Anion transport through the phosphate-specific OprP-channel of the *Pseudomonas aeruginosa* outer membrane: effects of phosphate, di- and tribasic anions and of negatively-charged lipids

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The mechanism of anion transport through the phosphate-starvation inducible OprP-channel of *Pseudomonas aeruginosa* outer membrane was studied in planar lipid bilayer membranes. The single-channel conductance of OprP was 160 pS in 100 mM chloride solution. Addition of other anions, in particular of phosphate, di and tribasic anions lead to a strong decrease of the chloride conductance. The decrease was used to calculate the stability constants for the binding of these ions to the binding site of the channel on the basis of a one-site two-barrier model. The stability constant of the binding of phosphate to the site was 11 000 M^{-1} at neutral pH. Surprisingly, di- and tribasic anions, such as sulfate and citrate had a much lower affinity to the binding site inside the channel. Although the single-channel conductance was dependent on the external pH, the stability constants for phosphate binding decrease only slightly for increasing the pH. The use of negatively-charged lipids instead of neutral ones in the planar lipid bilayers had no influence on the single-channel conductance of the OprP-channel, suggesting that the channel is shielded from the influence of surrounding molecules. Its permeability properties are probably not influenced by negatively-charged lipopolysaccharide molecules.

Introduction

The outer membrane of Gram-negative bacteria acts as a molecular filter with defined exclusion limits for substrates [1,2]. The active components of this sieve are the porins, a major class of outer membrane proteins [1–3]. Most porins act as general diffusion channels and form large water-filled pores with estimated diameters of 1.0–2.0 nm in vivo and in reconstituted systems [4–6]. Other porins act as substrate-specific channels, such as LamB and Tsx of *Escherichia coli* and OprP (protein P) of *P. aeruginosa* [7–9]. OprP is induced in the outer membrane of *P. aeruginosa* under the condition of phosphate starvation [9]. This channel is an exception among the substrate-specific channels of bacterial outer membranes because it is highly-specific for anions, especially for phosphate [9–11], whereas LamB and Tsx are selective for neutral solutes, such as sugars or nucleosides [7,8]. The permeation of anions especially that of phosphate across the outer membrane may represent a severe problem in general because of the high surface charge density of negatively charged groups attached to the lipopolysaccharides (LPS) [12].

The transport of a large variety of monobasic anions through OprP-channels has been studied in detail [11]. Their transport could be satisfactorily-well explained by a one-site two-barrier model. The results are consistent with a closely spaced binding site for anions inside the channel [11]. The channel discriminates among different anions according to their sizes, with the sole exception of fluoride. The transport of the halides follows the Eisenman sequence AVI [13]. The stability constants for their binding and those of other anions to the binding site inside the channel have been calculated from the single-channel data [11]. The OprP channel is not voltage-gated and has lifetimes of sev-

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Abbreviations: OprP, outer membrane protein P of *Pseudomonas aeruginosa*; LPS, lipopolysaccharide; GABA, γ-aminobutyric acid.

eral minutes. The current-voltage curves are linear for membrane potentials up to 150 mV that suggests that Nernst-Planck types of barriers rather than Eyring barriers are involved in movement of anions through the OprP-channel [11].

The knowledge of ion transport through anion-selective channels has increased in recent years (see Ref. 14 for a recent review). Many chloride-specific channels such as the glycine and the GABA-receptors and the chloride-specific channel from *Torpedo* have been characterized in patch-clamp experiments [15,16] and in lipid bilayer membranes [17] and their primary sequences have been derived from cDNA-sequencing [18,19]. Their secondary structures are different to those of outer membrane porins (including OprP), since they contain mainly α-helices, whereas the OprP-channel has according to its primary sequence and circular dichroism data the β-barrel stave structure of outer membrane porins [20].

