cell via the $\Delta\psi$-dependent system is delayed by the presence of SCN^-, one expects not to find such a delay at first addition of NO_2^-, because the latter anion does not have to enter the cell. This idea proves to be true, as can be seen in Table III.

In the presence of SCN^-, NO_3^- and NO_2^- will be reduced to N_2O on account of the complete

TABLE III

H_2 UPTAKE WITH NO_2^- AND NO_3^- BY *P. DENITRIFICANS* IN THE PRESENCE OF SCN^-

The reaction chamber contained 10 mg dry wt. cells from an H_2, CO_2 and NO_3^- chemostat culture in a volume of 2.0 ml. KSCN concentration was 87 mM. The suspension was incubated for 20 min under 100% H_2. The lag phases indicate the time (in s) between addition of oxidant and observed H_2 uptake. 1–6 are subsequent additions.

Addition (500 nmol)	Lag phase (s)
(1) NO_2^-	0
(2) NO_3^-	30
(3) NO_3^-	42
(4) NO_2^-	0
(5) NO_3^-	36
(6) NO_3^- [a]	30

[a] 1000 nmol.

inhibition of nitrous oxide reductase by SCN^- [12,30,31] (see also section about N_2O production). At first addition of NO_2^- an immediate H_2 consumption is observed, as predicted (Table III, row 1). Moreover, after introduction of NO_3^-, when reduction of NO_2^- by H_2 is finished, H_2 uptake shows a lag phase of half a minute (Table III, row 2). This is also observed at addition of a second amount of NO_3^-, after the preceding reaction has been completed (Table III, row 3). The whole picture can be repeated by subsequent addition of NO_2^- and twice of NO_3^- (Table III, rows 4–6). So addition of NO_2^- prior to NO_3^- is not able, unlike the situation in Fig. 2c, to abolish the lag phase in H_2 uptake, because in this case no NO_2^- is present at the moment of NO_3^- introduction.

When there indeed exists a $\Delta\psi$-dependent system for NO_3^- uptake, it must be possible to create a lag phase in H_2 uptake after addition of NO_3^- by $\Delta\psi$ dissipators other than SCN^-, for example, by

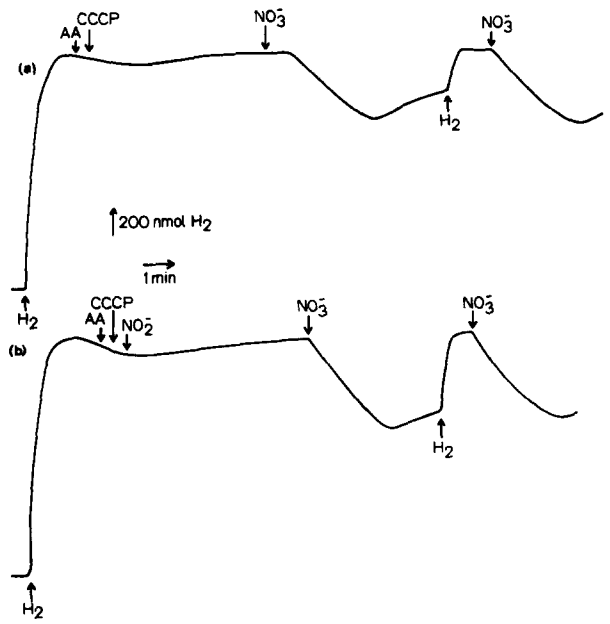

Fig. 3. Effect of CCCP on reduction of NO_3^- to NO_2^- by H_2 in *P. denitrificans* cells. The reaction chamber contained 4.6 mg dry wt. cells from an H_2, CO_2 and NO_3^- chemostat culture in 2.0 ml of 20 mM potassium phosphate buffer (pH 7.0). The concentration of antimycin A (AA) was 10 μg/ml and the concentration of CCCP was 3 μM. At the arrows 1000 nmol KNO_3 or KNO_2 was added. H_2 denotes the moment of saturation with 100% H_2.

