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Energy coupling to nitrate uptake into the denitrifying cells of *Paracoccus denitrificans*

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Abstract

This study deals with the effects of the agents that dissipate the individual components of the proton motive force (short-chain fatty acids, nigericin, and valinomycin) upon the methyl viologen-coupled nitrate reductase activity in intact cells. Substitution of butyrate or acetate for chloride in Tris-buffered assay media resulted in a marked inhibition at pH 7. In a Tris–chloride buffer of neutral pH, the reaction was almost fully inhibitable by nigericin. Alkalinisation increased the IC_{50} value for nigericin and decreased the maximal inhibition attained. Both types of inhibitions could be reversed by the permeabilisation of cells or by the addition of nitrite, and that caused by nigericin disappeared at high extracellular concentrations of potassium. These data indicate that nitrate transport step relies heavily on the pH gradient at neutral pH. Since the affinity of cells for nitrate was strongly diminished by imposing an inside-positive potassium (or lithium) diffusion potential at alkaline external pH, a potential dependent step may be of significance in the transporter cycle under these conditions. Experiments with sodium-depleted media provided no hints for Na^+ as a possible H^+ substitute.

Keywords: Nitrate transport; Proton symport; pH gradient; Ionophore; Bacteria

1. Introduction

Many organisms acquire actively nitrate from the environment and use it as a source of nitrogen (bacteria, cyanobacteria, fungi, and plants) or as a terminal electron acceptor (bacterial nitrate respirers and denitrifiers). Most nitrate transporters that have been sequenced to date are members of the nitrate/nitrite porter (NNP) family (TC#2.A.1.8), belonging to the major facilitator superfamily (MFS) (TC#2.A.1) or, much less frequently, of the proton-dependent oligopeptide transporter (POT) family (TC#2.A.17) (according to the Transporter Classification system, http://tcdb.ucsd.edu/index.php). They are generally assumed to work as secondary active transporters by employing the transmembrane proton electrochemical gradient ($\Delta\tilde{\mu}_H$) to drive nitrate uptake. On the contrary, the active nitrate transport system of the cyanobacterium *Synechococcus* sp. (TC#3.A.1.16.1) is an ATP-binding cassette (ABC) transporter (TC#3.A.1) powered by ATP [1].

The need for nitrate and nitrite transport in denitrification bacteria stems from the fact that the first reaction in the denitrification pathway, the reduction of nitrate to nitrite, takes place in the cytoplasm, while all three subsequent reactions are periplasmic. During early measurements of nitrate uptake by *Paracoccus denitrificans* with an ion-selective electrode, an insensitivity to an uncoupler of oxidative phosphorylation was noticed [2,3]. Later on, however, it was observed that uncouplers, in fact, produced a lag in the onset of nitrate reduction, but only when nitrite was initially absent [4]. The authors proposed the existence of two transport systems, one operating as a nitrate-proton symporter while the second as an energy-independent nitrate/nitrite antiporter. Recently, both these functions were suggested to be associated with one membrane protein in *Paracoccus pantotrophus*, a close relative of *P. denitrificans* [5].

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Some years ago, we introduced a spectrophotometric assay allowing continuous measurement of nitrate transport [6]. The assay takes advantage of the blue-coloured cation radical of methyl viologen as an electron donor for the membrane bound nitrate reductase. Since the nitrate transport step immediately preceding nitrate reduction is rate limiting, the rate of cation radical oxidation reflects the rate at which nitrate passes into the cells. This approach has proven effective in characterising nitrate transport in terms of rate–concentration relationships [6] and a sensitivity toward an uncoupler and an arginine-modifying reagent [7]. Using cells with the nitrate transporter blocked by phenylglyoxal, nitrate penetration through lipidic parts of the membrane was also studied in this way [8]. In the present work, the methyl viologen assay, in conjunction with various ionophores, is applied to gain insight into the role of individual components of the proton motive force (i.e., the transmembrane differences of pH and electric potential ψ) in the nitrate transporter-mediated process.

