

Mechanism of Ion Transport through the Anion-selective Channel of the *Pseudomonas aeruginosa* Outer Membrane

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ABSTRACT Protein P trimers isolated and purified from *Pseudomonas aeruginosa* outer membrane were reconstituted in planar lipid bilayer membranes from diphytanoyl phosphatidylcholine. The protein trimers formed highly anion-specific channels with an average single channel conductance of 160 pS in 0.1 M Cl solution. A variety of different nonvalent anions were found to be permeable through the channel, which suggests a channel diameter between 0.5 and 0.7 nm. The selectivity for the halides followed the Eisenman sequence AVI (without At⁻). The ion transport through the protein P channel could be explained reasonably well by a one-site, two-barrier model. The stability constant of the binding of Cl⁻ to the site was 20 M⁻¹ at neutral pH. The binding of anions to the site was pH dependent, which suggested that several charges are involved in the closely spaced selectivity filter. Permeability ratios for different anions as calculated from bi-ionic potentials showed agreement with corresponding ratios of single channel conductances. The protein P channels were not voltage-gated and had lifetimes of the order of several minutes. The current-voltage curves were linear for membrane potentials up to 150 mV, which suggested that Nernst-Planck-type barriers rather than Eyring barriers were involved in the movement of anions through the protein P channel.

INTRODUCTION

Whereas many cation-selective channels have been characterized in recent years (see the excellent textbook by Hille, 1984, or Auerbach and Sachs, 1984, for a recent review), the permeation mechanism of anions through channels is poorly understood at present. Only a few highly anion-selective channels like the Cl⁻ channel from the *Torpedo* electrical organ (Miller and White, 1980; White and Miller, 1981; Tank et al., 1982) and the glycine and γ -amino butyric acid (GABA) receptors from nerve cells (Hamill et al., 1983; Sakman et al., 1983) have been studied to a certain extent. Furthermore, the isolation, purification, and successful reconstitution of an intact, highly anion-selective channel has only

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been demonstrated for the phosphate-starvation-induced protein P trimer from the outer membrane of the gram-negative bacterium *Pseudomonas aeruginosa* (Hancock et al., 1982; Benz et al., 1983). This system is of special interest because it may serve as a model for anion transport and facilitated diffusion through channels.

The outer membranes of gram-negative bacteria such as *Escherichia coli*, *Salmonella typhimurium*, and *P. aeruginosa* act as molecular filters with defined exclusion limits for substrates (Nikaido and Nakae, 1979). The active components of these filters are a major class of proteins named porins (Nakae, 1976; Benz, 1985). Most porins form large, water-filled pores with measured diameters of 1.0–2.0 nm in reconstituted outer membrane vesicles (Hancock et al., 1979; Nikaido and Rosenberg, 1983) and in lipid bilayer membranes (Benz et al., 1978, 1985). Protein P is produced in the outer membrane of *P. aeruginosa* under the condition of phosphate starvation (Hancock et al., 1982). This channel is an exception among the pores of bacterial outer membranes because it has a small selectivity filter that is highly specific for anions (Benz et al., 1983). Only the sugar-specific LamB channel of *E. coli* has similar properties (Benz et al., 1986). The mode of induction of this protein as revealed by mutant (Poole and Hancock, 1984; Hancock et al., 1982) and physiological studies (Hancock et al., 1982; Poole and Hancock, 1983) and the recent isolation and characterization of insertion sequence mutants in the protein P gene (Poole and Hancock, 1986) suggest a role for protein P in phosphate transport. The pore-forming trimers of protein P (Angus and Hancock, 1983) are very stable and can be altered by chemical modification without inactivation of their channel-forming ability (Hancock et al., 1983). Chemical modification experiments and the pH dependence of anion flux indicated that several lysines located in or near the selectivity filter could be responsible for the anion selectivity.

In this publication, we have investigated the mechanism of anion translocation through the protein P channel in detail. The results were consistent with a binding site for anions inside the channel. The transport of anions could be satisfactorily explained by a one-site, two-barrier model. The channel was shown to discriminate among different anions according to their sizes, with the sole exception of F^- . The transport of the halides followed the Eisenman sequence AVI (without At^- , Eisenman, 1965; Diamond and Wright, 1969).

MATERIALS AND METHODS

Protein P Isolation

Protein P was purified exactly as described previously (Hancock et al., 1982).

Lipid Bilayer Experiments

The methods used for the lipid bilayer experiments have been described previously in detail (Benz et al., 1978). 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 either 2 mm² (in the case of the macroscopic conductance measurement and selectivity experiments) or ~0.1 mm² (for the single channel records). Black lipid bilayer membranes were obtained by painting, onto the hole, a 1% (wt/vol) solution of diphytanoyl phospho-

tidylcholine (Avanti Biochemicals, Inc., Birmingham, AL) in *n*-decane. The temperature was maintained at 25 °C during all experiments.

All salts (analytical grade) were obtained from Merck (Darmstadt, Federal Republic of Germany). The aqueous salt solutions were prepared with twice-quartz-distilled water. They were used unbuffered and had a pH between 5.5 and 6.5, unless indicated otherwise. To prevent protein inactivation, protein P was added to the aqueous phase from a stock solution (containing either 0.1% sodium dodecyl sulfate [SDS] or 0.1% Triton X-100) either before membrane formation or after the membranes had turned completely black. The single channel records were performed using calomel electrodes (with salt bridges) connected in series to a voltage source and a current amplifier (427, Keithley Instruments, Inc., Cleveland, OH). The amplified signal was monitored on a storage oscilloscope (5115, Tektronix, Inc., Beaverton, OR) and recorded on a strip-chart or tape recorder. Zero-current membrane measurements were performed as described previously (Benz et al., 1979). In brief, the membranes were formed in a 10 mM salt solution. After the membrane turned black, protein P was added to the aqueous phase in such a concentration that the membrane conductance increased ~100–1,000-fold above ground level conductance (10^{-8} – 10^{-7} S/cm²) within 20–30 min. Then the instrumentation was switched to allow measurements of zero-current potential (using a Keithley 610 electrometer). The salt concentration on one side of the membrane was raised by adding small amounts of concentrated solution to one side and corresponding amounts of 10 mM salt solution to the other side while stirring. The membrane potential reached its final value within ~5 min. A similar instrumentation was used for measurements of the bi-ionic potentials of two anions. In this case, the membranes were formed in 0.1 M salt solution. After a considerable membrane conductance was established, the salt was replaced on one side of the membrane by another salt of the same concentration. In this case, the potential also reached its final value within ~5 min.