OprP and the closely-related OprO-channel from *P. aeruginosa* [21] are still the only phosphate-specific channels known to date. In this investigation we studied the mechanism of anion transport through OprP in more detail. In particular, we investigated the effect of phosphate on the chloride conductance of the OprP-channel. Increasing concentrations of phosphate in the micromolar and millimolar range resulted in a dramatic decrease of the single-channel conductance of chloride. Experiments with other anions, such as sulfate and citrate suggested that the effect of phosphate is very specific and is not simply caused by the interaction of multibasic anions with the binding site inside the channel as has been found for PhoE of *Escherichia coli* [22]. The results could well be explained by the previously proposed one-site two-barrier model [11] and precise data for the binding of the different anions to the channel could be calculated. Furthermore, we demonstrate that negatively-charged lipids have no influence on the OprP-channel, which suggests that the channel forms its own sphere, and that its ion conducting properties would not be influenced by LPS.

Materials and Methods

Protein P isolation. Protein P was purified exactly as described previously [23]. It showed a single band on SDS-PAGE.

Lipid bilayer experiments. The methods used for the lipid bilayer experiments have been described in detail previously [24]. The experimental setup consisted of a Teflon cell with two water-filled compartments connected by a small circular hole. The hole had an area of about 0.2 mm². Black lipid bilayer membranes were obtained by painting, onto the hole, a 1% (w/v) solution of diphytanoylphosphatidylcholine or phosphatidylserine (Avanti Polar Lipids, Alabaster, AL, USA)

in n-decane. The temperature was maintained at 20°C during all experiments.

All salts were obtained from Merck (Darmstadt, Germany, analytical grade). They were used unbuffered (pH 4 and pH 6) or were buffered with cationic buffers (2 mM Tris at pH 8) to avoid any interference with the binding site inside the OprP-channel. Single-channel recordings were performed using calomel electrodes (with salt bridges) connected in series to a voltage source and a current-to-voltage converter made on the basis of a Burr Brown operational amplifier. The amplified signal was monitored on a storage oscilloscope (Tektronix 7633) and recorded on a strip chart or tape recorder.

Results

Single-channel conductance of phosphate, sulfate and citrate

We have shown previously that the single-channel conductance of the OprP-channel saturates for different anions when their concentration in the aqueous phase increases [11]. This is caused by binding of the anion to a binding-site inside the two-barrier one-site channel [25]. The movement of anions across the two barriers is given by the rate constant k_1 (jump of the anions from the aqueous phase to the central binding site) and k_{-1} (inverse movement). The stability constant of the binding between an ion and the binding site is $K = k_1/k_{-1}$. The fit of the single-channel conductance, $G_0(c)$, as a function of the anion concentration was calculated using the following equation [11]:

$$G_0(c) = G_{0,max} Kc / (1 + Kc) \qquad (1)$$

where $G_{0,max}$ is the maximum single-channel conductance at very high ion concentration:

$$G_{0,max} = e^2 k_{-1} / (2kT) \qquad (2)$$

where e (1.6 · 10⁻¹⁹ As) is the elementary charge, k (1.38 · 10⁻²³ J/K) is the Boltzmann constant and T the absolute temperature. $G_0(c)$ saturates for large anion concentration [11].

The fit allows the calculation of binding constants and of the maximum single-channel conductances. We performed similar experiments with phosphate, sulfate and citrate to evaluate the two parameters for these anions. Surprisingly, we did not observe any dependence of the single-channel conductance on the monobasic phosphate concentration (pH 6), since the single-channel conductance was approximately the same for 1 mM as compared with 1 M (see Table I). Below 1 mM salt concentration it was impossible to measure enough single-channel events to calculate precise numbers for the single-channel conductances. This

TABLE I

Average single-channel conductance, $G_0(c)$, of the OprP-channel as a function of the concentration, c, of different anions (the cation was in all cases potassium)

The membranes were formed from diphytanoyl phosphatidyl-choline/n-decane; T = 20°C, V_m = 50 mV. The aqueous salt solutions were used unbuffered (pH 6 and pH 4) or they were buffered with 2 mM Tris (pH 8). $G_0(c)$ was calculated as the average of at least 70 single events. The data for chloride were taken from Ref. 11.

