

## Outer Membrane Protein P of *Pseudomonas aeruginosa*: Regulation by Phosphate Deficiency and Formation of Small Anion-Specific Channels in Lipid Bilayer Membranes

ROBERT E. W. HANCOCK,<sup>1\*</sup> KEITH POOLE,<sup>1</sup> AND ROLAND BENZ<sup>2</sup>

*Department of Microbiology, University of British Columbia, Vancouver, British Columbia, Canada V6T 1W5,<sup>1</sup> and Fakultät für Biologie, Universität Konstanz, D7750 Konstanz, West Germany<sup>2</sup>*

Received 24 September 1981/Accepted 30 November 1981

A new major outer membrane protein, P, was induced in *Pseudomonas aeruginosa* PAO1 upon growth in medium containing 0.2 mM or less inorganic phosphate. Studies with media containing different levels of phosphate and with mutants of PAO1 suggested that protein P was coregulated with alkaline phosphatase and phospholipase C. Protein P was substantially purified and shown to form sodium dodecyl sulfate-resistant oligomers on polyacrylamide gels. The incorporation of purified protein P into artificial lipid bilayers resulted in an increase of the membrane conductance by many orders of magnitude. Single-channel experiments demonstrated that protein P channels were substantially smaller than all previously studied porins from *P. aeruginosa* and enteric bacteria, with an average single-channel conductance in 1 M NaCl of 0.25 nS. The protein P channel was apparently not voltage induced or regulated. The results of single-channel conductance experiments, using a variety of different salts, allowed a minimum channel diameter estimate of 0.7 nm. Furthermore, from these results it was concluded that the protein P channel was highly specific for anions. Zero-current potential measurements confirmed that protein P was at least 30-fold more permeable for Cl<sup>-</sup> than for K<sup>+</sup> ions. The possible biological role of the small, anion-specific protein P channels in phosphate uptake from the medium is discussed.

The outer membranes of gram-negative bacteria act as molecular sieves with defined exclusion limits for hydrophilic substances (17). This property results largely from integral outer membrane proteins called porins, which form large water-filled pores through the hydrophobic core of the outer membrane (12, 17). Porins, when purified and reconstituted into model membrane systems, form pores with an approximate cross-sectional area between 1.2 and 3.8 nm<sup>2</sup> and generally have a two- to fourfold preference for cations over anions (3, 6; Benz, unpublished data). This weak cation selectivity probably results from the presence of negatively charged groups in or on the mouth of the pore. For many of the porins studied, this weak selectivity is reflected in the *in vivo* properties of the porin (17). In contrast, the *lamB* porin shows a distinct preference for maltose and maltodextrins *in vivo*, which can probably be explained on the basis of weak binding sites in or near the pore and the strong binding sites of the maltose binding proteins present on the periplasmic side of the outer membrane (9).

*Pseudomonas aeruginosa*, a gram-negative opportunistic pathogen, contains one major spe-

cies of porin, protein F, under most growth conditions. This protein has two unusual properties when compared with the porins of enteric bacteria. It forms substantially larger pores than enteric porins, allowing the passage of significantly larger saccharides (up to approximately 6,000 daltons [12]). Despite this, it has low *in vivo* activity, with only 100 to 300 of the approximately  $2 \times 10^5$  porin molecules in the outer membrane being apparently functional at any given time (1, 3).

Consequently, the total area of pores available for diffusion is considerably reduced in *P. aeruginosa* compared with enteric bacteria, thus markedly reducing the rate of diffusion of hydrophilic substances into the periplasm. This low outer membrane permeability explains the high natural resistance of *P. aeruginosa* to hydrophilic antibiotics (1).

In addition to the major porin protein F, protein D1, which is only observable in the outer membranes of cells grown on glucose or related inducers as sole carbon source, has been demonstrated to have porin activity (11). The results suggest that this protein is closely analogous to the *lamB* protein of *Escherichia coli*.

In this paper we show that *P. aeruginosa* cells grown on low-phosphate medium produce a new outer membrane protein, P, which is apparently coregulated with alkaline phosphatase and phospholipase C. We have purified the protein and report on its rather unique properties in black lipid bilayers.

#### MATERIALS AND METHODS

**Strains and media.** *P. aeruginosa* PAO1 strain H103 (10) was used for most experiments described below. In addition, a series of strains, A50N, A18N, and B13, deficient in the induction of alkaline phosphatase and phospholipase C on phosphate-deficient media were a kind gift of G. L. Gray (University of Colorado, Denver). These strains were all derived by nitrosoguanidine mutagenesis from a rough, lipopolysaccharide-altered mutant of *P. aeruginosa* PAO1.

The medium used for induction of protein P contained 0.5 mM MgSO<sub>4</sub>–0.1 M sodium *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonate (HEPES) (pH 7.0)–7 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>–20 mM potassium succinate (pH 7.0)–0.1% (vol/vol) trace ion mixture, as previously described (14), and either 0.2 mM potassium phosphate buffer (pH 7.0) for phosphate-deficient medium or 0.6 to 62 mM potassium phosphate buffer (pH 7.0) for phosphate-sufficient medium. For routine maintenance of cultures, 1% proteose peptone no. 2 medium was used.

**Cell fractionation and SDS-polyacrylamide gels.** Outer membranes were isolated as previously described without lysozyme pretreatment (10). The technique for isolation of periplasmic enzymes by MgCl<sub>2</sub> treatment of cells was as described by Cheng et al. (8). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed as previously described, using a 14% (wt/vol) acrylamide running gel (10).