THEORY

Single Channel Conductance

The theoretical mechanism of ion translocation through channels has been described in detail by other workers (Läuger, 1973; Lieb and Stein, 1974; Cooper et al., 1985). Here we will only discuss the two-barrier, one-site model (Fig. 1), which provided an excellent description of the mechanism of anion transport through the protein P channel. This model assumes a binding site in the center of the channel. The rate constant k_1 describes the jump of the anions from the aqueous phase (concentration, c_M) across the barrier to the central binding site, whereas k_2 is the rate constant for the inverse movement. There is no evidence for asymmetry of the protein P channel; we therefore assumed symmetrical barriers with respect to the binding site (Fig. 1).

The stability constant of the binding between an ion and the binding site is $K = k_1/k_2$. It is assumed in Eq. 1 that only one ion can bind to the binding site at a given time and that no ion can pass the channel if the binding site is occupied. This means that an ion can enter the channel only when the binding site is free. The probability that the binding site is occupied is given by

$$p = \frac{K \cdot c_M}{1 + K c_M}, \quad (1)$$

and that it is free by

$$1 - p = \frac{1}{1 + Kc_M}. \quad (2)$$

The net flux of ions, Φ , through the channel under stationary conditions is equal to the net movement of ions across one barrier (for instance, across the left-hand barrier):

$$\Phi = k_1' c_M \frac{1}{1 + Kc_M} - k_2'' \frac{Kc_M}{1 + Kc_M}. \quad (3)$$

In this expression, the rate constants k_1' and k_2'' are multiplied by the probabilities that the binding site is free or occupied, respectively. Note that these probabilities are independent of the membrane potential if the top of the barriers is halfway between the binding site and the pore mouth.

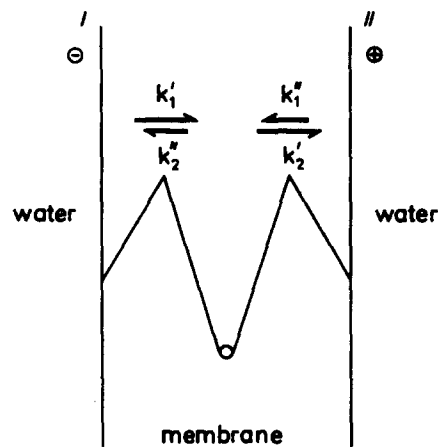


FIGURE 1. The one-site, two-barrier model used for the description of anion transport through the protein P channel.

The current through the pore, I , is given by

$$I = -e_0 \Phi = -e_0 \frac{Kc_M}{1 + Kc_M} [k_1'/K - k_2''], \quad (4)$$

where e_0 is the elementary charge. Eq. 4 can be used for the calculation of the single channel conductance, $\Lambda_0(c_M)$, in the limits of small voltages ($u \ll 1$, $V_m \ll 25$ mV) (Läuger, 1973; Latorre and Miller, 1983):

$$\Lambda_0(c_M) = \Lambda_{0,\max} \frac{Kc_M}{1 + Kc_M}, \quad (5)$$

where $\Lambda_{0,\max}$ is the maximum single channel conductance:

$$\Lambda_{0,\max} = \frac{e_0^2}{2kT} k_2, \quad (6)$$

where k is the Boltzmann constant and T is absolute temperature. Eq. 5 describes the single channel conductance as a function of the ion concentration in the aqueous phase. $\Delta_0(c_M)$ saturates for large anion concentrations, as was observed for the protein P channel (Benz et al., 1983; this study).

RESULTS

Macroscopic Conductance Data

When purified protein P from *P. aeruginosa* was added in small concentrations (10–100 ng/ml) to the aqueous phase bathing a black lipid bilayer membrane, the conductance of the membrane increased by several orders of magnitude. The time course of the conductance increase was similar to that described earlier for other bacterial porins, including protein P, in the presence of KCl (Benz et al., 1980; Hancock et al., 1982; Benz, 1984), with an initial rapid increase for 15–20 min followed by a slower rate of increase, which continued until membrane breakage occurred. When the rate of conductance increase was relatively slow (~30 min after the membrane turned black), it could be shown for different protein concentrations that the membrane conductance was a linear function of the protein concentration in the aqueous phase. The incorporation of the protein occurred at a reasonable rate at high ionic strengths (50 mM to 3 M). At lower ionic strengths, however, only poor reconstitution was observed, and large concentrations of the porin (1–10 $\mu\text{g/ml}$) were needed to obtain a 100-fold increase in conductance above the ground level conductance of the membranes (10^{-7} – 10^{-8} S/cm²). The reconstitution of protein P into the membrane was not a rare event. We would like to stress that with a salt concentration of 0.1 M and a protein concentration of 50 ng/ml, we were able to measure the incorporation of $>10^6$ – 10^7 channels/cm² into the lipid bilayer membranes. Furthermore, we could show that the addition of detergent alone in 100-fold-higher concentrations than those added together with the protein P caused no significant increase in membrane conductance.