c (mM)	Chloride pH 6	$G_0(c)$ (pS)				
		Phosphate			Sulfate pH 6	Citrate pH 6
		pH 4	pH 6	pH 8		
1	6	14	7	10	5	6
3	17	12	9	12	11	11
10	49	15	7	11	15	16
30	102	16	9	13	17	20
100	159	13	10	12	18	22
300	245	12	9	10	20	19
1000	260	16	10	12	17	21

means that the reconstitution rate decreased dramatically because of inactivation of the protein or because of Gouy-Chapman effects due to the many surface charges of OprP. The strong saturation indicated a considerable affinity of the channel for phosphate (i.e., the half-saturation constant was below 1 mM). Similar results were obtained when the pH was set to pH 4 and pH 8. In all cases we were not able to measure any dependence on the salt concentrations.

Similar experiments were performed for sulfate and citrate (see Table I). However, for these ions we observed saturation, indicating that the corresponding half-saturation constants were in the concentration range used in the single-channel experiments. This meant also that we could calculate the maximum single-channel conductance and the stability constant by
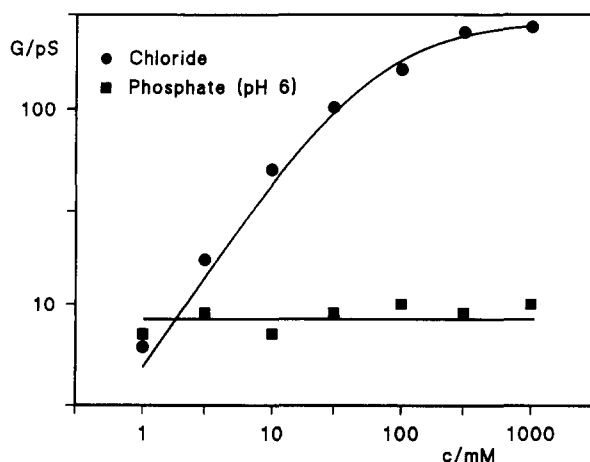

Fig. 1. Single-channel conductance of the OprP channel of *P. aeruginosa* as a function of the KCl concentration and the phosphate concentrations at pH 6 in the aqueous phase. The membranes were formed from diphytanoylphosphatidylcholine/n-decane; T = 20°C; V_m = 50 mV. The data for KCl (pH 6) were taken from Ref. 11.
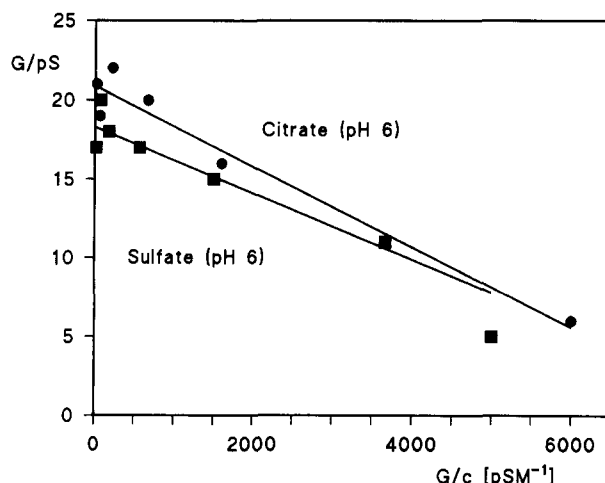

Fig. 2. Eadie-Hofstee plots of the single-channel conductances of the OprP-channel in citrate and sulfate given in Table I. The pH of the aqueous salt solutions was set to pH 6. The straight lines in the figure were drawn by linear regression of the data points and yielded the stability constants (K = 400 M^{-1} and 500 M^{-1}) and the maximum single-channel conductances ($G_{0,max}$ = 21 pS and 18 pS) for citrate and sulfate transport, respectively, through the OprP-channel.