2. Material and methods

2.1. Strain, growth conditions, and preparation of spheroplasts

P. denitrificans CCM 982 (NCIB 8944) was obtained from the Czech Collection of Microorganisms. Bacterial cells were cultured anaerobically from 2% inocula on 10 mM nitrate and 50 mM succinate in a mineral medium [6] at 30 °C. After 24-h growth, they were centrifuged at $7000\times g$ for 20 min at 4 °C, washed twice with and suspended in a medium to be used in a particular experiment. Conversion of cells into spheroplasts by lysozyme treatment exactly followed the procedure described previously [8].

2.2. Measurement of swelling

An aliquot of spheroplasts (0.9 mg of dry mass) was added to 2.5 ml of the swelling medium consisting either of water or of 0.25 M ammonium acetate, or of 0.25 M ammonium butyrate, buffered with 20 mM Tris–HCl to the pH indicated. Measurements were performed at 30 °C in 3-ml glass cuvettes with 1-cm path length. Swelling was quantified by the determination of the initial rate of the decrease of light absorbance at 500 nm, using a Cary 118C UV-visible spectrophotometer.

2.3. Measurement of intracellular K^+

Cell suspensions or blanks (1 ml) were decomposed by boiling with a mixture of concentrated sulfuric acid (2 ml) and nitric acid (4 ml) and then filled up to the final volume of 25 ml with demineralised water. The K content was determined by the inductively coupled plasma atomic emission spectrometry (ICP-AES) at an emission-line wavelength of 766.490 nm using an ICP emission spectrometer Jobin-Yvon Model 170 Ultrace (Jobin-Yvon, Longjumeau, France). The apparent intracellular concentration was calculated by assuming a cellular volume of 2 μl/mg dry weight [9].

2.4. Methyl viologen oxidation assay

With the aim to indirectly monitor nitrate transport, nitrate reduction within bacterial cells was coupled to the oxidation of the externally present coloured methyl viologen cation radical (MV^+). The assay was performed anaerobically at 30 °C in a reaction mixture (2.5 ml) containing the buffer and other additions mentioned in the figure legends, cells, and 1 mM methyl viologen. MV^+ was generated by injecting a freshly prepared solution of sodium dithionite in 10 mM NaOH to give an absorbance at 600 nm of 1.3, and then the reaction was started by the addition of a sodium nitrate solution. Initial velocity was determined from the slope of the initial part (first 10 s) of the progress curve using a millimolar absorption coefficient of 11.4. When the presence of sodium ions was to be avoided, $Na_2S_2O_4$ was replaced by titanium(III) citrate, prepared from solid $TiCl_3$ and sodium citrate solution under an atmosphere of argon essentially as described [10]. In this case, Tris–nitrate in place of $NaNO_3$ served to start the reaction.

2.5. Nitrite accumulation assay

The rate of nitrate reduction to nitrite by cell suspension was determined in a discontinuous assay. The magnetically stirred reaction mixture contained, in a total volume of 5 ml, 0.1 M Tris–HCl buffer, pH 7.0, 3.9 mg dry weight of cells, 10 μg of antimycin, and either no exogenous substrate or 10 mM sodium succinate. After preincubating the mixture for 10 min at 30 °C under atmosphere of argon, the reaction was initiated by adding 1 mM $NaNO_3$. Samples of 25 μl were removed at 2-min intervals and analysed for the content of nitrite using the standard diazotisation-coupling technique [11]. The rate was calculated by linear regression in Excel as the slope (±S.E.) of the time dependence of nitrite amount.

2.6. Data analysis

Maximal velocity V_{max} and apparent half-saturation (Michaelis) constant $K_{0.5}$ were determined from a nonlinear regression analysis, using the kinetic software EZ-FIT [12].

Inhibition data were analysed in Excel by fitting a logistic equation

$$v = v_{min} + (v_{max} - v_{min}) / \left(1 + 10^{\log[I] + pI_{50}}\right)$$

where v stands for the rate at the inhibitor concentration $[I]$, v_{max} for v at $[I]=0$, and v_{min} for v at $[I]\rightarrow\infty$; I_{50} represents

the concentration of inhibitor [I] that will produce a half-maximal effect on the reaction measured, $pI_{50} = -\log I_{50}$.

The comparison between a simple saturable (hyperbolic) kinetic model and its combination with a linear component employed the corrected Akaike Information Criterion (AIC_c) [13], which was computed as follows

$$AIC_c = N \times \ln(SS/N) + 2K + \frac{2K(K+1)}{N-K-1}$$

where N is the number of experimental observations, K the number of parameters in the model plus one, and SS is the sum of squares of residuals.