Single Channel Experiments

The addition of smaller amounts of protein P (~1 ng/ml) to lipid bilayer membranes of small surface area allowed the resolution of step increases in conductance. Fig. 2 shows a single channel record observed with 1 ng/ml of protein P using a diphytanoyl phosphatidylcholine membrane in the presence of 0.1 M KBr. The size of the current steps was fairly homogeneous as compared with the current steps observed with general diffusion porins from the outer membrane of *E. coli* (Benz et al., 1978) and *S. typhimurium* (Benz et al., 1980). Whereas with these other porins, a two- to threefold variation in the pore conductance has been observed, a fairly sharp histogram was obtained with protein P (Fig. 3).

The single channel record shown in Fig. 2 demonstrated that the conductance fluctuations were exclusively directed upward. In fact, terminating steps were only rarely observed. This indicated that the lifetime of the channels was long, usually exceeding 5–10 min. On the other hand, it can be seen from Fig. 2 that

the current noise increased with the number of channels inserted into the membranes. This noise was absent if the protein P-induced conductance increase was simply simulated with a resistor. We believe, therefore, that the current noise was due to small fluctuations in the barriers involved in the transport of anions, as has been discussed elsewhere (Sigworth, 1985). This presumed channel breathing was observed for most electrolytes. In the case of HCO_3^- , it was so large that it became impossible to detect the channel size when more than three channels were reconstituted into the lipid bilayer membrane (Fig. 4). Thus, the structure of the protein P channel may not be completely fixed and may undergo molecular fluctuations dependent on the type of electrolyte. On the other hand,

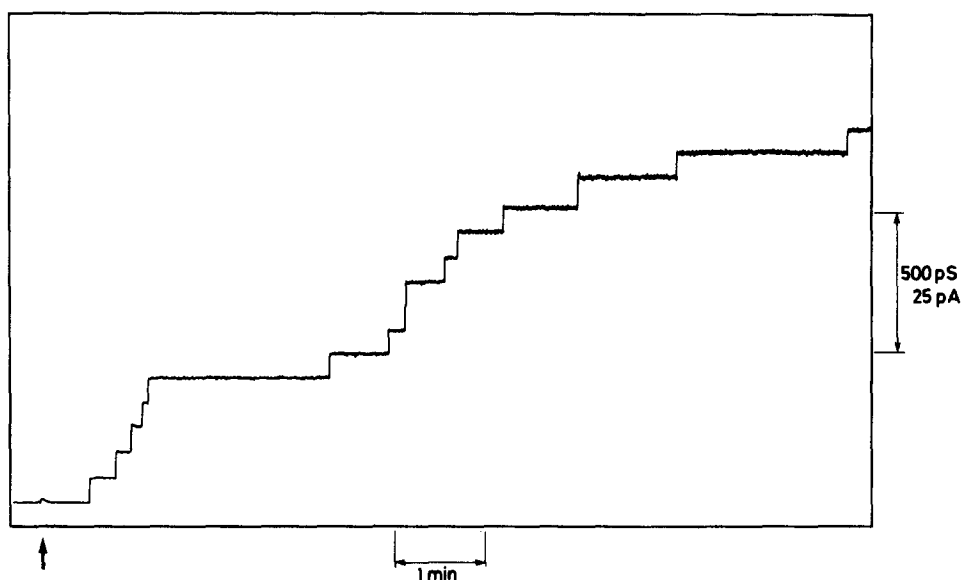


FIGURE 2. Stepwise increase of the membrane current in the presence of 1 ng/ml protein P of *P. aeruginosa* added to the aqueous phase containing 0.1 mM KBr, pH 6; $T = 25^\circ\text{C}$. The membrane was formed from diphytanoyl phosphatidylcholine/*n*-decane; $V_m = 50$ mV. The arrow indicates the blackening of the membrane.

it cannot be ruled out that divalent CO_3^{2-} (present in very small concentrations in the HCO_3^- solutions) blocked the channel, since divalent and trivalent anions show strong interactions with the selectivity filter (Benz, R., and R. E. W. Hancock, unpublished results).

Most porins form channels in which the ions move in a fashion similar to their movement through the aqueous phase (Benz et al., 1980, 1985; Benz and Hancock, 1981; Benz, 1984, 1985). In the case of the protein P channel, the single channel conductance was not dependent on the type of cation present in the aqueous phase (Table I). In the presence of 100 mM Cl solutions, the single channel conductance was ~ 160 pS, irrespective of the size and nature of the cation. Furthermore, the single channel conductance was found to be independent of the ionic strength of the aqueous phase if the anion concentration was

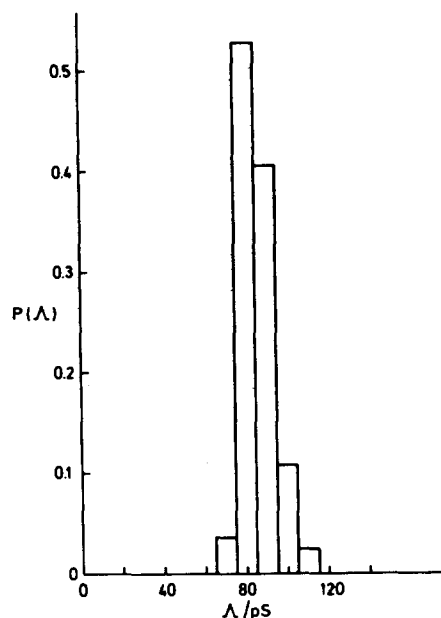


FIGURE 3. Histogram of the conductance steps observed with diphytanoyl phosphatidylcholine/*n*-decane membranes in the presence of protein P. The average single channel conductance was 86 pS for 180 steps. The aqueous phase contained 0.1 M KBr; $T = 25^\circ\text{C}$; $V_m = 50$ mV.

kept constant (Table I). This result indicated that cations are repelled from the selectivity filter and cannot enter the pore. This is consistent with previous measurements of zero-current potentials for protein P in the presence of KCl (Benz et al., 1983).