**Purification of protein P.** The solubilization in Triton X-100–EDTA of outer membranes from phosphate-deficient cells and chromatography on a DEAE-Sephacel column was exactly as described previously for protein D1 purification (11). However, proteins P and F eluted in the same fractions from this column. Therefore, these fractions were pooled and concentrated fivefold by dialysis against 20% (wt/vol) polyethyleneglycol 20,000 (Pharmacia Fine Chemicals AB, Uppsala, Sweden). To this pooled concentrate a fourfold excess of SDS (over Triton X-100; i.e., 2% SDS) was added, and the solution was made 3 mM for sodium azide. This solution was added to a Sepharose 4B column (46 by 2 cm) preequilibrated with 0.1% SDS–5 mM Tris-hydrochloride (pH 8.0)–3 mM azide (column buffer) and eluted with column buffer. Three-milliliter fractions were collected at 12 ml/h and tested for absorbance at 280 nm and for protein composition on SDS-polyacrylamide gels. Protein P, slightly contaminated with protein F, eluted just after the void volume, whereas the Triton X-100 eluted in subsequent fractions. The protein P-containing fractions were pooled, concentrated, and reappplied to the same Sepharose 4B column as described above. The resultant protein P peak was free of contamination by protein F (Fig. 1). Protein Ic (e, *phoE*) was a crude preparation isolated from an *ompB nmpA* double mutant of *E. coli* K-12 (containing Ic as the only porin), as previously described (3).

**Black lipid bilayer experiments.** The methods used for black lipid bilayer experiments have been described previously in detail (3–5). The apparatus consisted of a Teflon chamber with two compartments connected by a small hole (0.1 to 2 mm<sup>2</sup>). A membrane was formed across the hole by painting on a solution of 1 to 2% (wt/vol) oxidized cholesterol in *n*-decane. Bilayer formation was indicated by the membrane turning optically black to incident light. Conductance through the pores was measured after application of a given voltage, using a pair of Ag-AgCl electrodes inserted into the aqueous solutions on both sides of the membrane. The current through the pores was boosted by a preamplifier, monitored by a storage oscilloscope, and recorded on a strip chart recorder. Zero-current potential measurements were performed as described previously (3).

#### RESULTS

**Regulation by phosphate deficiency.** To study the influence of phosphate deficiency on the cellular protein composition, a medium based on BM2 succinate medium (10) was devised. Sodium HEPES (0.1 M; pH 7.0) was used as a buffer since we had previously shown that Tris buffer (commonly used for phosphate-limited media) was able to permeabilize *P. aeruginosa* outer membranes (14). In this medium, the yield of cells was dependent on the level of phosphate added below approximately 0.62 mM, which was taken as phosphate-sufficient medium. This concentration of phosphate allowed normal doubling times (around 45 min), as well as normal membrane protein composition and alkaline phosphatase levels (60 fmol of pNPP reduced min<sup>-1</sup> mg of cells<sup>-1</sup>) (data not shown). Supplementation of the HEPES-buffered medium with 0.2 mM phosphate had little effect on the doubling time (49 min) but reduced the final yield of cells twofold and increased the cellular alkaline phosphatase levels nearly three orders of magnitude to 33 pmol of pNPP reduced min<sup>-1</sup> mg of cells<sup>-1</sup>. Further reduction in phosphate supplementation caused a decrease in both cell yields and doubling times, but did not increase and in fact slightly decreased the levels of alkaline phosphate induced. Thus, in subsequent experiments medium containing 0.2 mM phosphate was used as phosphate-deficient medium. In the HEPES-based phosphate-deficient medium, little or no leakage of alkaline phosphatase occurred during logarithmic-phase growth and the enzyme was cryptic; i.e., the enzymatic activity was greater in broken cells than in intact cells, suggesting that the alkaline phosphatase was primarily located in the periplasm.

Comparison by SDS-polyacrylamide gel electrophoresis of the whole-cell protein compositions of cells grown in phosphate-sufficient and -deficient media revealed three major polypeptides which were induced by phosphate deficiency.