Eadie-Hofstee plots, as has been demonstrated previously for chloride and other anions [11]. Fig. 2 shows such a plot for the single-channel conductances of citrate and sulfate. The data for both ions (Table I) could be reasonably well fitted to Eqn. 1. This result indicated that a one-site two-barrier model provides also an excellent fit for sulfate and citrate transport through the OprP-channel.

Inhibition of the chloride conductance by phosphate

The experiments described above demonstrate that phosphate had a considerable affinity for the OprP-channel. Furthermore, we have noticed earlier that the addition of phosphate leads to the inhibition of the OprP-mediated macroscopic chloride conductance [10]. The influence of phosphate on the conductance of the OprP-channel was studied in more detail by measuring single-channel conductances in aqueous solutions containing 0.1 M KCl and increasing concentrations of phosphate at several different values of aqueous pH. The results are summarized in Table II. In additional experiments we studied also the inhibition of chloride conductance by sulfate and citrate (Table II).

When two anions (for instance chloride (Cl) and phosphate (P)) compete for the channel, the total single-channel conductance G_{tot} is given by the following equation derived from the model. It is similar to the treatment of the competitive enzyme inhibition by alternative substrates:

$$G_{tot} = G_{0,Cl} + G_{0,P} \tag{3}$$

$$G_{tot} = G_{0,max\,Cl}K_{Cl}c_{Cl}/(K_{Cl}c_{Cl} + (1 + K_P c_P))$$

$$+ G_{0,max\,P}K_P c_P/(K_P c_P + (1 + K_{Cl}c_{Cl})) \tag{4}$$

TABLE II

Inhibition of OprP-induced chloride conductance (G_{tot}) by increasing concentrations of phosphate, sulfate and citrate

The aqueous phase contained 0.1 M KCl and the indicated concentrations of phosphate, sulfate and citrate. The aqueous salt solutions were used unbuffered (pH 6 and pH 4) or they were buffered with 2 mM Tris (pH 8); $T = 20°C$, $V_m = 50$ mV. The membranes were formed from diphytanoyl phosphatidylcholine/n-decane; $T = 20°C$, $V_m = 50$ mV. G was calculated as the average of at least 70 single events.

| c (mM) | $G_0(c)$ (pS) | | | | |
| | Phosphate | | | Sulfate | Citrate |
	pH 4	pH 6	pH 8	pH 6	pH 6
0	290	180	140	155	165
0.1	220	140	110	160	150
0.3	140	95	77	150	150
1	75	40	40	149	138
3	40	25	27	126	125
10	22	14	16	61	105
30	15	12	15	58	65
100	15	10	15	23	30

Eqn. 4 can be used to calculate the binding constants (K_{Cl} and K_P) from single-channel experiments in the presence of different anions and the individual maximum single-channel conductances ($G_{0, max\ Cl}$ and $G_{0, max\ P}$).

The stability constant for phosphate binding, K_P, could thus be calculated from the data of Table II according to Eqn. 4. Fig. 3 shows the fit of the phosphate-mediated inhibition of chloride conductance at pH 6 as given in Table II by using Eqn. 4. The best fit was achieved for $G_{0, P} = 14$ pS (pH 4), 9 pS (pH 6) and 14 pS (pH 8). The stability constant K_P, for phosphate
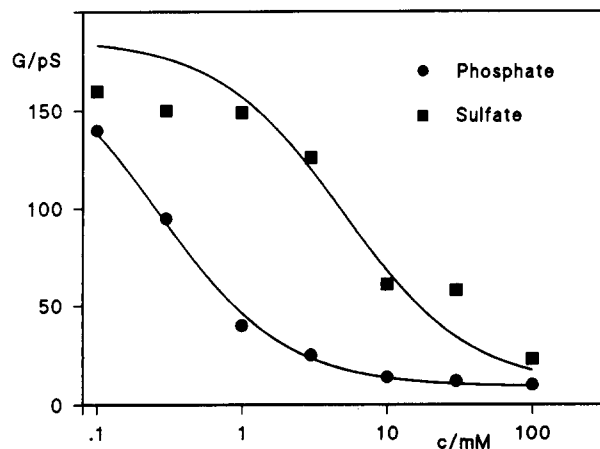