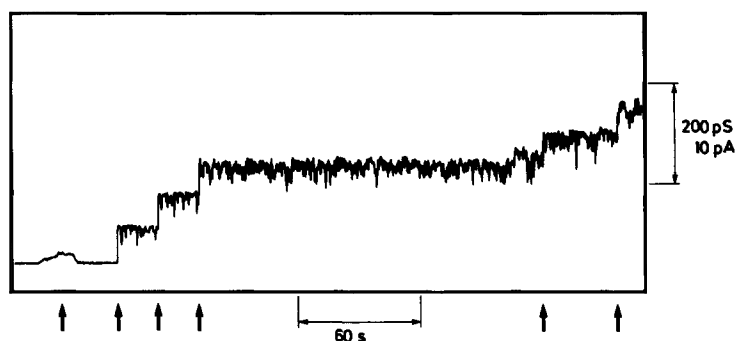


FIGURE 4. Single channel recording of protein P channels in the presence of 1 M KHCO_3 , pH 8; $T = 25^\circ\text{C}$. The membrane was formed from diphytanoyl phosphatidylcholine/*n*-decane. Note that the current noise increased with the number of channels incorporated into the membrane; $V_m = 50$ mV. The leftmost arrow indicates the blackening of the membrane. The others correspond to the opening of the pores.

Positively charged groups are presumably responsible for the anion selectivity of the protein P channel (Hancock et al., 1983). They form a binding site inside the pore that results in saturation of the single channel conductance with increasing anion concentration, as previously shown for Cl^- (Benz et al., 1983). Table II shows the single channel conductance as a function of the anion concentration for the halides and some other anions.

The data given in Table II could be reasonably well fitted to Eq. 5. This result indicated that a one-site, two-barrier model demonstrates the minimum requirements for the description of anion transport through the protein P channel. Fig. 5 shows an Eadie-Hofstee plot of the single channel conductances observed vs. salt concentration with F, Cl, and Br. The stability constant, K , for the binding of the halides to the binding site was found to be largest for Cl, whereas $\Lambda_{0,\text{max}}$ was maximal for F (Table III) and minimal for I. Thus, the $\Lambda_{0,\text{max}}$ followed the

TABLE I
Average Single Channel Conductance, $\bar{\Lambda}$, of the
Protein P Channel as a Function of a Variety of Salt Solutions

Salt	c_M mM	$\bar{\Lambda}$ pS	n
LiCl	100	165	85
KCl	100	159	127
CsCl	100	163	125
Choline Cl	100	156	104
Tris Cl	100	148	94
$\text{N}(\text{C}_2\text{H}_5)_4\text{Cl}$	100	155	122
KCl	30 (30)	102	202
CaCl_2	15 (45)	100	93
AlCl_3	10 (60)	98	72

The membranes were formed from 1% diphytanoyl phosphatidylcholine/*n*-decane; $T = 25^\circ\text{C}$; $V_m = 50$ mV. The pH of the unbuffered aqueous solutions was ~ 6 . n is the number of events used for the calculation of $\bar{\Lambda}$. The ionic strength is given in parentheses.

Eisenman sequence AVII, whereas the stability constant followed the sequence AVI (Eisenman, 1965; Diamond and Wright, 1969). Both Eisenman sequences are consistent with a closely spaced, positive binding site inside the channel.

Single Channel Conductance and Ion Radius

Many more monovalent anions are available than monovalent cations. These anions can be used to probe the diameter of the protein P channel. Table IV shows the single channel conductance of a number of different anions. The anion concentration was always 0.1 M in these experiments and the corresponding cation was, in all cases, K^+ . The pH of the unbuffered aqueous solutions was 6, unless otherwise indicated. Many anions were found to be permeable through the channel. However, single channel measurements could not be performed with large anions like HEPES^- and $\text{C}_3\text{H}_2\text{COO}^-$, since, for these ions, the single channel conductance was below 1–2 pS, the resolution of our single channel

instrumentation. The diameter of the selectivity filter of the protein P pore was found to be between 0.5 and 0.7 nm, as estimated from the observation that ClO_3^- (ionic radius, 0.20 nm; Stern and Amis, 1959), H_2PO_4^- , and BrO_3^- were permeable through the channel, whereas ClO_4^- ($r = 0.236$ nm), IO_3^- , and BF_4^- were not.

TABLE II
Average Single Channel Conductance, \bar{A} , of the Protein P Channel
as a Function of the Anion Concentration for Different Salts

Salt	c_M	\bar{A}
	mM	pS
KCl	3,000	270
	1,000	260
	300	245
	100	159
	30	102
	10	49
	3	17
	1	6
KF	3,000	466
	1,000	415
	300	240
	100	136
	30	50
KBr	10	17
	3,000	245
	1,000	223
	300	165
KI	100	86
	10	12
	1,000	64
	300	29
KNO ₂	100	13
	30	4
	3,000	243
	1,000	208
	300	153
KNO ₃	100	83
	30	37
	10	10
	1,000	94
	300	49
KHCOO (pH 7)	100	24
	30	7
	3,000	275
	1,000	234
	300	142
	100	75
	10	9

The membranes were formed from 1% diphytanoyl phosphatidylcholine/*n*-decane; $T = 25^\circ\text{C}$; $V_m = 50$ mV. The pH of the unbuffered aqueous solutions was 6 unless indicated otherwise. \bar{A} was calculated as the average of at least 70 single events. The data for Cl were taken from Benz et al. (1983).