Symbols in the figures represent means from at least four replicates ± S.E. Error bars are omitted if they are smaller than the heights of the symbols.

3. Results

Classical studies on mitochondrial swelling [14] have demonstrated that while the inner mitochondrial membrane is normally quite impermeable to acetate, the undissociated acetic acid crosses it rapidly via non-ionic diffusion. Spheroplasts derived from the anaerobically grown *P. denitrificans* cells followed the behaviour expected from published work on mitochondria. They swelled spontaneously not only in water (at a rate of 0.063 ± 0.001 s^{-1}) but also in the iso-osmotic solutions of ammonium acetate (0.004 ± 0.0005 s^{-1}) and ammonium butyrate (0.0047 ± 0.0001 s^{-1}), in support of the contention that protonated acids and NH_3 penetrate the membrane freely. The swelling rate declined as pH was raised from 7 to 9. Setting the value for the butyrate medium of pH 7 to 100%, the corresponding results for pH 8 and 9 were 89% and 19%, respectively. Collectively, these results confirmed the ability of short-chain fatty acids to carry protons through the *P. denitrificans* membrane and, hence, the applicability of these agents for the intended purpose, namely, modulation of the transmembrane pH gradient.

Fig. 1 shows the effect of varying external pH on the methyl viologen-linked nitrate reductase activity of *P. denitrificans* cells. In this experiment, the pH of the reaction medium was adjusted by adding Tris while maintaining a constant concentration of an anion, butyrate, or chloride. As can be seen, in the butyrate medium, there was relatively little methyl viologen oxidation at neutral pH. Similar experiments were carried out in Tris–acetate media, and the results (not shown) were entirely consistent with those for Tris–butyrate.

Suppression of nitrate reduction at neutral pH in the presence of weak permeant acids can occur in principle by at least three possible mechanisms. (1) A ΔpH-driven transport of nitrate into the cells slows down when diffusion of the protonated acid dissipates the pH gradient. (2) A drop in internal pH caused by the influx of H^+ restricts the reaction capacity of the nitrate reductase system. (3)

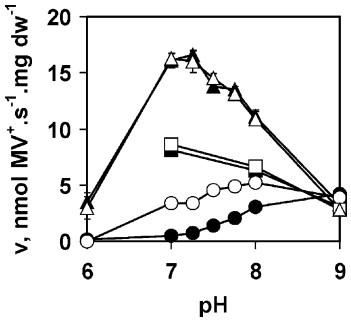

Fig. 1. pH profiles of the MV^+-dependent nitrate reduction in intact or Triton X-100-permeabilised cells and the effect of nitrite. The reaction mixture (2.5 ml) contained 0.1 M Tris–chloride (open symbols) or 0.1 M Tris–butyrate (solid symbols) of the pH indicated on the abscissa. 2.15 or 4.3 mg dry wt of bacteria was added from a thick stock suspension in 0.5 M sucrose plus 5 mM Tris–HCl, pH 7.0. The reaction was started by adding 1 mM $NaNO_3$. Circles, no further additions; triangles, 0.1% Triton X-100 present initially in the reaction mixture; squares, 2 mM nitrite added immediately prior to nitrate. A high background MV^+-nitrite reductase activity precluded experimentation with nitrite at pH 6, so the data for this pH are lacking in the figure.

Accelerated oxidation of physiological substrates produces a surplus of reducing equivalents that are fed into the respiratory chain with the concomitant decrease in the rate of MV^+ oxidation.

Explanation (2) became difficult to accept when it was learned that the pH optimum for nitrate reduction in Triton X-100-permeabilised cells lay at 7 (Fig. 1), which implied the negativity of the Δ(activity)/ΔpH quotient above this pH. Additional evidence against explanation (2) came from the observation that the reaction rates in Tris–butyrate and Tris–chloride did not differ significantly after inclusion of 2 mM nitrite (Fig. 1) to stimulate the putative nitrate/nitrite antiport.

