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Bordetella pertussis Major Outer Membrane Porin Protein Forms Small, Anion-Selective Channels in Lipid Bilayer Membranes

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The major outer membrane protein of molecular weight 40,000 (the 40K protein) of a virulent isolate of *Bordetella pertussis* was purified to apparent homogeneity. The purified protein formed an oligomer band (of apparent molecular weight 90,000) on sodium dodecyl sulfate-polyacrylamide gels after solubilization at low temperatures. The porin function of this protein was characterized by the black lipid bilayer method. The 40K protein formed channels smaller than all other constitutive major outer membrane porins studied to date. The average single-channel conductance in 1 M KCl was 0.56 nS. This was less than a third of the conductance previously observed for *Escherichia coli* porins. Zero-current potential measurements made of the porin to determine its ion selectivity revealed the porin to be more than 100-fold selective for anions over cations. The single-channel conductance was measured as a function of salt concentration. The data could be fitted to a Lineweaver-Burk plot suggesting an anion binding site with a K_d of 1.17 M Cl^- and a maximum possible conductance through the channel of 1.28 nS.

The outer membrane of all gram-negative bacterial species studied to date constitutes a size-dependent permeability barrier (12, 18). The major route of uptake of hydrophilic molecules smaller than the exclusion limit of the outer membrane is through the water-filled pores of a class of membrane proteins called porins (3, 18). Most porins studied to date have similar properties. They form oligomers (usually trimers) in their native state; these oligomers are stable to denaturation by the detergent sodium dodecyl sulfate (SDS) at temperatures below 60°C or so; the oligomers are often tightly associated with the underlying peptidoglycan; the monomer forms of porins have apparent molecular weights in the range of 30,000 to 40,000; and the purified porin oligomers can be reconstituted into lipid bilayers to form large, weakly selective channels (18). Nevertheless, there are exceptions to all of these general properties. For example, the phosphate-starvation-induced porin of *Pseudomonas aeruginosa*, protein P, has a monomer molecular weight of 48,000, an extremely weak association with the underlying peptidoglycan, and reconstitutes small, anion-specific channels in lipid bilayer membranes (3, 13). To date this protein is the only well-described porin which is capable of good discrimination between ions on the basis of charge.

Bordetella pertussis is a noninvasive pathogen, colonizing the ciliated epithelium of the human nasopharynx and elaborating a number of toxic substances believed to be responsible for the symptoms of whooping cough (20). Because the outer membrane comes in intimate contact with host epithelial and immune cells, outer membrane proteins are likely to be of great importance in colonization and subsequent immunity, in addition to playing a potential role in antibiotic susceptibility. Using SDS-polyacrylamide gel electrophoresis, Parton and Wardlaw (19) as well as others (5, 22) observed a major outer membrane protein with a molecular weight of approximately 40,000 (the 40K protein) in virulent phase I organisms as well as avirulent phase IV and C-mode (modulated) cells. The 40K major outer membrane protein was not easily observed in gels if the envelope sample was solubilized at 37°C (as opposed to 100°C) before electrophoresis (19).

As mentioned above, previous work has implicated the outer membrane of specific organisms in generalized resistance to antibiotics (2, 12, 18). We decided to characterize the major outer membrane protein of *B. pertussis* to see whether it could function as a porin and be a potential pathway for the uptake of antibiotics. In this paper, we describe the purification to apparent homogeneity and functional reconstitution into lipid bilayers of the 40K protein of *B. pertussis*. The properties of this porin were unlike those of any constitutive porin described to date, resembling those of the derepressible porin protein P of *P. aeruginosa*.

MATERIALS AND METHODS

Bacteria and culture conditions. *B. pertussis* UT25, a virulent clinical isolate minimally passaged (6) and stored in blood at −70°C, was grown in the defined liquid medium of Stainer and Scholte (24) as has been described (23). Purity of the cultures was determined by Gram staining and characteristic growth on Bordet-Gengou medium (6).