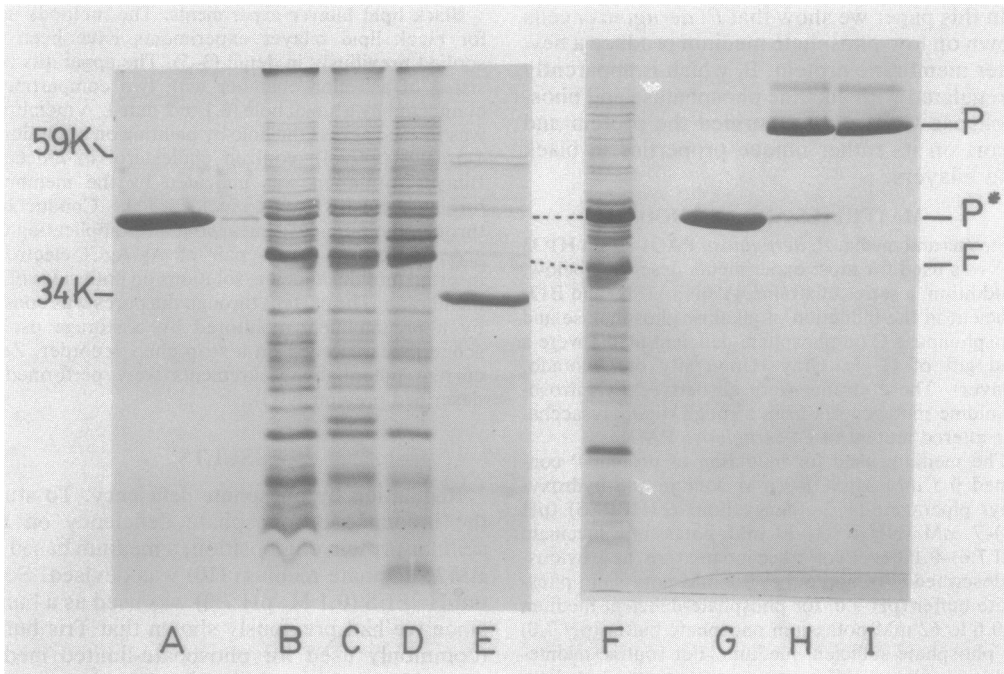


FIG. 1. SDS-polyacrylamide gel electrophoretogram of purified protein P and of outer membranes and  $MgCl_2$  washes of cells induced or not induced for P. Gels A and G, Purified protein P solubilized at  $75^\circ C$  (monomeric form of P is indicated as P\*); gels B and F, outer membranes of strain H103 grown on phosphate-deficient medium and harvested in mid- and late-logarithmic phases, respectively; gel C, outer membrane of strain H103 grown on phosphate-sufficient medium (the protein running at a slightly higher molecular weight than protein P is not heat modifiable); gel D, outer membrane of mutant strain B13; gel E,  $MgCl_2$  wash of H103 cells grown in phosphate-deficient medium (34K and 59K proteins are indicated); gels H and I, purified protein P solubilized at  $60$  and  $37^\circ C$ , respectively (oligomeric form of protein P is indicated as P; the small band running with a lower mobility than P may be aggregated oligomeric protein P). Unless otherwise stated, proteins were solubilized at  $88^\circ C$  before electrophoresis.

cy. Fractionation of cells revealed that two of these polypeptides of 59,000 and 34,000 daltons could be extracted from whole cells by 0.2 M  $MgCl_2$  treatment (Fig. 1, see gel E), suggesting they were periplasmic (8). Since alkaline phosphatase is periplasmic by the same criteria (8) and both alkaline phosphatase and phospholipase C are induced in low-phosphate media (8, 19), it seems possible that the above two polypeptides may be components of these two enzymes. An additional 48,000-dalton polypeptide, protein P (Fig. 1, gels B and F), was found in the outer membrane of cells grown on phosphate-deficient (0.2 mM) medium but not in cells grown on phosphate-sufficient medium (Fig. 1, gel C). It was found that the apparent molecular weight of protein P on SDS-polyacrylamide gel electrophoresis of whole outer membrane proteins was dependent on the temperature of solubilization (see below). By previously published criteria (13), it was not a peptidoglycan-associated protein.

Since we suspected from the above data that

protein P was coregulated with alkaline phosphatase and phospholipase C, we examined three mutants which were unable to respond to phosphate deficiency by induction of these two enzymes. All of these mutants lacked both the 59,000- and 34,000-dalton polypeptides (data not shown) and outer membrane protein P (Fig. 1, gel D) when grown on phosphate-deficient medium.

**Purification and properties of outer membrane protein P.** A highly purified preparation of protein P was obtained as outlined in Materials and Methods (Fig. 1, gels A and G). The apparent molecular weight on SDS-polyacrylamide gels of protein P after solubilization in SDS at  $75^\circ C$  was 48,000, corresponding exactly to the apparent molecular weight in outer membranes. However, solubilization of protein P in SDS at temperatures of  $<60^\circ C$  resulted in protein P running at an apparently higher molecular weight (Fig. 1, gels H and I). It seems likely that this form of the protein represents an oligomer, possibly a trimer by analogy with *E. coli* and *Salmonella typhi*-

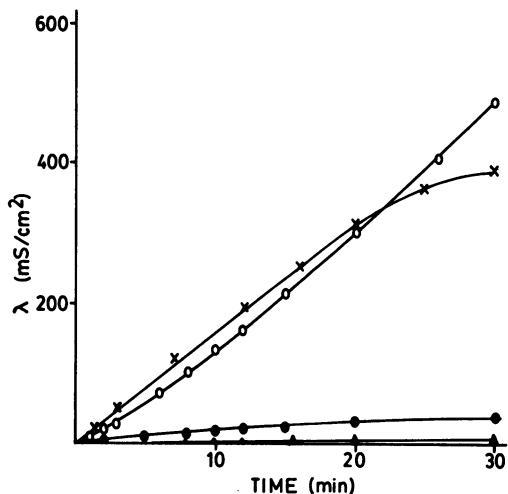


FIG. 2. Time course of increase of macroscopic conductance caused by the addition of 300 ng of protein F or 11.6 pg of protein P per ml to the aqueous solutions bathing a lipid bilayer membrane. Experiments were performed at 25°C, using membranes made from 1% oxidized cholesterol in *n*-decane, 0.1 M NaCl as the bathing solution, and an applied voltage of 10 (protein F) or 30 (protein P) mV. Symbols: ○, protein F; ●, protein F pretreated with 0.05% SDS for 10 s; ▲, protein F pretreated with 0.5% SDS for 1 min; ×, protein P (purified in the presence of SDS).

*murium* outer membrane porin proteins (16, 17), which behave quite similarly on SDS-polyacrylamide gels (16). Despite this apparent similarity to enteric porin proteins, we could clearly distinguish protein P from the previously described porins of *P. aeruginosa*, proteins F and D1. The most obvious difference was the lack of an oligomeric aggregate of proteins F and D1 after solubilization at low temperature; in fact, raising the temperature of solubilization increased the apparent molecular weight of proteins F and D1 (10).