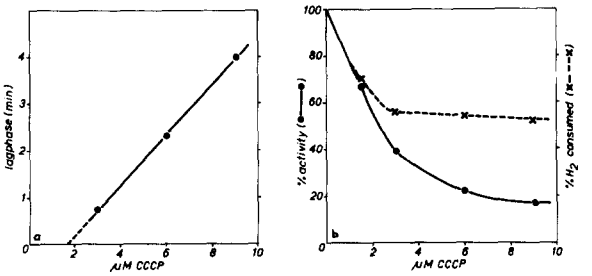

Fig. 4. Effect of increasing CCCP concentration on duration of the lag phase, the amount of H_2 consumed and the rate of H_2 uptake. Conditions were the same as in Fig. 3. Reaction was started with 1000 nmol KNO_3. (a) Relation between length of the lag phase and the CCCP concentration. (b) 100% H_2 consumed is equal to 1000 nmol H_2; 100% activity is equal to 152 nmol H_2/min per mg dry wt.

CCCP. This kind of experiment is shown in Fig. 3a. In the presence of antimycin A (to inhibit nitrite reductase activity) and CCCP injection of NO_3^- into the H_2-saturated cell suspension does not cause any H_2 uptake until about 1 min has passed. The second addition of NO_3^-, however, results in an immediate consumption of H_2. Addition of NO_2^- prior to NO_3^- does not bring about any H_2 uptake (Fig. 3b). But, in this case an immediate H_2 consumption is observed upon addition of not only the second but also the first amount of NO_3^-. The H_2/NO_3^- ratios, the rates of H_2 uptake and the lengths of the lag phases were calculated from experiments similar to that described in Fig. 3 and plotted against the concentration of CCCP (Fig. 4). From Fig. 4a, it is clear the the duration of the lag phase in H_2 uptake upon first introduction of NO_3^- increases with increasing CCCP concentration. Nevertheless, no lag phase is observed, at all concentrations of CCCP used, upon second addition of NO_3^-, at which time NO_2^- is present. So with CCCP an analogous picture in relation to NO_3^--dependent H_2 uptake is obtained to that with SCN^-. From Fig. 4b it can be seen that the H_2/NO_3^- ratio is always substantially lower than the theoretical value of 1.0 in the presence of CCCP. This seems to be related to the rate of H_2 uptake with NO_3^-, since it is also strongly inhibited by increasing concentrations of CCCP (Fig. 4b). Hence, an explanation for H_2/NO_3^- ratios lower than 1.0 may be that endogenous substrates contribute relatively

more to the reduction of NO_3^- at increasing CCCP concentrations.

Finally, we looked at the effect of yet another $\Delta\psi$ dissipator, the lipophilic cation $TPMP^+$ which has been succesfully used in earlier experiments as a substitute for SCN^- in determining respiration-driven proton translocation in denitrifying *P. denitrificians* [12]. Again it is possible to obtain a lag phase in H_2 uptake upon first addition of NO_3^- when cells are preincubated with antimycin A and $TPMP^+$. In contrast with SCN^- and CCCP the lengths of the lag phases obtained with $TPMP^+$ are not dependent on its concentration. Generally, a lag phase of about 20 s is observed in the range 5–35 mM $TPMP^+$. This lag phase again can be prevented by the presence of NO_2^- at the time addition of NO_3^-. Another difference is that $TPMP^+$, in contrast to SCN^- and CCCP, stimulates the NO_3^--dependent H_2 uptake. In the range 5–35 mM $TPMP^+$ the rate of H_2 uptake gradually increases to a value about 2-fold higher than that in absence of $TPMP^+$.