Purification of the 40K protein. A modification of the techniques of Gabay et al. (9) and Blake et al. (4) was used to purify the 40K protein of *B. pertussis* UT25. Approximately 2 g (wet weight) of harvested bacteria was suspended in 10 ml of cold 0.75 M sucrose–10 mM Tris hydrochloride (pH 8.0) containing 1 mg each of RNase and DNase. While the suspension was slowly swirled, 20 ml of 1.5 mM EDTA (disodium salt) was added dropwise. The cells were sonically disrupted at 0°C, and the unbroken cells were removed by centrifugation (23). The supernatant was centrifuged at 100,000 × *g* for 1 h to obtain cell envelopes. The cell

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envelope pellet was suspended in a solution of 5% (wt/vol) *N*-tetradecyl-*N*,*N*-dimethyl-3-ammonia-1-propane sulfonate (Zwittergent-3,14; Calbiochem-Behring, La Jolla, Calif.), 0.5 M $CaCl_2$, and 20 mM Tris hydrochloride (pH 8.0) and incubated for 1 h at 37°C. After incubation, the mixture was centrifuged at 100,000 × *g* for 1 h to pellet the detergent-insoluble material. Ice-cold absolute ethanol (4 volumes) was slowly added to the supernatant, which was then stored overnight at −20°C to precipitate the proteins. The precipitate was collected by centrifugation at 17,000 × *g* for 20 min and suspended in 0.5% Zwittergent-3,14–50 mM Tris hydrochloride (pH 8.0)–10 mM EDTA. This mixture was incubated at 37°C for 3 h and centrifuged at 17,000 × *g* for 10 min to pellet any insoluble material. The supernatant was briefly sonicated and applied to a DEAE-Sephacel (Pharmacia, Inc., Piscataway, N.J.) anion-exchange column (2.5 by 5.5 cm) equilibrated with 0.5% Zwittergent-3,14–50 mM Tris hydrochloride (pH 8.0)–10 mM EDTA. The column was washed with the buffer until the absorbance of the column effluent at 280 nm returned to base level. Three bed volumes of the same buffer containing 0.1 M NaCl were applied to the column (flow rate, 20 ml/h) to elute the 40K protein. Other Zwittergent-soluble proteins were found to elute at higher salt concentrations. To identify fractions containing the 40K protein, samples were treated as above with absolute ethanol to precipitate the proteins which were then suspended in electrophoresis solubilization buffer and examined by SDS-polyacrylamide gel electrophoresis. The protein precipitates of 40K protein-containing fractions were pooled, dissolved in a buffer solution of 1% SDS–0.4 M NaCl–10 mM Tris (pH 8.0)–5 mM EDTA–0.05% NaN_3, briefly sonicated, and applied to a gel filtration column (1.5 by 66 cm) of Sephacryl S-200 (Pharmacia) equilibrated with the same SDS buffer. The sample was chromatographed at a flow rate of 3 ml/h, and fractions of 1 ml were collected and analyzed by SDS-polyacrylamide gel electrophoresis. The porin preparation used in these studies was greater than 97% pure as judged by densitometer analysis of SDS-polyacrylamide gels stained with Coomassie blue.

SDS-polyacrylamide gel electrophoresis. A modified Laemmli (15) procedure was performed for electrophoretic analysis of proteins. A 12% separating gel was made from a stock solution of 30% (wt/vol) acrylamide and 0.54% *N*,*N*'-methylene-bisacrylamide. Samples were solubilized in digestion buffer containing 12.5% glycerol, 1.25% SDS, 1.25% 2-mercaptoethanol, 0.005% bromophenol blue, and 0.25 M Tris hydrochloride (pH 6.8), at the temperature stated in the legend to Fig. 1, and loaded onto a 3% stacking gel.