Fig. 3. Fit of the phosphate- and sulfate-mediated inhibition of OprP-induced chloride conductance. The data were taken from Table II. The solid lines were drawn according to Eqn. 5 by using the following parameters: $G_{0, P} = 9$ pS, $K_P = 11000$ M^{-1}, $G_{0, Cl} = 280$ pS, $K_{Cl} = 20$ M^{-1}, (pH 6); and for sulfate $G_{0, S} = 20$ pS, $K_S = 600$ M^{-1}, $G_{0, Cl} = 280$ pS, $K_{Cl} = 20$ M^{-1}, (pH 6).

TABLE III

Stability constants K and maximum single-channel conductance $G_{0, max}$ for the transport of different anions through OprP

The parameters were derived in part from a fit of the data for sulfate and citrate (given in Table I) to Eqn. 1 by using Eadi-Hofstee-plots (see Fig. 2). The stability constant K and the maximum single-channel conductance $G_{0, max}$ for phosphate were taken from a fit of the phosphate-induced block of chloride conductance to Eqn. 5 (given in Table II; see also Fig. 3). The data for the halides were taken from Ref. 11.

Anion	K (M^{-1})	$G_{0, max}$ (pS)
Fluoride	3.5	515
Chloride	20	280
Bromide	4.7	265
Iodide	1.3	110
Phosphate (pH 4)	13000	14
(pH 6)	11000	9
(pH 8)	8000	14
Sulfate (pH 6)	500	18
Citrate (pH 6)	400	21

binding was close to 10000 M^{-1} (13000 M^{-1} at pH 4, 11000 M^{-1} at pH 6 and 8000 M^{-1} at pH 8) at all pH values studied. The parameters for chloride transport, as derived from the multiparameter fit, were similar to those obtained previously from single-channel experiments at different pH [11]. Analogous results were obtained for the fit of the single-channel data derived for sulfate (see Fig. 3) and citrate (data not shown) using Eqn. 4 (Table III).

Table IV shows the values of k_1 and k_{-1} as calculated according to Eqn. 2 and $K = k_1/k_{-1}$ from the data given in Table III. The rate constant for the jump from the external solution to the binding site inside the OprP-channel, k_1 (which is equal to two times the maximum permeability), had a maximum for monobasic phosphate followed by sulfate, citrate and chloride, whereas the inverse reaction rate k_{-1} (i.e. the maximum turnover number of ions through a one-site two-barrier channel) was largest for fluoride. This means that at very small anion concentrations the permeabil-

TABLE IV

Rate constants k_1 and k_{-1} as calculated from the data of Table III using Eqn. 2 and $K = k_1/k_{-1}$

Anion	k_1 (ms^{-1} ×10^7)	k_{-1} (s^{-1} ×10^7)
Fluoride	60	17
Chloride	180	9.0
Bromide	40	8.5
Iodide	4.6	3.5
Phosphate (pH 4)	5700	0.44
(pH 6)	3500	0.28
(pH 8)	3100	0.44
Sulfate (pH 6)	310	0.62
Citrate (pH 6)	260	0.64

Effects of negatively charged membranes on OprP-induced single-channel conductance in potassium chloride and potassium phosphate

The membranes were formed from phosphatidylsering/n-decane; $T = 20°C$. $V_m = 50$ mV. G was calculated as the average of at least 70 single events.

c (mM)	$G_0(c)$ (pS)	
	Chloride (pH 6)	Phosphate (pH 6)
1	5	10
3	15	8
10	54	11
30	107	9
100	164	10
300	250	7
1 000	270	10

ity of the channel is highest for phosphate, whereas it conducts F^- or Cl^- best at large anion concentrations.