Reduction of NO_3^- and NO_2^- to N_2O by endogenous substrates

In order to be sure that the observed lag phases in H_2 uptake really are a consequence of retardation in the entry of NO_3^- and not of some peculiar property of the hydrogenase, we have tried to show the occurrence of lag phases in NO_3^- reduction by other means. For that purpose cell preparations from NO_3^--limited continuous culture with succinate as carbon and energy source are used. With these cells formation of N_2O from reduction of NO_3^- or NO_2^- by endogenous substrates is followed in time. Acetylene, a potent inhibitor of nitrous oxide reductase, [21,30,32,33], is used to block reduction of NO_3^- and NO_2^- at the level of N_2O. In Fig. 5 it can be seen that addition of NO_3^- in the presence of acetylene brings about an immediate production of N_2O without any lag phase. A second addition of NO_3^- yields the same picture. This instantaneous production of N_2O is expected, because NO_3^- uptake can be started via the $\Delta\psi$-dependent system. After abolition of $\Delta\psi$ by SCN^-, introduction of NO_3^- results only in N_2O formation after a lag phase of about 1 min. This lag phase is found not only at the first but also at the second, third and fourth addition of NO_3^-. Again as expected, since there is no NO_2^- accumulation. Injection of NO_2^-, however, leads to immediate production of N_2O. Moreover, the rate of N_2O production from NO_2^- (30 nmol N_2O/min per mg dry wt.) is about 2.5-times as fast as that from NO_3^- (12 nmol N_2O/min per mg dry wt.), hence the lag phase observed upon addition of NO_3^- is not due to a relatively slow formation of N_2O from NO_2^-. Calculation of the N_2O/NO_2^- and N_2O/NO_3^- ratios from Fig. 5 gives values between 0.46 and 0.50, which are close to the theoretical value of 0.50 for complete reduction of NO_3^- and NO_2^- to N_2O. So the occurrence of a lag phase in N_2O

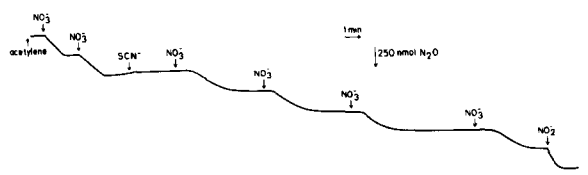

Fig. 5. Effect of SCN^- on reduction of NO_3^- or NO_2^- to N_2O by endogenous substrates in *P. denitrificans* cells. Cells from a succinate and NO_3^- chemostat culture were used. The reaction chamber contained 9.6 mg dry wt. cells in 2.0 ml. Acetylene concentration was 4.15 mM. KSCN was added in a concentration of 57 mM. 500 nmol KNO_3 or KNO_2 were added at the arrows.

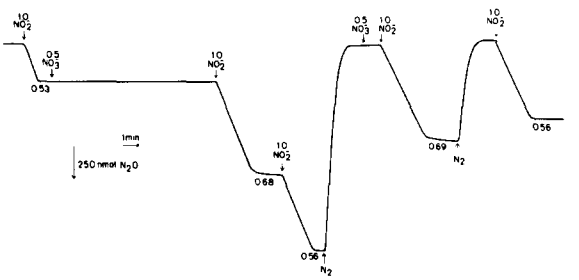

Fig. 6. Effect of NO_2^- on N_2O production from NO_3^- by *P. denitrificans* cells in the presence of SCN^-. The reaction chamber contained 9.4 mg dry wt. cells from a succinate and NO_3^- chemostat culture. KSCN concentration was 114 mM. At the moment indicated by arrows the following amount of KNO_3 or KNO_2 were added, respectively: 500 nmol KNO_2, 500 nmol KNO_3, 1000 nmol KNO_2, 1000 nmol KNO_2, 500 nmol KNO_3, 1000 nmol KNO_2 and 1000 nmol KNO_2. The numbers above the N_2O trace are relative numbers. The numbers under the N_2O trace denote the calculated N_2O/NO_2^- values, which are in the case of complete conversion to N_2O, half the relative number above the trace.

production from reduction of NO_3^- by endogenous substrates rules out the possibility of a hydrogenase-specific effect.