A test on the contribution of physiological substrates metabolism involved measurements of nitrate reduction from the appearance of nitrite, with antimycin added to block further conversions. As harvested, *P. denitrificans* contained endogenous substrate which promoted reaction at a specific rate of 0.18 ± 0.01 nmol nitrite s^{-1} mg dw^{-1} (0.1 M Tris–chloride, 10 mM $NaNO_3$, pH 7.0, 30 °C). The presence of 10 mM sodium succinate caused an increase to 0.60 ± 0.01 nmol nitrite s^{-1} mg dw^{-1}. When the same cells were assayed for the nitrate-dependent MV^+ oxidation activity, the resulting specific rate amounted to 4.8 ± 0.2 nmol MV^+ s^{-1} mg dw^{-1} and remained unaffected by 10 mM succinate. In light of these results, it appears that electron supply from MV^+ is too great for the oxidation of physiological substrates to proceed effectively, and hence, alternative (3) seems also to be unlikely.

To investigate further the possibility that ΔpH drives nitrate transport at neutral pH (mechanism No. 1 above), dose–response studies with nigericin were carried out. The cells indeed exhibited a high sensitivity to nigericin when assayed in 0.1 M Tris–chloride of pH 7.0 (Fig. 2). On the basis of comparison of kinetic parameters obtained by curve fitting, it was found that a shift of pH from 7.0 to 8.0 had

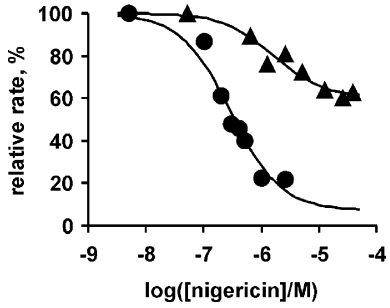

Fig. 2. Inhibition by nigericin of the MV^+-nitrate reductase of intact cells at pH 7 (circles) and 8 (triangles). Experimental conditions: 0.1 M Tris–chloride, 3.7 mg of cells, varying concentrations of nigericin, and 10 mM $NaNO_3$ added to start the reaction. Relative rates are given as percentages of the rate in the absence of ionophore. The curves represent the best fit of the data sets to the logistic equation, with $v_{max}=100$, $v_{min}=7.1$, $pI_{50}=6.53$ (pH 7) and $v_{max}=100$, $v_{min}=60.3$, $pI_{50}=5.79$ (pH 8).

two consequences: (i) the concentration of ionophore required for 50% maximal effect increased by about one order of magnitude (from 0.29 μM to 1.6 μM); and (ii) there was a marked depression of the highest attainable degree of inhibition (from 93% to 40%). As in experiments with butyrate described above, the simultaneous presence of 2 mM nitrite or 0.1% Triton X-100 prevented nigericin from exerting any inhibitory effect. Hence, it was concluded that the basis for butyrate and nigericin action is probably similar.

If the inhibition of nitrate transport by nigericin results from the entry of H^+ in exchange for intracellular K^+, then it should disappear as the K^+ concentration added outside approaches that within the cell. This was found to be the case, as demonstrated in Fig. 3, for a bacterial culture in which the intracellular concentration of K^+ was estimated to be 98.6 mM by ICP-AES analysis. No discernible activation by K^+ could be detected in the absence of nigericin, in confirmation that external K^+ acts merely by changing the pH gradient via the nigericin-mediated H^+/K^+ antiport.

From the point of view of the formal enzyme kinetics, nigericin may affect nitrate utilisation in two ways, either by decreasing the limiting velocity (V_{max}) or by increasing the apparent half-saturation concentration ($K_{0.5}$). Examination

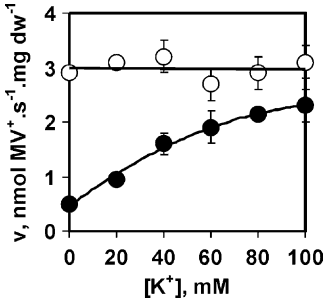

Fig. 3. Protective effect of extracellular potassium against nigericin inhibition. The medium contained 0.1 M Tris–chloride, pH 7.0, 3.2 mg of cells, and varying concentrations of potassium in the form of KCl. Nigericin was applied at 1.3 μM (closed circles) or omitted as a control (open circles). The reaction was started by 10 mM $NaNO_3$.

of the nitrate concentration dependence demonstrated changes in both these kinetic parameters. For example, in 0.1 M Tris–chloride of pH 7.3 the V_{max} dropped from 3.9 ± 0.2 to 1.9 ± 0.2 nmol MV^+ s^{-1} mg dw^{-1} while the $K_{0.5}$ rose from 0.025 ± 0.009 to 0.15 ± 0.08 mM as the result of the addition of 2.6 μM nigericin. Clearly, the before-described nigericin inhibitions at 10 mM nitrate were mainly due to the V_{max} effect.