**Lipid bilayer experiments.** Good separation of protein F and protein P was achieved by gel-sieving chromatography in SDS solution. Treatment of protein F with SDS caused dissociation of putative oligomeric pores, as judged by the appearance of the monomer form of protein F in SDS-polyacrylamide gels after solubilization at room temperature (10) and the loss of porin activity in the black lipid bilayer system after brief exposure to SDS (Fig. 2). In contrast, protein P oligomers were stable to SDS (Fig. 1), and the channel-forming properties described below were resistant to treatment with 0.5% (wt/vol) SDS for 10 min at 55°C.

**Macroscopic conductance measurements.** The conductance of lipid bilayer membranes increased at least three orders of magnitude when

purified protein P was added to the aqueous phase before membrane formation or after the membranes had turned black (Fig. 2). The increase in current continued steadily for 20 to 30 min and thereafter at a slower rate until membrane breakage. The increase in conductance presumably reflected self-assembly of protein P into the lipid bilayer membrane in a time-dependent process. In agreement with this, we observed that the rate of rise in conductance increased with increasing protein in concentration added to the aqueous phase. Protein P was two to three orders of magnitude more active than protein F in the bilayer system (in current per unit weight of protein (Fig. 2).

These experiments were performed with oxidized cholesterol since previous experiments (3–6) had demonstrated that the type of lipid used to form bilayers influenced the rate of incorporation of a variety of porins into the membrane bilayer, but did not affect the properties of the individual conductance unit.

Our previous results have suggested that porins are not voltage gated in that it does not require a fixed voltage to "open" porin pores (2–4), although Schindler and Rosenbusch (18), using a slightly different technique, have arrived at a different conclusion for matrix protein of *E. coli* B. A plot of the current measured versus the voltage applied for three different membranes (measured 30 min after addition of protein P to the aqueous phase) passed through zero current at zero voltage, suggesting that it did not require a given voltage to initiate a current (under such circumstances the current voltage curves would be expected to pass through the "switch on" voltage at zero current). Furthermore, it did not require application of a voltage across the membrane for protein P incorporation to occur, since the current level was approximately the same when a voltage of 10 mV was first applied 30 min after protein addition as when the voltage was continuously applied over the 30-min period.

**Single-channel experiments.** When purified protein P from a stock solution in SDS was added in small quantities (1 to 5 pg/ml) to the aqueous solutions bathing the membrane, membrane conductance started to increase in a stepwise fashion (e.g., Fig. 3). The occurrence of these steps was related to the presence of protein P since the steps were not observed with analogous SDS-treated fractions isolated from cells grown in phosphate-sufficient medium. By analogy with other porins (2–4, 6) the steps probably represent incorporation of single protein P oligomers into the membrane. The steps were not observed with concentrations of SDS 1,000-fold higher than those present in single-channel experiments. In addition, purified *P. aeruginosa* lipopolysaccharide and purified ma-

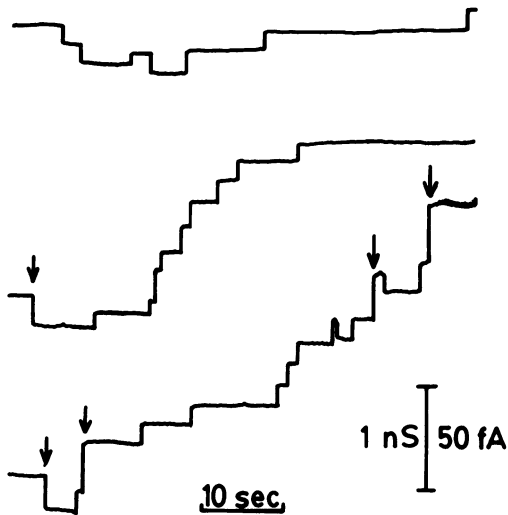


FIG. 3. Conductance steps caused by the addition of 5  $\mu\text{g}$  of protein P per ml to the aqueous solution (1 M KCl) bathing a small ( $0.1\text{-mm}^2$ ) lipid bilayer membrane formed of 1% oxidized cholesterol in *n*-decane. The applied voltage was 50 mV and the temperature was  $25^\circ\text{C}$ . Three separate sections from strip chart recordings of the conductance steps are shown. The arrows indicate larger steps caused by the simultaneous incorporation of two channel-forming units of protein P.

for outer membrane protein H1 or I failed to cause such step conductance increases.

The conductance increments were frequently directed upwards, although sometimes as many as 30% of the increments in a given experiment were terminating (i.e., conductance decrease) events. The nature of these terminating events is unclear but they could represent migration of protein P out of the bilayer membrane into the surrounding solvent-rich torus or into micro-lenses (solvent-rich bubbles) in the bilayer. The frequency of terminating events has been shown to be quite variable among the studied porins (3, 4, 6). From our traces we could estimate a channel lifetime in the order of 1 to 2 min, although this lifetime varied greatly with individual channels. Both initiating and terminating events were of the same magnitude (see below). When greater than 10 upwards-directed conductance increments occurred, the level of noise rose markedly, making it difficult to judge the size of the conductance step. Only rapid clearly identifiable increases (such as those shown in Fig. 3) were used for studying the properties of protein P.