Lipid bilayer methods. The methods used for the characterization of the pore-forming ability of the *B. pertussis* porin have been previously described in detail (2, 3, 13). The lipid used to form the membrane in the Teflon chamber was 1.5% oxidized cholesterol in *n*-decane, a generous gift from R. Benz, University of Konstanz, Federal Republic of Germany. The experiments were done at 24°C. Electrical measurements were made by immersing the Ag-AgCl electrodes into the aqueous solutions on either side of the Teflon divider which was perforated by a small 0.1- to 2-mm^2 hole over which a membrane was painted. Bilayer formation was recognized by the membrane turning optically black when viewed with incident light. Current fluctuations were amplified 10^9- or 10^{10}-fold with a Keithley (Cleveland, Ohio) 427 preamplifier and monitored by a Tektronix (Beaverton, Ore.) 5115 storage oscilloscope (plug-in amplifier 5A22). Observations were recorded on a strip chart recorder for further analysis. Bulk conductance experiments were monitored with a Keithley 610 C electrometer. Zero-current potential measurements were performed exactly as described previously (3).

RESULTS

Purification of the *B. pertussis* 40K protein. Preliminary experiments showed that the 40K protein, as well as other outer membrane proteins, formed SDS-resistant complexes with the underlying peptidoglycan. These proteins were released from the peptidoglycan by the addition of 0.1 M NaCl–SDS to SDS-treated cell envelopes (S. K. Armstrong and C. D. Parker, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, D108, p. 72). This crude protein mixture, highly enriched in 40K protein, possessed pore-forming activity in black lipid membrane experiments (data not shown). Gel filtration of this mixture on Sephacryl S-200, although resulting in a greatly purified preparation, failed to resolve the 40K protein from a contaminant of molecular weight 18,000 which consistently copurified with it, even after multiple chromatographic steps. The presence of this contaminant necessitated the ion-exchange technique described herein.

When cell envelopes were treated with 5% Zwittergent-3,14 and 0.5 M $CaCl_2$ in 20 mM Tris (pH 8.0), virtually all the 40K protein, in addition to other minor envelope proteins, was released from the insoluble peptidoglycan. Application of this material to an ion-exchange column and elution with buffer containing 0.1 M NaCl yielded fractions of highly purified 40K protein. When a salt gradient (0.1 to 0.4 M NaCl) was subsequently used, other envelope proteins eluted from the column. Gel filtration chromatography of the 40K protein-containing-fractions from the ion-exchange column resulted in further purification of the 40K protein. The apparent molecular weight of the 40K protein was found to be dependent upon the solubilization temperature before SDS-polyacrylamide gel electrophoresis (Fig. 1). If solubilized at 27°C, the 40K protein migrated as a presumed oligomer of 90,000 daltons; when the 40K protein was boiled for 10 min before electrophoresis, it migrated as a single 40,000-dalton monomeric species. The purified 40K protein did not contain lipopolysaccharide as detected by immunoblotting with the *Bordetella* lipopolysaccharide-specific monoclonal antibody P5H7 (8) (data not shown). No lipopolysaccharide was detected in purified protein preparations analyzed in silver-stained polyacrylamide gels (25) (data not shown).

Black lipid bilayer experiments. Addition of the 40K protein to the aqueous phase (1 M KCl) bathing a lipid bilayer membrane resulted in a time-dependent increase in membrane conductance similar to that noted for other porins. As little as 5 ng of protein per ml caused an increase of 3 orders of magnitude in conductance over a period of 30 min. At this time, the conductance (current divided by voltage) was independent of the applied voltage up to 180 mV, suggesting that the conductance channels were not voltage regulated or gated.

When smaller amounts of protein were added to the aqueous phase and the resolution of the current-measuring device was increased, it could be observed that the conductance increase involved a series of steps (Fig. 2). By analogy with other lipid bilayer experiments, these steps were considered to involve single channels incorporating into the membrane and thus allowing the passage of ions through the membrane. A histogram of the conductance increments in 1 M KCl demonstrated a relatively narrow distribution of sizes of the step increase with an average single-channel conductance of 0.56 nS. This is smaller than all other major porins

studied to date, and only the phosphate-starvation-inducible porin protein P of *P. aeruginosa* has been shown to have a smaller single-channel conductance.