Given the apparent failure of butyrate and nigericin to inhibit nitrate transport at alkaline pH values, a question inevitably arises as to whether H^+ also remains as the coupling ion when its concentration in the environment is very low, or whether its role can be overtaken by another ion, e.g., Na^+. This would be a situation not unprecedented in bacterial bioenergetics [15,16]. However, the use of titanium(III) citrate in place of sodium dithionite as a reducing agent for methyl viologen enabled to show that the utilisation of Tris–nitrate by intact cells in 0.1 M Tris–chloride buffer of pH 9.0 proceeds at comparable rates in the absence of any added sodium ion and in the presence of 10 mM Na^+ (results not shown). Furthermore, it turned out that the nitrate-dependent MV^+ oxidation under alkaline conditions could still be fully inhibited by CCCP, although the inhibitory potency of the uncoupler decreased gradually with increasing pH (in 0.1 M Tris–chloride buffer, the I_{50} values were determined to be 0.42 μM, 1.4 μM, and 6.4 μM for pH 7, 8, and 9, respectively).

Since uncouplers are known to dissipate both the ΔpH and $\Delta\psi$ components of the proton electrochemical gradient, the sensitivity to CCCP (but not to nigericin) persisting in alkaline pHs might reflect a more pronounced contribution of $\Delta\psi$. Based on earlier observations of a partial depolar-

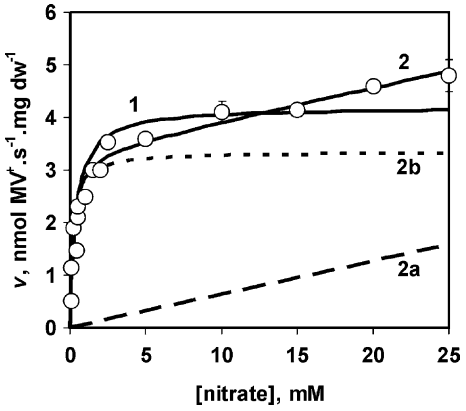

Fig. 4. Concentration dependence of the rate of MV^+ oxidation by nitrate in depolarised cells at pH 9. Bacteria were suspended in 0.2 M Tris–borate, pH 9, and preincubated with valinomycin (0.13 mg ml^{-1}) for 3 h prior their transfer to the reaction mixture containing 0.2 M potassium borate of the same pH. The reaction was initiated with the indicated concentrations of $NaNO_3$. The fits are to the laws for a simple saturable system (1) and a composite model (2) consisting of linear (2a) and hyperbolic (2b) parts. V_{max}, $K_{0.5}$, and AIC_c converged to be 4.2 nmol MV^+ s^{-1} mg dw^{-1}, 0.37 mM, and −21.9 in the former and 3.3 nmol MV^+ s^{-1} mg dw^{-1}, 0.19 mM, and −41.3 in the latter case, respectively. A significantly lower AIC_c score suggests that model 2 describes much better the data.

isation of *P. denitrificans* cells by external K^+, the possible role of $\Delta\psi$ was checked by depolarising cells by inwardly oriented gradients of K^+ or Li^+, with and without pretreatment with an appropriate ionophore (valinomycin or Lithium Ionophore VI). The sole substitution of 0.2 M potassium borate for 0.2 M Tris–borate (pH 9) brought about a decrease in the V_{max} (from 6.6±0.3 to 4.3±0.2 nmol MV^+ s^{-1} mg dw^{-1}), with a lesser change in the $K_{0.5}$ (from 50±9 to 70±10 μM). For cells pretreated with valinomycin, the assay in K buffer produced a more complex nitrate concentration dependence that could be resolved by non-linear regression into a saturable and a linear component (Fig. 4). The latter was probably due to an increased passive permeation of nitrate across the phospholipid bilayer after collapsing $\Delta\psi$. The saturable component was characterised by a $K_{0.5}$ value about 2- to 3-fold higher than that relative to nitrate utilisation by untreated cells. A comparable increase in $K_{0.5}$ was obtained with Li ionophore and lithium borate as the assay buffer (results not shown). On the other hand, when valinomycin action was assessed in the same way at pH 7 using buffers containing phosphate instead of borate, the $K_{0.5}$ and V_{max} values were unaffected, suggesting that the effect of valinomycin is pH specific.