As previously found for other porins (3, 4, 6), the single-conductance increments for protein P were distributed about a mean. Figure 4 shows a histogram observed for protein P in 1 M NaCl. Only 72% of the 502 conductance increments

measured apparently represented single channels entering the membrane. The remaining 28% of the conductance increments were distributed around means two-, three-, and fourfold greater than the mean observed for single channels (see Fig. 3 for examples of such events). The proportion of single channels observed varied from 71 to 89% for the salts described in Table 1. It was considered that the larger conductance increases represented two or more oligomers of protein P loosely associated with one another. In agreement with this, reducing the salt concentration of the solution bathing the membrane resulted in more than 50% of the steps being larger than the single-channel size, suggesting that aggregation was influenced by the ionic strength.

The average conductance for single channels of protein P in 1 M NaCl was 253 pS (Fig. 4; Table 1). This value was only 5.6% of the average conductance for pores formed by porin protein F of *P. aeruginosa* (3) and about 20% of the increase observed for the previously small-

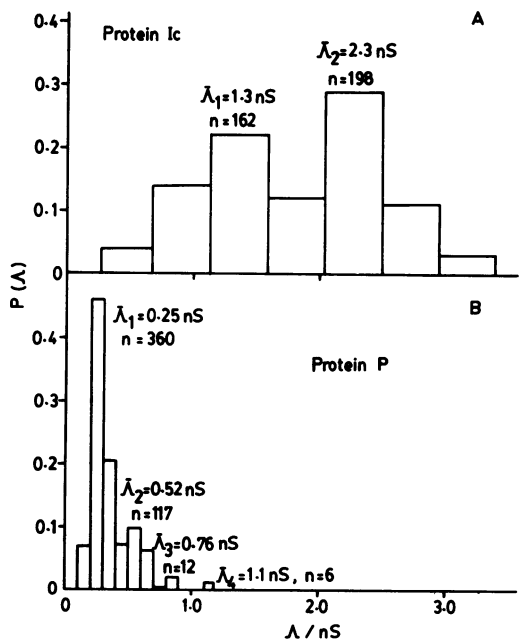


FIG. 4. Histogram of the conductance fluctuations observed with membranes from oxidized cholesterol dissolved in *n*-decane in the presence of protein Ic (e) of *E. coli* K-12 (Benz, unpublished data) (A) or protein P of *P. aeruginosa* (B). The aqueous phase contained 1 M NaCl (pH 6.5), the temperature was  $25^\circ\text{C}$ , and the applied voltage was 50 mV.  $P(\lambda)$  is the probability of a given conductance increment  $\lambda$  taken from recorder traces such as those shown in Fig. 3.  $\bar{\lambda}_1$ ,  $\bar{\lambda}_2$ , etc. demonstrate the mean conductances for single channels, groups of two channels entering the membrane simultaneously, etc.;  $n$  is the number of steps averaged to give the appropriate  $\bar{\lambda}$  values.

est outer membrane porin, protein Ib of *E. coli* K-12 (3). The data presented in Fig. 4 and Table 1 are thus consistent with the assumption that protein P channels are by far the smallest outer membrane channels demonstrated to date. An outer membrane protein Ic, analogous to protein P, has been recently described in *E. coli* K-12 (20). Its mode of induction, coregulation with alkaline phosphatase, and behavior on SDS-polyacrylamide gels were quite similar to protein P. However, the pores formed by crude preparations of protein Ic were significantly larger (average conductance, 1,300 pS) (Fig. 4). Interestingly enough, a significant number of protein Ic conductance increases apparently represented the simultaneous incorporation of two or more oligomers into the lipid bilayer membrane.

Single-channel conductance measurements were performed for a wide variety of salts (Table 1). When chloride was the anion and the cation was varied, the average channel conductance changed less than 40% despite a 10-fold range of cation radii. This suggested that the protein P channel was quite anion selective. This was further suggested by comparison of the results obtained with  $K^+ Cl^-$  and  $K^+ HEPES^-$  as the salts (Table 1). We were unable to observe even a small conductance increase when the large-ion HEPES was the anion, despite the addition to the bathing solution of concentrations of protein P 10-fold higher than those used to observe frequent conductance steps when KCl was the

bathing solution. To exclude the possibility that the HEPES anion was blocking protein P channels, we performed an experiment in which the concentration of HEPES was 1 M, that of  $K^+$  was 1.1 M, and that of  $Cl^-$  was 0.1 M (Table 1). Under these circumstances the size of the average conductance steps (36 pS) equalled those in the presence of 0.1 M KCl (38 pS). Thus, protein P channels were highly chloride or anion specific. The small differences in average channel conductance of the various chlorides noted in Table 1 can be partially explained on the basis of the differing ion activities (dissociation in water) of the salts.

To examine further the properties of protein P channels, we studied the single-channel behavior in the presence of anions of different sizes. In the series  $Cl^-$ ,  $NO_2^-$ ,  $NO_3^-$ , we observed progressively decreasing average single-channel conductance with increasing ionic size (Table 1). For the anions  $H_2PO_4^-$  and  $HPO_4^{2-}$ , an increase in macroscopic conductance could be observed but the individual conductance steps (<10 pS) could not be resolved with the apparatus used. However, larger conductance increases of varying magnitude were occasionally observed. Presumably these increases represented the simultaneous incorporation of large aggregates of protein P oligomers into the lipid bilayer. As noted above, no increases in either step or macroscopic conductance were observed with HEPES as the anion. Although most experi-

TABLE 1. Single-channel conductances of various salts through protein P channels<sup>a</sup>

