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Purification and Characterization of Protein H, the Major Porin of *Pasteurella multocida*

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Protein H (B. Lugtenberg, R. van Boxtel, D. Evenberg, M. de Jong, P. Storm, and J. Frik, Infect. Immun. 52:175–182, 1986) is the major polypeptide of the outer membrane of *Pasteurella multocida*, a bacterium pathogenic for humans and animals. We have purified this protein to homogeneity by size exclusion chromatography after selective extraction with surfactants and demonstrated its pore-forming ability after reincorporation into planar lipid bilayers. In these experiments, the current through the pores was a linear function of the applied voltage in the range of −50 to +50 mV. Voltages beyond ±50 mV tended to partially close the channels, giving rise to apparent negative resistances. These observations suggest that protein H channels are probably not voltage regulated in vivo. With the patch clamp technique, single-channel conductance fluctuations of 0.33 nS were recorded in 1 M KCl. Electrophoretic and circular dichroism analyses showed that protein H forms homotrimers stable in sodium dodecyl sulfate at room temperature, with a high content of β-sheet secondary structure. Upon boiling, the trimers were fully dissociated into monomers with an increase of α helix and irregular structure, at the expense of β sheets. The apparent molecular mass of fully denatured monomers ranged between 37 and 41.8 kDa, depending on the electrophoretic system used for analysis. The trimeric arrangement of protein H was confirmed by image analysis of negatively stained, two-dimensional crystal arrays. This morphological study revealed, in agreement with electrophoretical data, a trimeric structure with an overall diameter of 7.7 nm. Each monomer appeared to contain a pore with an average diameter of 1 nm. Quantitative comparisons revealed that the amino acid composition (hydropathy index of −0.40) and the N-terminal sequence (determined over 36 residues) of protein H are similar to those of bacterial general porins, notably porin P2 of *Haemophilus influenzae*. We conclude from this set of structural and functional data that protein H of *P. multocida* is a pore-forming protein related to the superfamily of the nonspecific bacterial porins.

Porins are channel-forming proteins found in gram-negative eubacteria, mitochondria, and chloroplasts (for recent reviews, see references 2, 15, 16, and 22). In gram-negative bacteria, porins are usually in the form of homotrimers and allow the diffusion through the outer membrane of small hydrophilic solutes, including hydrophilic antibiotics, up to an exclusion limit of about 600 Da. Though most of the presently known porins function as nonspecific molecular sieves, some of them exhibit some specificity. In addition to their pore function, bacterial porins serve as receptors for bacteriophages and bacteriocins, and in the case of pathogenic bacteria, they also appear to be targets of the immunological system. As polytopic membrane proteins, porins are unorthodox in the sense that, in contrast to their plasma membrane counterparts, they are devoid of long stretches of apolar residues capable of forming transbilayer α helices and contain a high proportion of β-sheet conformation. Though their general structure has been inferred from the large amount of data accumulated over the years by chemical, physical, genetic, and immunological methods (22), the work performed on the *Rhodobacter capsulatus* porin constitutes a real breakthrough in the elucidation of the structure of porins. Indeed, the porin of *R. capsulatus* has been crystallized and analyzed by X-ray diffraction (54), and its primary structure has recently been determined (50). This analysis revealed that each subunit composing the trimer contains 16 antiparallel β strands forming a transmembrane β barrel (53).

Pasteurella multocida is a typical gram-negative eubacterium belonging to the γ-3 subdivision of the purple bacteria phylum (9, 55). This microorganism is pathogenic for humans and a wide variety of mammals and birds (35) and is often associated with *Bordetella bronchiseptica* in atrophic rhinitis of swine (46). Lugtenberg et al. (30, 31) presented evidence for the presence in the envelope of *P. multocida* of a 37.5-kDa major protein (protein H) which, owing to its high immunogenicity and exposure on the cell surface, is considered an attractive vaccine candidate. This protein has been partially purified and shown to share several properties with porins of enterobacteria, namely, resistance to denaturation by sodium dodecyl sulfate (SDS) and noncovalent association with murein (31).

The aim of this study was to determine whether protein H is a porin. The protein was purified from two strains of *P. multocida*, and its function was assayed in planar lipid bilayers. As it appeared that protein H is actually a porin, its amino acid composition and N-terminal sequence were compared with those of a series of bacterial porins. Protein H was further characterized at the structural level by electrophoresis, electron microscopy, and circular dichroism analyses.

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MATERIALS AND METHODS

Bacterial strains, growth conditions, and preparation of cell envelopes. Strains 7473 and 9222 of *P. multocida* (serotype D2) were obtained from M. Kobisch (Station de Pathologie Porcine, Centre National d'Etudes Vétérinaires et Alimentaires, Ploufragan, France). They were grown at 37°C in a medium containing, per liter, 20 g of Tryptose (Difco), 1 g of D-glucose, 5 g of NaCl, and 2.5 g of yeast extract (Difco) (pH 7.4). The cells were harvested at the end of the exponential growth phase, which was usually reached after 12 h under gentle agitation. Four liters of a culture containing 6.2×10^9 cells per ml was centrifuged at $12,000 \times g$ for 15 min at 4°C. The pellets were dispersed in 300 ml of 0.1 M sodium phosphate buffer (pH 7.4), centrifuged as described above, and dispersed in 20 ml of phosphate buffer containing 0.4 mM phenylmethylsulfonyl fluoride. Cell envelopes were prepared as described by Nikaido (42).