Effect of negatively-charged lipids

The surface of Gram-negative bacteria contains many negatively charged groups attached to LPS. These groups could influence ion transport through OprP on the basis of Gouy-Chapman (surface charge) effects. To test this we measured the single-channel conductance of the OprP-channel in membranes formed of the negatively-charged lipid phosphatidylserine. First, we varied the KCl-concentration from 1 mM to 1 M (Table V). Surprisingly, we did not detect any appreciable difference compared to the results obtained previously for neutral membranes (cf. Tables I and V). Then we measured the single-channel conductance as a function of the phosphate concentration at pH 6. Again we did not notice any difference to the data obtained from neutral membranes. These results indicate that the anion transport through the OprP-channel is not influenced by negative surface charges attached to the lipids.

Discussion

Here we have studied the transport of phosphate, sulfate and citrate through the phosphate-starvation-inducible outer membrane channel OprP of *P. aeruginosa*. It was possible to measure for all three anions the single-channel conductance. The saturation of the single-channel conductance for sulfate and citrate allowed the calculation of $G_{0, max}$ and the stability constants using Eqn. 1 derived from the two-barrier one-site model (Table III). This model is justified by the experimental result that the conductance vs. concentration relationships measured for a variety of different anions always showed saturation and never a maximum followed by a decrease at even larger anion concentra-

tions. This would be expected, in principle, for the existence of a second binding site inside the channel [11]. The results for sulfate and citrate are given in Table III together with the same parameters for the transport of other anions taken from a previous publication [11]. Interestingly, the dibasic anion sulfate had a higher affinity for the binding site than the tribasic anion citrate. On the other hand, the maximum single-channel conductance was very similar for both anions, a result that may reflect their similar size.

The results of the single-channel experiments demonstrate that the OprP-channel has a considerable affinity for the binding of phosphate. This result is consistent with its in-vivo role [26]. The stability constant for the binding of phosphate to the binding site inside the channel could not be derived from single-channel experiments in different phosphate solutions because the conductance was independent of the phosphate concentration over the range of concentrations used. Alternatively, the binding constant for phosphate could be calculated from the inhibition of the chloride conductance, since phosphate and chloride apparently competes for the same binding site of the one-site two-barrier channel. In this respect the OprP channel shares some similarity to calcium channels of muscle membranes, since it has been demonstrated [27] that a variety of alcaline cations are permeable through the calcium channel in the absence of divalent cations. The flux of monovalent cations, in particular of sodium, is blocked when calcium is present probably because calcium has a much higher affinity towards the binding site(s) inside the channel than the alcaline cations. It has to be noted, however, that the calcium transport through the calcium channels cannot be explained by a two-barrier one-site model [28] and more complicated models such as a three-barrier two-sites model is required to explain Ca^{2+}-transport.