Salt	Concn (M)	Avg conductance (pS)	Ion radius (nm)		n
			Anion	Cation	
LiCl	1.0	223	0.181	0.068	132
NaCl	1.0	253	0.181	0.097	360
KCl	1.0	238	0.181	0.133	763
KCl, pH 8.5	1.0	250	0.181	0.133	116
RbCl	1.0	260	0.181	0.147	79
CsCl	1.0	228	0.181	0.167	142
NH <sub>4</sub> Cl	1.0	225	0.181	0.188	142
Tris <sup>+</sup> Cl <sup>-</sup>	1.0	189	0.181	0.67	161
MgCl <sub>2</sub>	0.5	166	0.181	0.066	287
NaNO <sub>2</sub>	1.0	111	0.20	0.097	379
KNO <sub>3</sub>	1.0	32	0.24	0.133	123
K <sub>2</sub> SO <sub>4</sub>	1.0	<10	0.26	0.133	
K <sub>2</sub> HPO <sub>4</sub> , pH 9.0	1.0	<10	0.28	0.133	
KH <sub>2</sub> PO <sub>4</sub> , pH 5.0	1.0	<10	0.28	0.133	
K <sup>+</sup> HEPES <sup>-</sup>	1.0	<2	1.4 × 0.6 × 0.5	0.133	
KCl	0.1	38	0.181	0.133	244
KCl and K <sup>+</sup> HEPES <sup>-</sup>	0.1 } 1.0 }	36 }	0.181 } 1.4 × 0.6 × 0.5 }	0.133	105

<sup>a</sup> Average conductance increment was measured on membranes from oxidized cholesterol-*n*-decane in the presence of protein P. The pH of the salt solutions was between 6 and 7 unless otherwise indicated. The applied voltage was 50 mV and the temperature was 25°C. The average conductance increment was determined by recording a large number (n) of conductance steps and drawing a histogram (Fig. 4). The single-conductance increases were then averaged. The ion radii were taken from published data or established by model building, using CPK Precision Molecular Models (Ealing Co., Watford, England).

ments were performed at a pH of 6 to 7, raising the pH to 8.5 had little or no effect on the single-channel conductance in KCl.

The channels were apparently symmetrical in that switching the voltage from +50 to -50 mV produced an equivalent current of opposite sign through the channels. In addition, the average current per channel was directly proportional to the applied voltage (Fig. 5); i.e., the conductance (current per unit voltage) and thus the properties of the individual channel were independent of the voltage across the membrane. As expected for a small channel, the conductance through protein P channels was saturable at higher salt concentrations such that the average conductance changed only 10% between 1 and 3 M KCl.

**Zero-current potential measurements.** After the incorporation of protein P into oxidized cholesterol in the presence of 0.1 M KCl, the concentration of KCl on one side of the membrane was increased, and the steady-state potential ( $V_m$ ) caused by the balancing of the osmotic potential difference across the membrane according to the specific properties of the protein P channel was measured. This potential was found to be negative on the more dilute site of the membrane, implying that the channel was anion selective (i.e., anions were preferentially migrating to the dilute side). The results for different applied concentration gradients ( $c''/c'$ ) measured

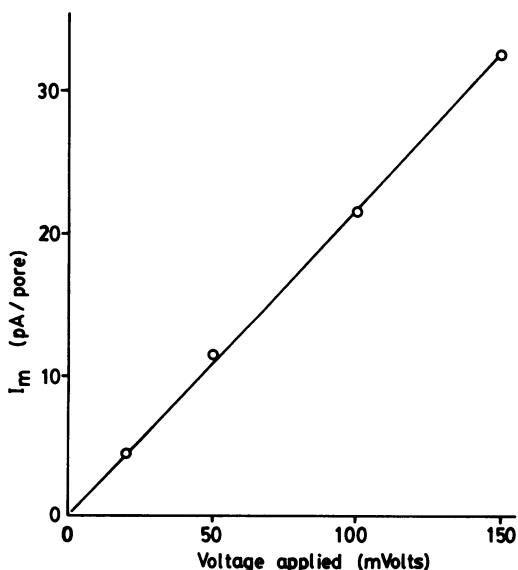


FIG. 5. Relationship between the mean current through single channels ( $I_m$ ) averaged for a large number of steps ( $n = 135$  to 320) and the voltage applied across the membrane. Otherwise conditions were the same as noted in the Fig. 3 legend.

on five separate membrane are presented in Fig. 6. Also illustrated are theoretical lines drawn by using the Goldman-Hodgkin-Katz equation, assuming that the permeability ratio for anions and cations ( $P_a/P_c$ ) across protein was either 30 or infinite. This latter case ( $P_a \gg P_c$ ) is a specialized case of the Goldman-Hodgkin-Katz equation commonly called the Nernst equation. The data suggest that the protein P pores are specific for anions, with the  $P_c/P_a$  lying between the two theoretical values.

Not all membranes tested gave results identical to those noted in Fig. 6. Occasionally much lower anion selectivity was noted, especially when a large number of protein P oligomers were incorporated into the membrane (as measured by the absolute conductance level). This might represent nonspecific ion leakage at the borders of the protein oligomers and the membrane.