Because of the immediate reduction of NO_2^- to N_2O it is not possible to determine whether NO_2^- is able to abolish the lag phase in N_2O production from NO_3^- in the presence of SCN^- by addition of NO_2^- prior to the moment of NO_3^- introduction. However, with a somewhat different approach the stimulation of NO_3^- uptake by NO_2^- can still be demonstrated, as shown in Fig. 6. In this experiment a higher concentration of SCN^- is used than that in Fig. 5 and acetylene is omitted, since SCN^- is present from the beginning. At this SCN^- concentration no N_2O production is observed for as long as 10 min after the first addition of NO_3^-. However, when NO_2^- is introduced after this period instantaneous formation of N_2O can be seen, as was the case upon first addition of NO_2^-. However, a striking difference between the events induced by first and second addition of NO_2^- is that at second addition of NO_2^- the amount of N_2O produced after the reaction has stopped significantly exceeds the quantity expected on the basis of merely reduction of NO_2^-. This is illustrated by the calculated N_2O/NO_2^- ratios at first and second addition of NO_2^-, which have values of 0.53 and 0.69, respectively. At third addition of NO_2^- also more N_2O is produced than expected. Thus, the explanation must be that reduction takes place not only of NO_2^- but also of NO_3^- after the second and third addition of NO_2^-, which means that NO_2^- can overcome the uptake barrier to NO_3^- imposed by dissipation of $\Delta\psi$ by SCN^-. Summation of the N_2O/NO_2^- ratios yields a value of 1.24 that does not fit with the amount of NO_2^- added (2.00) but is in agreement with the introduced amount of NO_2^- and NO_3^- together (2.50). Moreover, the whole picture can be reproduced a second time. The added value of the N_2O/NO_2^- ratios then amounts to 1.25, which is exactly equal to half the amount of NO_2^- and NO_3^- injected (2.50).

Discussion

In a previous article [12], we have proposed a scheme for anaerobic electron transport and associated proton translocation in *P. denitrificans*. Based on this scheme, four protons are translocated per transfer of two electrons through the traditional site II region. Hence, together with the knowledge that H_2 donates its electrons at the level of ubiquinone and its protons to the cytoplasm, we expected to find the same stoicheiometry of 4 in measurements of respiration-driven proton translocation with H_2 as substrate and NO_2^-, N_2O or O_2 as electron acceptor. This is indeed what we have found (Table II).

The insensitivity of reduction of NO_3^- to NO_2^- by H_2 to antimycin A indicates that electrons destined for nitrate reductase branch off before the cytochrome b-c_1 complex. Taking this into account, the measured $\vec{H}^+/NO_3^-$, for reduction of NO_3^- to N_2 by H_2, is also in agreement with above-mentioned scheme. Combination of these old and new data gives a picture of the anaerobic

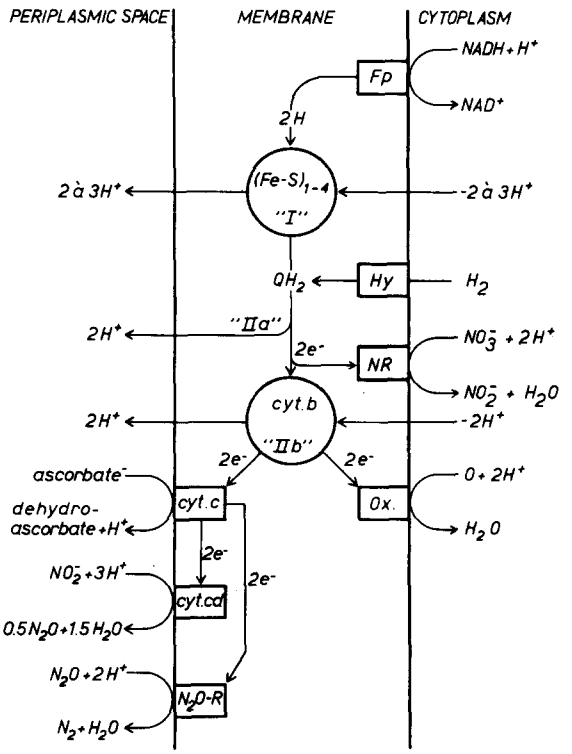

Fig. 7. Anaerobic respiratory chain of *P. denitrificans* and its proton-translocating properties. Fp, flavoprotein; Fe-S, iron-sulphur centre; QH_2, ubiquinol; Hy, hydrogenase; NR, nitrate reductase, cyt., cytochrome; N_2O-R, nitrous oxide reductase; Ox, alternative oxidase. I and IIa + IIb are the traditional sites of energy conservation.

electron-transport chain and its proton-translocating properties as shown in Fig. 7.