Similar single-channel conductance experiments with the 40K protein from virulent UT25 cells were performed for a variety of salts. The data suggested that the average single-channel conductance increase was quite similar for a variety of different Cl^--containing salts (Table 1). This suggested that the channel was strongly selective for anions. To confirm this, zero-current potential measurements were performed. A concentration gradient of KCl was formed across a lipid bilayer membrane into which 100 or more channels had been incorporated. Under the influence of such a concentration gradient, ions will diffuse across the membrane according to the ion preference of channel. The preferential movement of, e.g., anions for an anion-selective channel will create a negative potential on the dilute side of the membrane which will oppose the driving force provided by the concentration gradient. The potential created for a given concentration gradient at which ions cease to move is called the zero-current potential. Application of the Goldman-Hodgkin-Katz equation (3) to these data allows calculation of the relative permeability of anions and cations. When such an analysis was performed for the 40K protein, the protein was found to be more than 100-fold selective for anions over cations (Fig. 3).

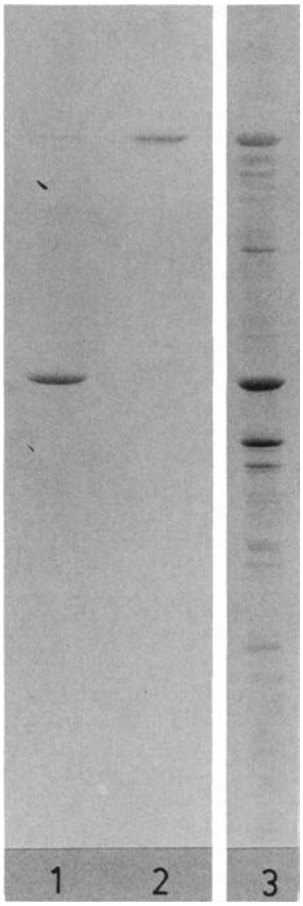

FIG. 1. SDS-polyacrylamide gel electrophoresis of purified 40K protein and of cell envelopes of *B. pertussis* UT25. Lanes: 1, purified 40K protein solubilized in digestion buffer at 100°C for 10 min; 2, 40K protein solubilized in digestion buffer at 27°C; 3, the cell envelopes from which the 40K protein was purified, solubilized at 100°C for 10 min. Molecular weights cited in the text were estimated by reference to the following standards: phosphorylase *b*, 92,500; bovine serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; and lysozyme, 14,400.

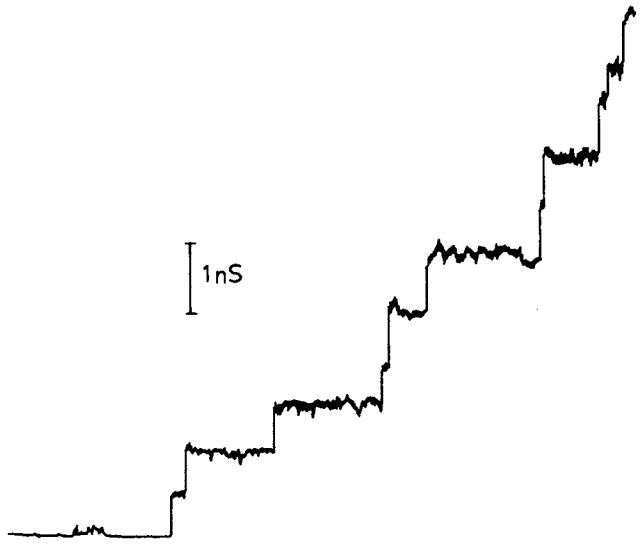

FIG. 2. Stepwise increase in membrane conductance after the addition of 6 ng of *B. pertussis* 40K protein per ml (final concentration) to the aqueous phase consisting of 1 M KCl, pH 6.0. The membrane was formed from a 1.5% solution of oxidized cholesterol in *n*-decane. The applied voltage was 10 mV.