## DISCUSSION

The results presented in this paper suggest that outer membrane protein P of *P. aeruginosa* is a porin which forms relatively small, anion-specific channels in lipid bilayers. It differs substantially from other outer membrane porins, from both *P. aeruginosa* and enteric bacteria, which have been described as forming pores with single-channel conductances in 1 M KCl 5- to 20-fold larger than protein P (3). In addition, these larger porins are generally weakly cation selective, with a 2- to 4-fold selectivity for cations over anions (3, 5; Benz, unpublished data), whereas protein P has at least a 30-fold higher specificity for anions over cations. Only two porins are known to have even weak (1.5- to 4-fold) selectivity for anions: the voltage-dependent anion channels of mitochondrial outer membranes (7), and protein Ic of *E. coli* (Benz and Henning, unpublished data). As pointed out above, protein Ic has a number of properties in common with protein P, including mode of induction.

The small conductances stimulated by protein P oligomers in lipid bilayers and the high specificity suggest that the correct terminology for such conductance units is channel rather than pore. The biophysical mechanism will be discussed in detail in a future communication (Benz, Poole, and Hancock, manuscript in preparation).

The data in Table 1 allow a minimal size estimate for the diameter of protein P channels. These results favor the assumption that phosphate (diameter, 0.56 nm) is one of the largest anions capable of passing through protein P pores. Since atoms cannot approach closer than their van der Waals repulsion radii, a minimum diameter estimate of 0.7 nm would seem war-

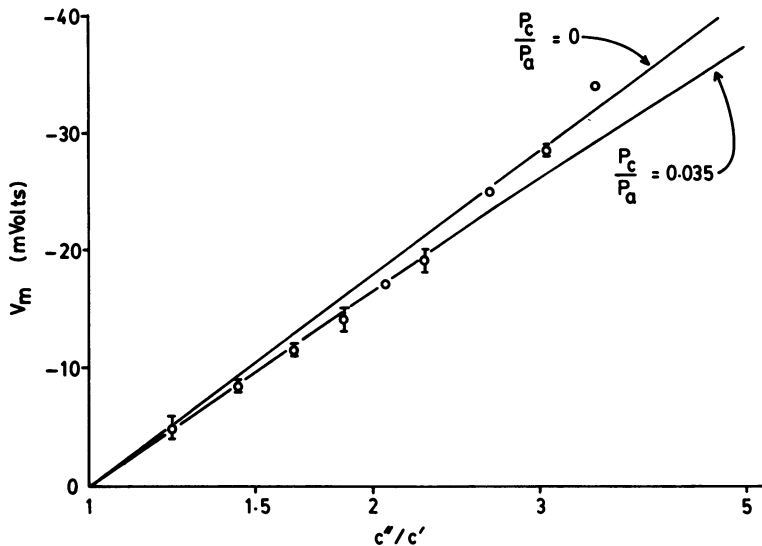


FIG. 6. Zero-current potentials  $V_m = \psi' - \psi''$  as a function of the ratio  $c''/c'$  of the salt concentrations on both sides of the membrane.  $c'$  was fixed to  $10^{-2}$  M MCl, and  $c''$  was varied between  $10^{-2}$  and  $10^{-1}$  M KCl. The aqueous phase contained 3  $\mu$ g of protein P per ml, which formed channels in the oxidized cholesterol-*n*-decane membrane (as shown by a steady current increase when a voltage was applied across the membrane for 30 min before the start of the experiment).  $V_m$  was more negative on the dilute ( $c'$ ) side of the membrane. The error bars on each point represent the range of results obtained (maxima and minima for up to five different membranes). The lines were drawn according to the Goldman-Hodgkin-Katz equation (5), with  $P_{K^+}/P_{Cl^-} = 0.035$  or 0. This second condition is a specialized form of the above equation known as the Nernst equation. The results were obtained from five different membranes.

ranted. Such a size might be too small to allow even monosaccharides through.

It would seem worthwhile to ask the question, Why does *P. aeruginosa* produce small anion-specific channels? In enteric bacteria such as *E. coli* and *S. typhimurium*, with more than  $10^5$  large hydrophilic pores in their outer membranes (7), the production of a small channel such as that formed by protein P would be superfluous. Despite their slight cation selectivity, the large size and number of enteric porins would ensure that the flow of anions would exceed the flow through an equal number of protein P channels. The major porin of *P. aeruginosa*, protein F, forms larger pores than the enteric porins (12, 16) but the low *in vivo* activity of protein F (1, 3) would result in an approximately 100-fold lower rate of anion flow across the outer membrane of *P. aeruginosa* than in enteric organisms. Thus, protein P induction should considerably boost the rate of movement of anions into the periplasm. One advantage of forming such small channels is that protein P induction does not result in enhanced susceptibility to such anionic antibiotics as carbenicillin (Poole, unpublished data), presumably because such antibiotics would be too large to pass through protein P channels.

Since protein P is induced in low-phosphate

medium and is apparently coregulated with two periplasmic phosphate-scavenging enzymes, alkaline phosphatase and phospholipase C, one possibility for the biological role of protein P is as a mediator of phosphate uptake from the medium. The rate of movement of phosphate through protein P channels is at least  $10^2$ - to  $10^4$ -fold lower than the rate of movement of chloride under ideal conditions. However, with the low levels of phosphate present in media inducing protein P production, this relatively low rate of uptake (in the order of  $10^2$  to  $10^3$  ions channel $^{-1}$  s $^{-1}$ ) may not be a serious drawback. Indeed this rate, equivalent to 1 to 10  $\mu$ mol of phosphate taken up mg of cell dry weight $^{-1}$  min $^{-1}$  (assuming  $3 \times 10^4$  open channels per cell), is far greater than the  $V_{max}$  for most microbial uptake systems. The efficiency of phosphate uptake from dilute environments would be considerably enhanced by the strong anion selectivity of protein P channels and would be further enhanced if there were specific phosphate binding sites on the mouth of protein P channels or a periplasmic phosphate binding protein or both. Analogous binding sites for maltose and maltodextrins have been postulated to considerably enhance the specificity of the *lamB* outer membrane porin of *E. coli* K-12 (9), since *in vitro* model membrane studies with this porin have demonstrated faster



rates of permeation of monosaccharides or serine than its *in vivo* maltodextrin substrates (15). We are currently searching for a periplasmic phosphate binding protein which might be coregulated with protein P in an attempt to gain further evidence for the biological role of protein P.