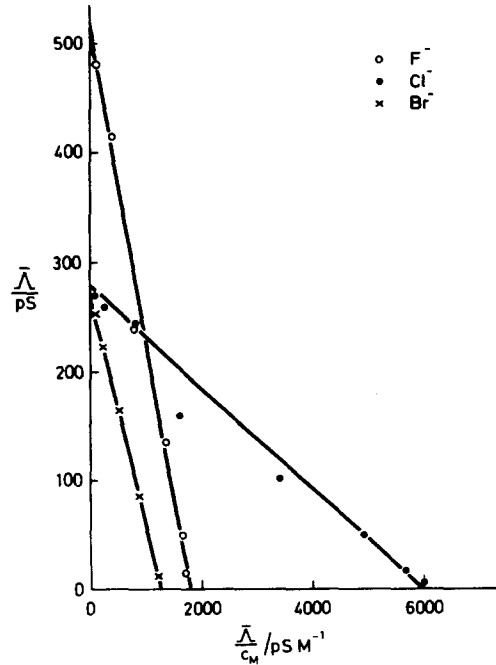


FIGURE 5. Eadie-Hofstee plot of the concentration dependence of the single channel conductances given in Table II for F, Cl, and Br. The straight lines in the figure were drawn by linear regression of the data points and yielded the stability constants and maximum single channel conductances given in Table III.

Unfortunately, the radii of only a few anions are available from the literature. Thus, it was possible to plot the single channel conductance as a function of the van der Waals radius (Pauling, 1960; Stern and Amis, 1959; Fig. 6) for only a few of the anions tested. The radii of some of the others were derived from

TABLE III
Stability Constant, K , and Maximum Single Channel Conductance, $\Lambda_{0,max}$

Anion	K	$\Lambda_{0,max}$
	M^{-1}	pS
F ⁻	3.5	515
Cl ⁻	20	280
Br ⁻	4.7	265
I ⁻	1.3	110
NO ₂ ⁻	4.6	263
NO ₃ ⁻	2.0	140
HCOO ⁻ (pH 7)	3.0	310
CH ₃ COO ⁻ (pH 8)*	2.5	70
N ₃ ⁻ (pH 8)*	5.9	205

Values were derived from a fit of the data given in Table II to Eq. 6 using Eadie-Hofstee plots (see Fig. 5). The pH was 6 unless otherwise indicated. The data denoted with an asterisk were derived from experimental results at 1 M and 10⁻¹ M only (not included in Table II).

space-filling models. The data shown in Fig. 6 were consistent with the above assumption that the limiting diameter of the porin protein P channel is between 0.5 and 0.7 nm. Thus, the diameter of this channel is considerably smaller than those of the general diffusion porins (Benz et al., 1985; Benz, 1985, 1986).

pH Dependence of Single Channel Conductance

In a previous publication (Hancock et al., 1983), we showed that lysines are presumably responsible for the anion selectivity of the protein P channel. In addition, chemical cross-linking indicated that the active form of protein P is

TABLE IV
Average Single Channel Conductance, $\bar{\Lambda}$, of the Protein P Channel of *P. aeruginosa* as a Function of Different Anions

Anion	$\bar{\Lambda}$	r
	ρS	nm
F ⁻	136	0.133*
Cl ⁻	159	0.181*
Br ⁻	86	0.195*
I ⁻	13	0.216*
NO ₂ ⁻	83	—
NO ₃ ⁻	24	0.198 [‡]
ClO ₃ ⁻	23	0.200 [‡]
ClO ₄ ⁻	<2	0.236 [‡]
BrO ₃ ⁻	14	0.214 [‡]
IO ₃ ⁻	<2	0.225 [‡]
HCO ₃ ⁻ (pH 8.5)	35	0.133 [‡]
CN ⁻ (pH 10)	7	—
SCN ⁻ (pH 7)	13	0.21 [‡]
OCN ⁻ (pH 7)	44	0.17 [‡]
N ₃ ⁻ (pH 8)	76	—
HCOO ⁻ (pH 7)	75	0.18 [‡]
CH ₃ COO ⁻ (pH 8)	14	0.21 [‡]
C ₂ H ₃ COO ⁻ (pH 8)	3	0.25 [‡]
BF ₄ ⁻ (pH 7)	<2	0.28 [‡]
HEPES ⁻ (pH 9)	<2	—

All anions were present at a concentration of 0.1 M. The corresponding cation was, in all cases, K⁺. The membranes were formed from diphytanoyl phosphatidylcholine/*n*-decane; $T = 25^{\circ}C$; $V_m = 50$ mV. The aqueous pH was 6 unless otherwise indicated. r was the anion radius according to Pauling (1960),* Stern and Amis (1959),[‡] or from model building using CPK Precision Molecular Models (Ealing Co., Watford, England).[‡]

formed by three identical polypeptide subunits (Angus and Hancock, 1983). This means that at least three lysines could be involved in the selectivity filter if one channel per trimer is assumed. Three lysines in a closely spaced environment should lead to different pK_a values for the individual lysines. To test this hypothesis, we performed single channel experiments at 30 and 300 mM KCl as a function of the aqueous pH. The aqueous solutions were used either unbuffered or buffered with cationic buffers like Tris⁺ to avoid any direct influence of the buffer molecules on the channel properties.

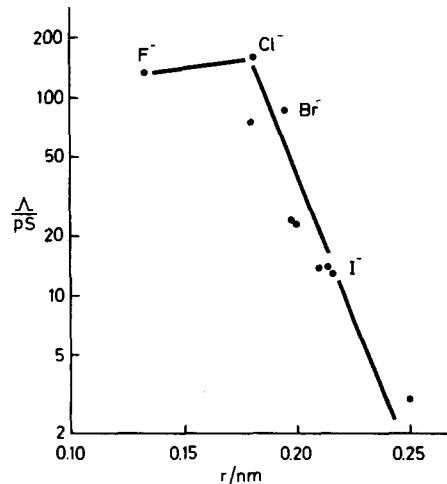


FIGURE 6. Single channel conductance of the protein P channel in 0.1 M salt solution as a function of the anion radii. The data were taken from Table IV.