Only one other bacterial outer membrane porin, *P. aeruginosa* protein P (3, 13), has been shown to demonstrate very strong ion selectivity similar to that of the *B. pertussis* 40K porin. In this case, it has been demonstrated that the basis of selectivity is an anion-binding site within the channel. To investigate such a possibility for the *B. pertussis* 40K porin, the single-channel conductance was measured as a function of salt concentration. Unlike other bacterial porins which demonstrate a linear relationship between salt concentration and conductance (3, 13), but like protein P, the 40K protein showed saturation kinetics (Fig. 4). The data could be fitted to a Lineweaver-Burk plot, suggesting an anion binding site with a K_d of 1.17 M Cl^- and a Λ_{max} (maximal possible conductance through the channel) of 1.28 nS.

DISCUSSION

The data presented in this paper demonstrated that the *B. pertussis* 40K major outer membrane porin has properties

TABLE 1. Single-channel conductances of UT25 porin in different salt solutions

Salt	Concn (M)	Avg conductance (nS)	No. of events
KCl	1.0	0.56	428
NaCl	1.0	0.62	142
	0.5	0.43	169
LiCl	1.0	0.55	104
$MgCl_2$	1.0	0.61	106
	0.5	0.40	119
$Tris^+Cl^-$	0.5	0.27	142

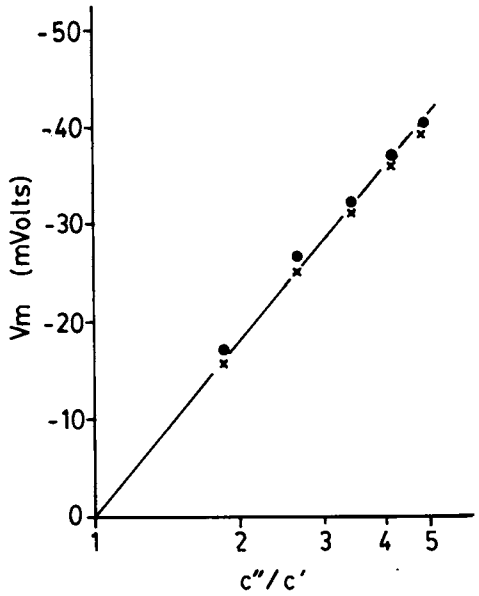

FIG. 3. Ionic selectivity of *B. pertussis* 40K porin. Zero-current membrane potentials (V_m) across a membrane of diphytanoyl phosphatidylcholine *n*-decane in the presence of porins as a function of the KCl gradient c''/c' across the membrane. The dilute side of the membrane (c') was 50 mM, while c'' was varied by the addition of increasing amounts of 3 M KCl. The aqueous phase contained 30 ng of 40K protein per ml (●) or 10 ng of protein P per ml (×).

unlike any constitutive porin described to date. In fact, only the *P. aeruginosa* phosphate-starvation-inducible porin protein P (13) and the *Escherichia coli* maltose-inducible LamB porin (R. Benz, personal communication) have similar properties, i.e., low single-channel conductance, strong ion preference, and a binding site within the channel. However, these two channels are somewhat unique in that they are inducible and can be considered to be special-purpose porins since they bind phosphate (R. E. W. Hancock and R. Benz, manuscript in preparation) and maltose (18), respectively. This raises the possibility that the *B. pertussis* 40K porin has a function in binding an important anionic substrate, e.g., nicotinic acid or nucleosides. This would allow concentration at the cell surface and consequently more efficient uptake of these substrates. The size of substrate permitted might be quite large. Although the single-channel conductance of the *B. pertussis* 40K porin is smaller than that of other constitutive porins at 1 M KCl (0.56 nS compared with 2.1 nS for the *E. coli* OmpF porin [3] and 5.0 nS for *P. aeruginosa* porin protein F [2]), it is more than double that of protein P (0.25 nS [13]). This larger size is reflected in the binding affinity for Cl^- since protein P binds Cl^- with a K_d of 40 mM (13), whereas *B. pertussis* 40K porin binds Cl^- with a K_d of almost 1.2 M. Thus, quite large anions and even uncharged hydrophilic compounds of moderate size should pass through the 40K porin. Although cations will be excluded, this could be easily compensated for by the presence of an alternative, cation-selective channel in the *B. pertussis* outer membrane.