Kristjansson et al. [34] were able to measure an $\vec{H}^+/2e^-$ ratio for reduction of NO_3^- to N_2 by endogenous substrates and found a value of 4.3. Correction of this ratio for scalar proton consumption yields a stoicheiometry of 5.9, which is in between theoretical values of 5.2 and 6.2 calculated on the basis of Fig. 7. Moreover, the same laboratory has determined $\vec{H}^+/2e^-$ ratios for reduction of NO to N_2O or N_2 of 3.6 and 3.7, respectively, which are believed to be underestimated by about 10% [35]. Hence, after this correction and correction for scaler proton consumption values of 6.0 and 6.1 are found, which are also consistent with the theoretical value of 6.0–7.0 for both reductions. Furthermore, the $\vec{H}^+$/oxidant stoicheiometries for reduction of N_2O to N_2 and NO_2^- to either N_2O or N_2 by endogenous substrates are in reasonable agreement with Fig. 7 [31,34]. $\vec{H}^+/O$ ratios of 8, reported by the latter authors, for cells grown under denitrifying condition are substantially higher than is expected on the basis of Fig. 7, i.e., 6.0–7.0. This finding is possibly related to the presence of *a*-type cytochromes in their cell preparations [31]. In agreement with this suggestion, $\vec{H}^+/O$ measurements with a cytochrome *c*-deficient mutant of *P. denitrificans*, which uses the alternative oxidase, yield ratios of 6.0–7.5 [36].

Porte and Vignais [28] have proposed a scheme for the respiratory chain of *P. denitrificans* grown on H_2, CO_2 and O_2, in which they postulate an energy-transducing site between H_2 and ubiquinone to account for a $\vec{H}^+/O$ of 6–7 for reduction of O_2 via cytochrome *o* by H_2. However, we have found an $\vec{H}^+/O$ of about 4. Hence, there is no need to assume the presence of such an extra site of energy conservation in cells grown with H_2, CO_2 and NO_3^-.

Taking into account the different orientations of the oxidative or reductive half-reactions with respect to the cytoplasmic membrane, the number of charges translocated across the membrane per electron can be calculated on the basis of the number of protons translocated. In Table IV these calculations have been performed for NADH or H_2 as substrate and O_2, NO_3^-, NO_2^- or N_2O as electron acceptor. It is clear that the efficiency of energy conservation, which is directly related to the $\vec{q}^+/e^+$ value, is equal for the different acceptor couples of nitrogenous oxides when compared for the same reductant. So the five electrons needed for reduction of NO_3^- to $0.5N_2$ are energetically equivalent for a specified substrate. The efficiency of oxidative phosphorylation during denitrification is 67–71% or 50% of the efficiency of O_2 respiration with, respectively, NADH or H_2 as substrate.

In the present work we have studied the kinetics of NO_3^- reduction in whole cells of *P. denitrificans* by measuring H_2 consumption of N_2O production with electrodes. An important finding was the occurrence of lag phases in H_2 uptake or N_2O production after addition of NO_3^- under appropriate conditions. These lag phases are most likely related to the entry of NO_3^- into the cell. More specifically, several experimental data indi-

TABLE IV

$\vec{H}^+/e^-$ AND $\vec{q}^+/e^-$ RATIOS CALCULATED FOR REDUCTION OF DIFFERENT ELECTRON ACCEPTOR COUPLES BY NADH OR H_2.