In confirmation of previous experiments performed at 300 mM KCl (Hancock et al., 1983), the average single channel conductance, $\bar{\Delta}$, was found to be a function of the aqueous pH at both 30 and 300 mM KCl (Fig. 7). $\bar{\Delta}$ decreased with increasing pH from 3 to 5. Between pH 5 and pH 7, the single channel conductance was approximately independent of pH. Above pH 7, $\bar{\Delta}$ decreased again. Since the channel remained anion selective in the whole pH range (see below), we believe that the data in Fig. 7 may reflect how many charges were involved in the selectivity filter (see Discussion).

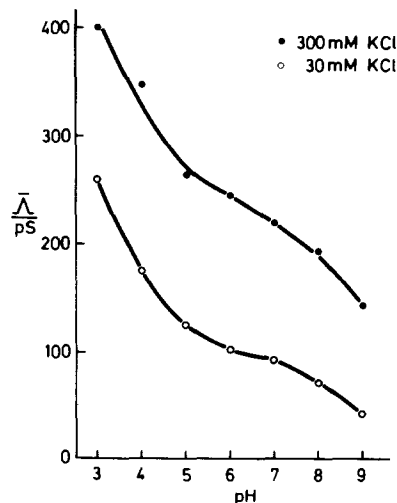


FIGURE 7. Average single channel conductance of the protein P in 30 and 300 mM KCl as a function of the aqueous pH. The membranes were formed from diphtanoyl phosphatidylcholine/*n*-decane; $T = 25^\circ C$; $V_m = 50$ mV.

TABLE V
 Maximum Single Channel Conductance, $\Delta_{0,\max}$, and Stability Constant, K , for
 Cl^- Transport through the Protein P Channel as a Function of the Aqueous pH

pH	$\Delta_{0,\max}$	K
	pS	M^{-1}
3	420	60
4	390	28
5	300	24
6	280	20
7	260	18
8	240	14
9	190	10

$\Delta_{0,\max}$ and K were obtained by a fit of the data given in Fig. 7 to Eq. 5. $T = 25^\circ\text{C}$;
 $V_m = 50 \text{ mV}$.

Fig. 7 clearly confirms that pH influenced the transport parameters. This could also be observed by calculating $\Delta_{0,\max}$ and K from the data of Fig. 7 using Eq. 5 (Table V). K decreased about sixfold between pH 3 and pH 9, whereas $\Delta_{0,\max}$ decreased more than twofold over this pH range.

Current-Voltage Curves

The current-voltage behavior of diphytanoyl phosphatidylcholine/*n*-decane membranes in the presence of small amounts of porin protein P was investigated (Fig. 8). Current-voltage curves were obtained in 0.1 M KCl, with six pores incorporated into the membrane (curve I, Fig. 8), and in 10^{-2} M KCl, with 14 pores (curve II, Fig. 8). The membrane current observed in both cases was approximately a linear function of the applied voltage up to 120 mV when these voltages were maintained for ~ 5 s. For larger voltages, the current-voltage

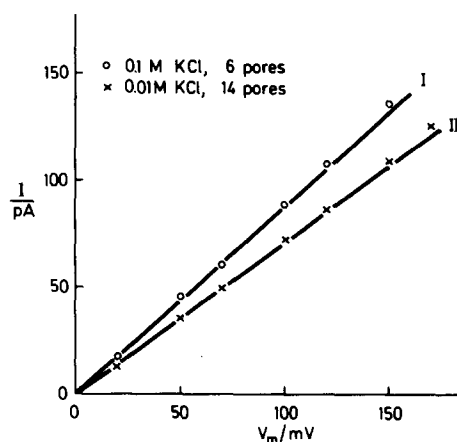


FIGURE 8. Current-voltage curves of diphytanoyl phosphatidylcholine/*n*-decane membranes in which 6 protein P channels (curve I, 0.1 M KCl) or 14 protein P channels (curve II, 10^{-2} M KCl) were incorporated. The aqueous pH was 6; $T = 25^\circ\text{C}$.

curves became slightly superlinear (Fig. 8). This result suggested that the protein P pores were not voltage-gated up to 150 mV. Substates of the pores were occasionally observed but only at applied voltages >200 mV.

Zero-Current Membrane Potentials

In a previous publication (Benz et al., 1983), we reported that protein P forms highly Cl⁻-selective channels at pH 6. Here we studied in more detail the pH dependence of the selectivity and measured zero-current membrane potentials in the presence of anions other than Cl⁻. The zero-current membrane potentials were always found to be negative on the more dilute side of the membranes. This result indicated that protein P formed in all cases anion-selective pores. The results are summarized in Table VI. The zero-current membrane potentials ranged between -58 and -55 mV for a 10-fold salt gradient. Analysis of the

TABLE VI
Zero-Current Membrane Potentials, V_m , of Membranes from Diphytanoyl Phosphatidylcholine/n-Decane Membranes in the Presence of Protein P Measured for a 10-fold Gradient of Different Salts

Salt	pH	V_m mV
KCl	3	-56
"	6	-58
"	9	-57
KF	6	-56
KBr	6	-57
KNO ₂	6	-56
KNO ₃	6	-55
KHCOO	7	-57
KCH ₃ COO	8	-56

V_m is defined as the difference between the potential on the dilute site (10^{-2} M) and the potential at the concentrated side (10^{-1} M). Means of at least three membranes are given; $T = 25^\circ\text{C}$.

data of Table VI using the Goldman-Hodgkin-Katz equation (Benz et al., 1979) suggested that cations also have a certain permeability through the protein P channel because the ratios of the permeabilities P_a for anions and P_c for cations ranged between ~60 and 250. However, control experiments with the ideal K⁺-selective valinomycin system also resulted in a smaller zero-current membrane potential of 56 mV (mean of six membranes) for a 10-fold K⁺ gradient instead of the expected 59 mV (Benz, R., unpublished results). The results of the control experiments indicated that a potential smaller than the expected -59 mV does not mean that the protein P channel is permeable for cations at an appreciable rate. The reduction of the potential is most probably caused by the flux of water through the membranes, which lowers the salt gradient across the membranes (Benz, R., unpublished results). On the other hand, it cannot be completely ruled out that the discrepancy is created by the use of ion concentrations instead of their activities.