Our data suggest that the binding of phosphate to the OprP-channel is independent of pH. This means that the affinity is not simply caused by the charge density of the anions, since monobasic phosphate has a higher affinity than the dibasic sulfate and the tribasic citrate. Furthermore, phosphate is predominantly monobasic at pH 4 and 6, but it is preferentially dibasic at pH 8 with very little influence on its affinity towards the binding site within OprP. This means also that OprP has a completely different channel structure from the other well-studied phosphate-starvation-inducible outer membrane protein PhoE of *E. coli*. The affinity of PhoE for di and tribasic anions is very similar to that for phosphate [22] and it acts as a general diffusion pore with an exclusion limit of about 600 Da [29]. OprP is not a general diffusion channel, since its selectivity filter is narrow and large anions with diameters greater than 0.6 nm are not permeable through the channel [11].
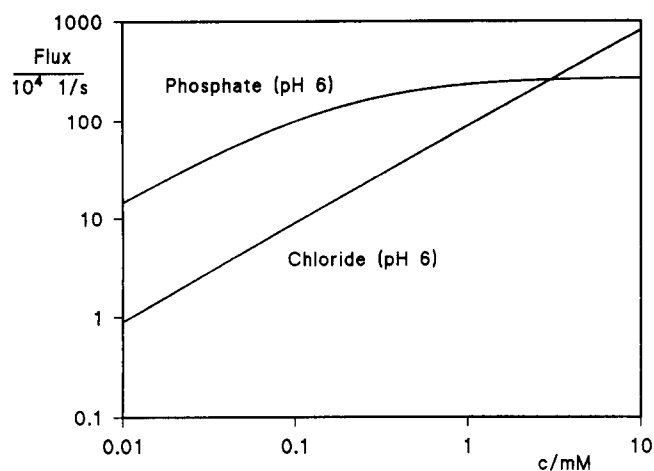
Fig. 4. Flux of chloride and phosphate through one single OprP channel as a function of the corresponding ion concentration, c, on one side of the channel. The concentration on the other side was set to zero. The flux was calculated using Eqn. 5 and the rate constants given in Table IV.

The OprP-channel conducts at high salt concentration chloride much better than phosphate. This argues in principle against a phosphate-specific channel. However, phosphate limitation means that the phosphate concentration in the growth media is below 1 mM. At these concentrations the channel offers a considerable advantage for the transport of phosphate as the following consideration demonstrates. The net flux of ions (Φ) through the two-barrier one-site channel in the case of an ion gradient across the membrane ($c'' = 0$, $c' = c$) and in the absence of a membrane potential is given in Ref. 11:

$$\Phi = \Phi_{max} Kc /(2 + Kc) \qquad (5)$$

where $\Phi_{max} = k_{-1}$ is the maximum flux of ions at very high ion concentrations (i.e., the turnover number of the channel). The maximum permeability of a one-site two-barrier channel is $k_1/2$ at very low ion concentration [11]. Fig. 4 shows the net flux of cloride and phosphate (pH 6) through the OprP-channel as a function of the ion concentration on one side of the channel (the concentration on the other side is set to zero) as calculated according to Eqn. 5. The flux of phosphate is starting at higher mM concentrations than the flux of chloride. Furthermore, the phosphate permeability is in the linear range of the flux vs. concentration curve, approx. 20-times higher. This is again consistent with the in-vivo situation of phosphate starvation. The channel should have a high permeability at very low external phosphate, which together with the sink provided by the high-affinity phosphate-binding protein within the periplasmic space [30] should provide maximal phosphate transport and presentation to the cytoplasmic membrane phosphate transport system.

The results of the experiments with negatively-charged lipids suggest that the ion transport through the OprP-channel is not influenced by negatively-charged groups in the vicinity of the channel. This means also that the phosphate transport through OprP in vivo is not limited by the negatively-charged LPS. Furthermore, the single-channel conductance of the OprP-channel in 0.1 M chloride solution was about 160 pS. Thus, the current through the pore at a membrane potential of 100 mV is equivalent to about half of the maximum flux of ions through a spherical sink of 0.3 nm radius (i.e., the pore radius estimated previously on the basis of single-channel experiments with different anions [11]). Nevertheless, we did not observe any indication of diffusion limitations, even at 1 mM phosphate where the flux of ions would be far above diffusion limitation of a sink of 0.3 nm radius [31]. Thus, the protein P channel is probably not long and narrow, a result more consistent with a one-site two-barrier than a two-site three-barrier or more complicated models. The channel has to be rather short to account for the large phosphate flux at small concentrations. Furthermore, it is required that the channel should have large capture radii, which could be positively charged and thus would form a sink for anions, especially for phosphate. This would also mean that the movement of anions towards the binding site cannot be described by a simple jump across a barrier but is analogous to a diffusion process. This can be understood by consideration of the large 'on' rates for the movement of the ions into the binding sites that are close to those of diffusion controlled reaction processes [32].

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