The $\vec{H}^+/e^-_{corr.}$ values are calculated from the scheme of anaerobic electron transport in Fig. 7.

Acceptor couple	Reductant			
	NADH		H_2	
	$\vec{H}^+/e^-_{corr.}$	$\vec{q}^+/e^-$	$\vec{H}^+/e^-_{corr.}$	$\vec{q}^+/e^-$
NO_3^-/NO_2^-	2.0–2.5	2.0–2.5	1.0	1.0
NO_2^-/N_2O	3.0–3.5	2.0–2.5	2.0	1.0
N_2O/N_2	3.0–3.5	2.0–2.5	2.0	1.0
O/H_2O	3.0–3.5	3.0–3.5	2.0	2.0

cate the involvement of the membrane potential in transport of NO_3^- across the cytoplasmic membrane. (1) In the presence of SCN^-, CCCP or $TPMP^+$, which all share the property of decreasing $\Delta\psi$, a lag phase in H_2 uptake upon first addition of NO_3^- can be seen when NO_3^- is reduced to NO_2^-. (2) A lag phase in H_2 uptake is not only observed upon first addition but also upon all subsequent additions of NO_3^- when it is reduced to N_2O in the presence of SCN^-. (3) Measurements of N_2O production from NO_3^- in the presence of SCN^- revealed also lag phases in N_2O production upon first and subsequent additions of NO_3^- being reduced by endogenous substrates from either H_2-grown (not shown) or succinate-grown cells. (4) In the absence of $\Delta\psi$ dissipators a lag phase is never found in H_2 uptake or N_2O production after addition of NO_3^-, that is reduced by, respectively, H_2 in H_2-grown cells or endogenous substrates in succinate-grown cells. Because cells incubated without any electron acceptor can retain an appreciable $\Delta\psi$ [37], the absence of lag phases in these experiments also proves a role for $\Delta\psi$ in NO_3^- uptake. (5) Whether or not $\Delta\psi$ is decreased by SCN^-, a lag phase neither in H_2 uptake nor in N_2O production is observed upon first and subsequent addition of NO_2^-, that is reduced by, respectively, H_2 in H_2-grown or endogenous substrates in succinate-grown cells. Since NO_2^- is reduced at the periplasmic side of the cytoplasmic membrane and therefore does not enter the cell, one indeed does not expect to find an involvement of $\Delta\psi$ in NO_2^- reduction.

So the relation between the occurrence of a lag phase and absence or presence of $\Delta\psi$ seems to be well established. However, the lag phases did not last indefinitely, but H_2 uptake and N_2O production start after a certain period upon addition of NO_3^-. This means that by that time NO_3^- has found its way into the cell, probably by passive diffusion down the NO_3^- gradient. Nevertheless, this cannot be the whole explanation, because the rate of NO_3^- reduction, measured as rate of H_2 uptake or N_2O production, is too fast to be merely accounted for by passive diffusion into the cell. Considerable evidence has been gathered for the functioning of an NO_3^-/NO_2^- antiport system, that is thought to take over uptake of NO_3^- as soon as a sufficient concentration of NO_2^- has built up intracellularly by reduction of the low amount of NO_3^- that enters the cell passively. Experimental data that support this view can be summarised as follows. (1) Addition of NO_2^- prior to NO_3^- in experiments where $\Delta\psi$ is abolished by SCN^-, CCCP or $TPMP^+$ results in an immediate uptake of H_2 upon first addition of NO_3^-, being reduced to NO_2^-, where in parallel experiments without prior addition of NO_2^- a lag phase is observed. (2) In all cases when NO_3^- was reduced to NO_2^- and there was a lag phase in H_2 uptake upon first addition of NO_3^-, no lag phase was observed after second and all subsequent additions of NO_3^-. (3) It is possible to initiate N_2O production from NO_3^- by addition of NO_2^- while $\Delta\psi$ is dissipated by SCN^-. (4) In the case of reduction of NO_3^- to N_2O, without accumulation of NO_2^-, lag phases in H_2 uptake and N_2O production appear in the presence of SCN^- after first and subsequent additions of NO_3^-.