In another set of experiments, we measured bi-ionic potentials. In these experiments, protein P-containing membranes separated 0.1 M salt solutions containing either F^- , Br^- , NO_2^- , NO_3^- , or CH_3COO^- from a salt solution containing 0.1 M Cl. Table VII shows the results of these experiments together with the permeability ratio, P_{Cl}/P_a , calculated from the bi-ionic potential, V_m , according to the equation:

$$V_m = -\frac{RT}{F} \ln \frac{P_{Cl}c_{Cl}}{P_a c_a}, \quad (7)$$

using $c_{Cl} = c_a = 0.1$ M. The permeability ratios show some analogy to the ratios of the single channel conductances and to the ratio k_1/k_2 of the corresponding anions (see Discussion).

TABLE VII
Bi-ionic Potentials of Membranes from Diphytanoyl Phosphatidylcholine/n-Decane Membranes in the Presence of Protein P Measured in 0.1 M Asymmetric Salt Solutions

Anion on the anionic side	V_m mV	P_{Cl}/P_a	$\bar{\Lambda}_{Cl}/\bar{\Lambda}_a$	k_1^Cl/k_1^a
F^-	-21	2.2	1.2	3.0
Br^-	-29	3.1	1.9	4.5
NO_2^-	-31	3.3	1.9	4.6
NO_3^-	-61	11	6.6	20
$HCOO^-$	-33	3.6	2.1	6.0

V_m is defined as the difference between the potential on the anionic side and the potential on the Cl side. P_{Cl}/P_a was calculated from Eq. 7. Means of at least three membranes are given; $T = 25^\circ C$. The ratios of the corresponding single channel conductances and of the corresponding rate constants, k_1 (Table VIII), are given for comparison.

DISCUSSION

In this study, we confirmed that protein P from the outer membrane of *P. aeruginosa* forms highly anion-selective pores in lipid bilayer membranes. Cations had no access to the pore, as demonstrated by the observation that variations in the ionic strength caused by the valence of the cations had essentially no effect on the single channel conductance of 0.1 M Cl solution (see Table I). Furthermore, the size of the cation had no influence whatsoever on the single channel conductance. On the other hand, the channel was permeable for a large number of different monovalent anions, which seemed to move through the channel with conductances related to their size.

We propose here a one-site, two-barrier model for the mechanism of anion transport through the protein P channel. This model seems justified by the experimental result that the conductance vs. concentration relations measured for a variety of different anions always showed saturation and never a maximum, followed by a decrease at higher anion concentrations. Thus, in all cases, the

experimental data fitted well to Eq. 5. It is also evident that a two-site, three-barrier model would have provided a good fit of the experimental data if we had assumed that only one site of both binding sites is occupied at a given time, as has been shown for the well-studied gramicidin channel using the small cations Li^+ and Na^+ (Andersen, 1983; Hladky and Haydon, 1972; Urban et al., 1980). We consider such a possibility rather unlikely for two reasons. First, any additional barrier would tend to lower the rate of ion flux through the channel and we could not explain the rather high observed flux of ions through the protein P pore. Second, even in the case of the rather narrow gramicidin A channel, the second site becomes occupied at high concentrations of K^+ , Rb^+ , and Cs^+ (Hladky and Haydon, 1972; Finkelstein and Andersen, 1981), and it is difficult to imagine why this should not be the case for the larger protein P channel. On the other hand, the permeability ratio $P_{\text{Cl}}/P_{\text{a}}$, calculated from the bi-ionic potentials (Table VI), does not completely coincide with the ratio k_1/k_2 , as predicted by the theory

TABLE VIII
Rate Constants k_1 and k_2 as Calculated from the
Data of Table III Using Eq. 6 and $K = k_1/k_2$

Anion	k_1 $10^7 \text{ M}^{-1} \text{ s}^{-1}$	k_2 10^7 s^{-1}
F^-	60	17
Cl^-	180	9.0
Br^-	40	8.5
I^-	4.6	3.5
NO_2^-	39	8.4
NO_3^-	9.0	4.5
HCOO^- (pH 7)	30	10
CH_3COO^- (pH 8)	5.5	2.2
N_3^- (pH 8)	35	6.6

(Läuger, 1973). So far it is not clear whether this result is caused by an asymmetry of the barriers of the protein P channel or by additional barriers.

Table VIII shows the values of k_1 and k_2 calculated according to Eqs. 5 and 6 from the data given in Table II. The rate constant for the jump from the external solution to the binding site inside the channel, k_1 , had a maximum for Cl, whereas the inverse reaction rate, k_2 , was largest for F. At low anion concentrations, the specificity of the protein P channel followed the Eisenman sequence AVI (without At^- , Eisenman, 1965; Diamond and Wright, 1969). On the other hand, the limiting single channel conductance, $\Lambda_{0,\text{max}}$, followed the Eisenman sequence AVII. Both results are consistent with the assumption that the selectivity filter inside the channel is closely spaced and contains positively charged groups. In this case, the selectivity follows the size of the ions. For F only, the hydration energy seems to play a certain role, which may indicate that the anions become partially dehydrated while entering the selectivity filter.

This publication represents the first complete study of anion permeation through a highly anion-selective channel. The Cl^- channel reconstituted from the electroplax of *Torpedo* into lipid bilayer membranes allows the resolution of

single channel recordings only in Cl and Br (White and Miller, 1981; Tank et al., 1982; Hanke and Miller, 1983) and the protein has not been isolated and purified. The selectivity of the GABA and glycine receptors from nerve tissue has the permeability sequence $\text{Br}^- > \text{J}^- > \text{Cl}^- > \text{F}^-$ (Hamill et al., 1983; Eisenman sequence AII, Eisenman, 1965), while the conductance sequence is AV. This result is very confusing and cannot be understood on the basis of the simple model presented here, in which the permeability sequence and conductance sequence should be similar. On the other hand, it cannot be ruled out that a rather complicated model is valid for the ion transport through the GABA and glycine receptors. The selectivity sequence found here for the protein P channel ($\text{Cl}^- > \text{F}^- > \text{B}^- > \text{I}^-$) has also been described for the permeability of muscle membranes for anions (Edwards, 1982).