#### ACKNOWLEDGMENTS

We thank Michaela Gimple for expert technical assistance. This work was supported by grants to R.E.W.H. from the National Scientific and Engineering Research Council of Canada and the Canadian Cystic Fibrosis Foundation and by grants from the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 138 and Be865/1-1). In addition, some of this work was performed with the aid of an N.S.E.R.C. International Collaborative Research Grant to R.E.W.H. in the laboratory of R.B.

#### LITERATURE CITED

- Angus, B. L., A. M. Carey, D. A. Caron, A. M. B. Kropinski, and R. E. W. Hancock. 1981. Outer membrane permeability in *Pseudomonas aeruginosa*: comparison of a wild-type with an antibiotic-supersusceptible mutant. *Antimicrob. Agents Chemother.* **21**:299-309.
- Benz, R., B. A. Boehler-Kohler, R. Dieterle, and W. Boos. 1978. Porin activity in the osmotic shock fluid of *Escherichia coli*. *J. Bacteriol.* **135**:1080-1090.
- Benz, R., and R. E. W. Hancock. 1981. Properties of the large ion permeable pores formed from protein F of *Pseudomonas aeruginosa* in lipid bilayer membranes. *Biochim. Biophys. Acta* **646**:298-308.
- Benz, R., J. Ishii, and T. Nakae. 1980. Determination of ion permeability through the channels made of porins from the outer membrane of *Salmonella typhimurium* in lipid bilayer membranes. *J. Membrane Biol.* **56**:19-29.
- Benz, R., K. Janko, W. Boos, and P. Lauger. 1978. Formation of large ion-permeable membrane channels by the matrix protein (porin) of *Escherichia coli*. *Biochim. Biophys. Acta* **511**:305-319.
- Benz, R., K. Janko, and P. Lauger. 1979. Ionic selectivity of pores formed by matrix protein (porin) of *Escherichia coli*. *Biochim. Biophys. Acta* **551**:238-247.
- Columbini, M. 1979. A candidate for the permeability pathway of the outer mitochondrial membrane. *Nature* (London) **279**:643-645.
- Cheng, K. J., J. M. Ingram, and J. W. Costeron. 1970. Release of alkaline phosphatase from cells of *Pseudomonas aeruginosa* by manipulation of cation concentration and of pH. *J. Bacteriol.* **104**:748-753.
- Ferenci, T., and W. Boos. 1980. The role of *Escherichia coli*  $\lambda$  receptor in the transport of maltose and maltodextrins. *J. Supramol. Struct.* **13**:101-116.
- Hancock, R. E. W., and A. M. Carey. 1979. Outer membrane of *Pseudomonas aeruginosa*: heat- and 2-mercaptoethanol-modifiable proteins. *J. Bacteriol.* **140**:902-910.
- Hancock, R. E. W., and A. M. Carey. 1980. Protein D1—a glucose-inducible, pore-forming protein from the outer membrane of *Pseudomonas aeruginosa*. *FEMS Microbiol. Lett.* **8**:105-109.
- Hancock, R. E. W., G. M. Decad, and H. Nikaïdo. 1979. Identification of the protein producing transmembrane diffusion pores in the outer membrane of *Pseudomonas aeruginosa* PAO1. *Biochim. Biophys. Acta* **554**:323-331.
- Hancock, R. E. W., R. T. Irvin, J. W. Costeron, and A. M. Carey. 1981. *Pseudomonas aeruginosa* outer membrane: peptidoglycan-associated proteins. *J. Bacteriol.* **145**:628-631.
- Hancock, R. E. W., V. J. Raffle, and T. I. Nicas. 1981. Involvement of the outer membrane in gentamicin and streptomycin uptake and killing in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **19**:777-785.
- Luckey, M., and H. Nikaïdo. 1980. Specificity of diffusion channels produced by  $\lambda$  phage receptor protein of *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **77**:167-171.
- Nakae, T., J. Ishii, and M. Tokunaga. 1979. Subunit structure of functional porin oligomers that form permeability channels in the outer membrane of *Escherichia coli*. *J. Biol. Chem.* **254**:1457-1461.
- Nikaïdo, H. 1980. Nonspecific transport through the outer membrane, p. 361-407. *In* M. Inouye (ed.), *Bacterial outer membranes*. Wiley-Interscience, New York.
- Schindler, H., and J. P. Rosenbusch. 1978. Matrix protein of *Escherichia coli* outer membranes forms voltage-controlled channels in lipid bilayers. *Proc. Natl. Acad. Sci. U.S.A.* **75**:3751-3755.
- Stinson, M. W., and C. Hayden. 1979. Secretion of phospholipase C by *Pseudomonas aeruginosa*. *Infect. Immun.* **25**:558-564.
- Tommassen, J., and B. Lugtenberg. 1980. Outer membrane protein e of *Escherichia coli* K-12 is co-regulated with alkaline phosphatase. *J. Bacteriol.* **143**:151-157.