In conclusion, we propose that two systems exist for the uptake of NO_3^- into the cell. One system is dependent on $\Delta\psi$ and its function is probably to initiate NO_3^- uptake. We speculate that NO_3^- uptake will occur in symport with more than one proton. This implicates also an involvement of Δ pH in uptake of NO_3^-. So uptake will be dependent on $\Delta\tilde{\mu}_{H^+}$. The other system functions as an NO_3^-/NO_2^- antiporter and its function is to take over NO_3^- uptake from the first system as soon as possible. Since even a very low concentration of exogenously added NO_2^- (50 μM), which has first to move inside the cell down its concentration gradient in its protonated form HNO_2, is able to prevent the lag phase in H_2 uptake upon first addition of NO_3^- (not shown), we suggest that NO_3^- uptake in vivo will proceed mainly via the antiporter. The use of this system has the following advantages.

(1) Intracellular NO_2^- accumulation is prevented, since the entry of every NO_3^- is coupled to the exit of one NO_2^-.

(2) Electroneutral antiport resolves the problem of transport of NO_3^- across the cytoplasmic membrane against the negative membrane potential inside the cell.

(3) Uptake via the antiporter is energetically more favourable than via the $\Delta\mu_{H^+}$-dependent system.

Kristjansson et al. [34] have measured to pH change induced by introduction of NO_3^- in a suspension of polarized *P. denitrificans* and *Pseudomonas denitrificians* cells and concluded from the observed H^+/NO_3^- ratio of -1.0 that NO_3^- is taken up in symport with one proton. We were able to reproduce an H^+/NO_3^- value of -1.0 (unpublished results), but we interpret this finding as follows. In our opinion this ratio of -1.0 is merely a reflection of the proton consumed in the overall reaction when NO_3^- is reduced to N_2 by endogenous substrates ($2.5SH_2 + NO_3^- + H^+ \rightarrow 2.5S + 0.5N_2 + 3H_2O$). An analogous reasoning gives also an explanation of the observed H^+/NO_2^- ratio of -1.0, the H^+/O and H^+/N_2O of 0.0 in this polarized system. Moreover, in *Escherichia coli* the same author has found an H^+/NO_3^- ratio of only -0.07 in polarized cells. This value is close to zero, which is expected when NO_3^- is reduced to NO_2^- in these cells [7].

The effect of SCN^- on nitrate reduction is more complex than was thought previously. Now it appears that SCN^- only inhibits nitrate reductase activity in whole cells at high concentrations. The concentration of SCN^- that actually causes inhibition depends on the culture condition of the cells. Cells grown on succinate and NO_3^- are more susceptible to SCN^- than cells grown with H_2, CO_2 and NO_3^-. With the latter cells the inhibiting effect of SCN^- on the nitrate reductase enzyme could be observed separately from its effect on NO_3^- uptake by virtue of its $\Delta\psi$-dissipating property. Nevertheless, several purified respiratory nitrate reductases were much more sensitive to SCN^- as indicated by K_i values of less than 1.5 mM [29,38,39]. An explanation might be that the SCN^- concentration is much lower in the inner than in the outer bulk phase, because of its anionic character. In agreement with this fact it has been found that N_2O production from NO_3^- in the presence of moderate concentrations of SCN^- is completely inhibited by addition of 0.01% Triton X-100, which makes the membrane permeable to most ions (unpublished results).

Something similar has been found with CCCP. In the presence of CCCP concentrations that produced a lag phase in NO_3^- uptake, also the rate of H_2 uptake upon addition of NO_3^- was inhibited. Inhibition of denitrification by uncouplers has been described earlier by Walter et al. [40] for *Ps. denitrificans* and *Ps. aeruginosa*.

At the moment experiments are in progress to give a further characterization of the mechanism of NO_3^- uptake and NO_2^- excretion in *P. denitrificans*.

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