The single channel conductance of the protein P channel in 0.1 M Cl solution is ~ 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) (Moore and Pearson, 1981; Hille, 1984). Nevertheless, we did not observe any indication of diffusion limitations even at 1 mM Cl, where the flux of ions would be far above the diffusion limitation of a sink of 0.3 nm radius (Luger, 1976). Thus, the protein P channel cannot be long and narrow. The channel has to be rather short to account for the large ion flux at low anion concentrations, in strong support of the one-site, two-barrier model. Furthermore, the channel would have to have large capture radii, which would be positively charged and would thus form sinks for anions. Similar arrangements have also been discussed for K^+ channels with extremely large single channel conductances (Marty, 1981; Latorre and Miller, 1983; Moczydlowski and Latorre, 1983; Vergara and Latorre, 1983; Auerbach and Sachs, 1984). In this respect, it is interesting to note that the 2-nm-wide protein F channel of the *P. aeruginosa* outer membrane has the same single channel conductance at 1 mM KCl as the 0.5–0.7-nm-wide protein P channel (Benz and Hancock, 1981; see also Fig. 9).

Concerning the number of charged groups involved in the selectivity filter, we can only speculate. The channel-forming unit is a trimer of identical subunits (Angus and Hancock, 1983). The trimeric structure of the channel would in principle require that three charges or multiples of three charges, most probably lysines (Hancock et al., 1983), be involved in the selectivity filter. However, these lysines would be very close to one another and would thus presumably not all be charged at neutral pH. The pH dependence of the single channel conductance supports this view and could be interpreted by assuming that three lysines are present in the selectivity filter, three are charged at acid pH, two at neutral pH, and one at alkaline pH. In fact, the chemical modification of lysines by acetylation resulted in a substantial decrease of the single channel conductance and in the absence of any ion binding (Hancock et al., 1983; see also Fig. 9). The decreased single channel conductance was presumably only partially due to steric hindrance by the acetyl groups and probably reflected, to a greater extent, the absence of the binding site. This would mean that the binding site and its ready accessibility for anions plays an important role in ion permeation through the protein P channel.

The current-voltage curves were found to be linear up to ~ 150 mV. Our simple model would in principle predict a somewhat stronger voltage dependence and more superlinear current-voltage relationships than that demonstrated in Fig. 8. This result could mean that more than one binding site or two barriers are involved in ion transport. In fact, Barry and Gage (1984) have presented evidence that the current-voltage curve of channels becomes more and more linear as the number of sites (and barriers) is increased. However, as we have already discussed above, we consider the existence of more than one site in the protein P channel rather unlikely, although we cannot exclude such a model on the basis of our experimental results. If we assume that the barriers are not located midway between the binding site and membrane-solution interface but are both shifted toward the binding site, the one-site, two-barrier model could

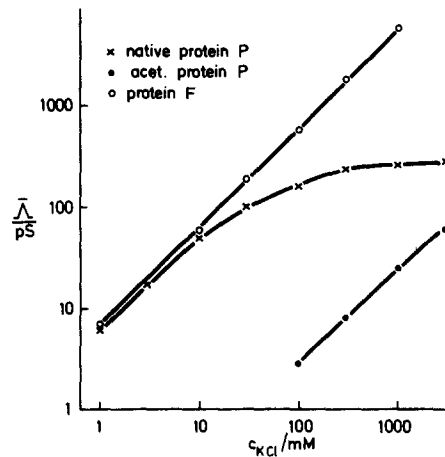


FIGURE 9. Single channel conductance of the protein F pore, the protein P channel, and the acetylated protein P channel as a function of the KCl concentration in the aqueous phase. The data for the protein F pore of *P. aeruginosa* were taken from Benz and Hancock (1981), and the data for the acetylated protein P channel were taken from Hancock et al. (1983). The aqueous pH was 6; $T = 25^\circ\text{C}$. The curve for protein P was calculated according to Eq. 5 assuming $K = 20 \text{ M}^{-1}$ and $\Lambda_{0,\text{max}} = 280 \text{ pS}$.

also account for a linear current-voltage curve. The stability constant would in this case be voltage dependent, and linear current-voltage curves are expected for $Kc_M > 1$ and voltages up to 150 mV. For $Kc_M < 1$, the current-voltage curves would become superlinear, which we did not observe (see Fig. 8). Thus, we have to consider this possibility rather unlikely. Another possible explanation for a linear current-voltage curve consists of an assumption of trapezoidal barriers instead of Eyring barriers. Hladky (1974) has presented evidence that trapezoidal barriers lead to a reduced voltage dependence of carrier-mediated ion transport and of the transport of lipophilic ions as compared with that of an Eyring barrier (Benz and McLaughlin, 1983). Square barriers would lead to linear current-voltage curves even for very high voltages (see also Cooper et al., 1985). This would mean that the movement of the ions toward the binding site cannot be

described by a 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, which are close to those of diffusion-controlled reaction processes (Eigen et al., 1964).

Further interest arises from the study of the permeation of divalent and trivalent anions through the protein P channel and their interaction with the binding site. Large trivalent anions may lead to the complete blockage of the channel, as observed for citrate (Benz, R., and R. E. W. Hancock, unpublished results). Chemical acetylation experiments have already led to interesting insights into the properties of the selectivity filter (Hancock et al., 1983). Further chemical modifications of the channel-forming protein may give a more detailed picture of the channel interior and the topology of the binding site (Hancock and Benz, 1986).

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