4. Discussion

Previous claims on the existence of a nitrate-proton symport concern mostly plant cells and are based on indirect measurements of membrane depolarisation [17–23], extracellular alkalinisation [18,24,25], or intracellular acidification [26] elicited by nitrate addition. The kinetic approach employed here, albeit indirect too, uncovers a similar type of information about denitrification bacteria, which have been so far much less studied in respect to nitrate translocation mechanisms. The time scale of measurements allows for a separate analysis of the early, energy-dependent phase of nitrate import, which provides the cell with nitrate to initiate nitrate respiration, and is clearly distinquishable from the subsequent, as yet putative, nitrate/nitrite exchange, triggered only after a sufficient amount of nitrite is generated from nitrate in the cytoplasm or when nitrite is added externally [4,6,7].

Although the previously noticed sensitivity to an uncoupler of the unidirectional nitrate transport in *P. denitrificans* [4,6,7] suggests its active nature, it by itself cannot help in discriminating among several possible modes of energy coupling, e.g., a symport with H^+, a symport with Na^+, or even an uptake directly linked to ATP hydrolysis. Now, the first of the three possibilities receives strong support from the finding that selective dissipation of the chemical (ΔpH) component of the proton motive force suffices to block the initial phase of transport at neutral pH. Since the ΔpH generally represents only a lesser part of the pmf in *P. denitrificans* [9], its manipulation is not expected to cause massive de-energisation of respiring cells. Thus, if the transport process had been powered by ATP, it would have still proceeded despite of the presence of the ΔpH dissipators. A conversion of the pmf into a sodium motive force, which then would drive the transport, also does not seem to be a valid explanation, given the facts that the presence of extracellular Na^+ apparently did not weaken the inhibition produced by nigericin and that the rate of nitrate utilisation did not change significantly following sodium depletion. A point to be dealt with in future work is why measurements of spheroplasts swelling in iso-osmotic ammonium nitrate have so far failed to provide an independent evidence for parallel movements of proton and nitrate across *P. denitrificans* cells membrane [3,27].

Kinetic models developed previously for nitrate transport in *Arabidopsis thaliana* [20], *Neurospora crassa* [21], and *Emericella* (*Aspergillus*) *nidulans* [23] postulate a transporter existing in an anionic form (C^-), which, upon the binding of nitrate anion and two protons at the external side of the membrane, gives rise to the elecroneutral complex $C^-(NO_3^-)(H^+)_2$. A conformational change then presumably allows the bound ligands to move towards the cytoplasmic surface, where they dissociate. Finally, the transport cycle is completed through another conformational change, causing back reorientation of the empty transporter. Since the latter transition is thought to be associated with the outward movement of a negative charge, the model predicts a decrease in the apparent affinity for nitrate with decreasing membrane voltage. This is actually the behaviour observed in experiments with valinomycin at pH 9 (Fig. 4). The observations showing that the pH component of the pmf predominantly, if not solely, drives the transport in the neutral pH range (Figs. 1–3) seem to be more difficult to reconcile with the above mechanism. A hypothetical explanation is to propose that one of the two essential proton-binding residues does not deprotonate at all during the whole transport cycle under these conditions due to its rather high p*K* value. In this way, a lowered transport stoichiometry of one proton for one nitrate anion and, hence, the exclusive ΔpH dependency could arise. Two mobile forms of a carrier, differing in their degree of protonation (e.g., C^- and CH), have been included by Rottenberg [28] in the models for proton-metabolite symport with pH-dependent variable stoichiometry. However, as demonstrated by others [29] using kinetic modelling, the operation of such a proton symporter would confront the cell with an increased continuous influx of protons even in the absence of the transported substrate. Clearly, detailed analyses of the actual H^+ to NO_3^- ratios are needed, preferably in systems allowing for defined manipulations of pH and ionic composition on both sides of the membrane.

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