It has been postulated that the outer membrane permeability of gram-negative bacteria may determine their susceptibility to certain chemical compounds and antibiotics (12, 18). Although specific studies of the outer membrane permeability of *B. pertussis* have not been performed, the in vitro antibiotic susceptibility of this organism has been examined (1, 7). *B. pertussis* was shown to be quite sensitive to the hydrophobic antibiotic erythromycin (MIC, 0.02 to 1.56 μg/ml), which is the antibiotic of choice in the treatment of the disease, and also demonstrates good susceptibility to the amphiphilic antibiotics tetracycline and chloramphenicol. The raises the possibility that *B. pertussis*, like *Neisseria gonorrhoeae*, has a hydrophobic diffusion pathway (12, 18), in contrast to most gram-negative bacteria studied to date.

From the data described here, it is possible that the *B. pertussis* outer membrane presents a considerable barrier to the penetration of β-lactam antibiotics and that only small anionic β-lactams would penetrate the 40K channel at significant rates. In agreement with this, hydrophilic antibiotics such as penicillins, cephalothin, lincomycin, and the aminoglycosides streptomycin and gentamicin demonstrate poor efficacy against *B. pertussis* (1, 7).

A major outer membrane protein of approximately 40,000 daltons has been observed in all virulent and avirulent isolates examined (5, 19, 22) as well as *Bordetella bronchiseptica* and *Bordetella parapertussis*, where it is seen to migrate as a slightly higher-molecular-weight species in SDS-polyacrylamide gels (5, 22). Preliminary studies with the partially purified 40,000-dalton proteins from an avirulent phase IV UT25 derivative (6, 16) and UT25 modulated with nicotinic acid (14, 21, 23) showed similar conductance measurements in black lipid membrane experiments (S. K. Armstrong, and T. Parr, unpublished data).

Bacterial culture conditions have been shown to affect the expression of several porins from gram-negative species (13, 17). Although the organisms from which the major outer membrane protein was purified were cultured in the defined Stainer and Scholte medium (24), the same strain grown on Bordet-Gengou medium also produced this protein. It is not known if the organism produces the 40K protein in vivo, but preliminary evidence indicates that human convalescent sera recognize both the oligomeric and monomeric forms of this protein in Western immunoblots (Armstrong and Parker, in preparation). Because this porin is a major protein observed in all *Bordetella* strains and species, secretory antibody

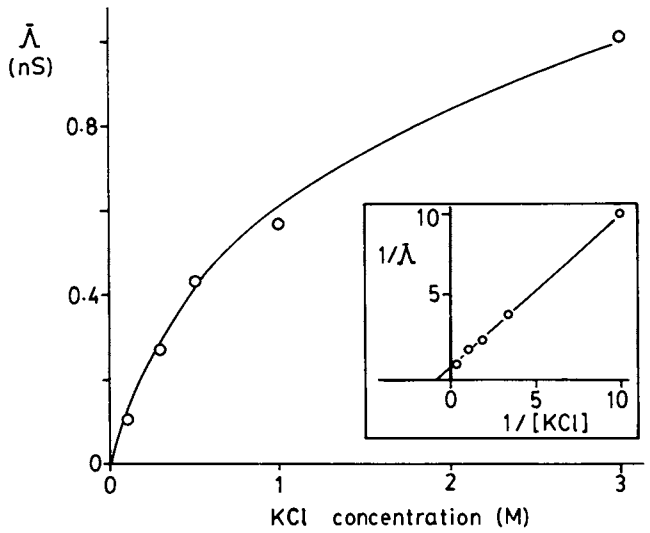

FIG. 4. Dependence of average single-channel conductance ($\bar{A}$) on the concentration of KCl in the aqueous phase. A concentration of 6 ng of 40K *B. pertussis* protein per ml was added, and 100 to 200 stepwise conductance increases similar to those seen in Fig. 2 were measured. The Lineweaver-Burke plot (inset) suggested an anion-binding site with a K_d of 1.17 M Cl^-.

reactive with it may be protective against colonization and infection. Other investigators have shown that porins are capable of activating complement (10) and may be useful as vaccine components (11). These possibilities are currently being investigated.

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