Purification of protein H. All centrifugations in the procedures described below were performed for 15 min at 265,000 $\times g$ and at 15°C. Incubations with detergents were done at 37°C for 1 h unless indicated otherwise. The buffer referred to as Tris buffer was 50 mM Tris-HCl buffer (pH 7.4).

For the purification of protein H monomers, isolated *P. multocida* envelopes (2.5 ml containing 45 mg of protein) were treated with 2% sodium *N*-lauroyl sarcosinate (Sarkosyl) in Tris buffer. The pellet obtained by centrifugation was dispersed in 2 ml of Veronal buffer (pH 8.6; ionic strength = 0.03) containing 0.2 M sodium deoxycholate. The insoluble material recovered by centrifugation was then incubated with 2 ml of 1% SDS in Tris buffer. The new pellet obtained by centrifugation was then dispersed in 2 ml of 1% SDS in Tris buffer containing 5 mM EDTA and incubated for 5 min at 100°C. The supernatant (2 ml containing 5.3 mg of protein) recovered after centrifugation was applied on top of an AcA44 column (height, 60 cm; diameter, 3.2 cm; void volume, 156 ml; IBF, France) equilibrated and eluted with 50 mM sodium phosphate buffer (pH 7.4) containing 0.1% SDS.

For the purification of protein H trimers, cell envelopes (50 mg of protein) were incubated in 2 ml of Tris buffer containing 2% Sarkosyl and centrifuged. This step was repeated once, and the insoluble material was dispersed in 2 ml of Tris buffer containing 2% SDS, 0.5 M NaCl, and 5 mM EDTA. After incubation and centrifugation, the supernatant (2 ml containing 9.3 mg of protein) was subjected to size exclusion chromatography as described above in phosphate buffer containing 0.1% SDS and, to avoid oligomer dissociation, 0.3 M NaCl.

In both procedures, the separations by size exclusion chromatography were performed at 25°C to prevent SDS crystallization. Purification of the protein was monitored by UV absorption at 280 nm. Buffer flow through the column was 10.5 ml/h, and 3.5-ml fractions were collected. The fractions containing protein H were identified by SDS-polyacrylamide gel electrophorésis (PAGE) and pooled. In the case of the oligomeric form of protein H, the pooled fractions were further dialyzed for 48 h at room temperature against 50 mM sodium phosphate buffer (pH 7.4) containing 0.3 M NaCl and 0.02% NaN_3. Removal of SDS resulted in the precipitation of the protein, which was then recovered by centrifugation at $120,000 \times g$ for 1 h at 4°C. The protein was finally dispersed in 0.1 M sodium phosphate buffer (pH 7.4) and stored at −80°C.

Determination of protein and of KDO. Protein was determined by the method of Lowry et al. (29), with serum albumin as a standard. Specific titration of protein H within complex protein mixtures was performed by scanning densitometry of electrophoregrams, using purified protein H as a standard. Purified protein H was determined by the method of Moore and Stein (39). Ketodeoxyoctulosonic acid (KDO) was determined as described by Osborn (43), with the use of commercial KDO as a standard.

SDS-PAGE. SDS-PAGE was performed according to the method of Laemmli (26). The compositions of the stacking gel and of the separating gel were as follows: for the stacking gel, T = 4.8% and C = 2.6%; for the separating gel, T = 12.5% and C = 2.6%, where T is the total monomer (acrylamide and *N,N'*-methylene bisacrylamide) concentration and C is the amount of *N,N'*-methylene bisacrylamide relative to that of total monomers. Some separations were also performed in the presence of 8 M urea (37) or in 6 to 30% polyacrylamide gradients (C = 3.5%) in 40 mM Tris–20 mM CH_3COONa buffer (pH 7.4) containing 2 mM EDTA and 0.1% SDS. Proteins were stained with Coomassie brilliant blue R250 in acetic acid-methanol-water (1:4:5, vol/vol/vol) or silver stained (51).

Amino acid analysis and protein sequencing. Protein hydrolysis and determination of amino acid compositions were performed as described previously (57) with the exception of tryptophan, which was spectrophotometrically determined by the method of Edelhoch (10) after solubilization of protein H in 6 M guanidinium chloride (pH 7.0). The index of Marchalonis and Weltman (33), $S\Delta Q = 10^4\Sigma(X_{IA} - X_{iB})^2$, was used to assess the relatedness of porin amino acid compositions. When two proteins A and B are compared with this method, X_{iA} and X_{iB} are the mole fractions of the amino acid i in protein A and protein B, respectively.

Automated sequence analyses were performed in an Applied Biosystems liquid-phase Sequenator (model 475) equipped with an on-line analyzer (model 120A) with a 5-μm phenylthiohydantoin C_{18} high-pressure liquid chromatography column (220 by 2.1 mm). Amino acid sequence data were analyzed using DNA Strider version 1.0 (34).

Circular dichroism spectroscopy. Circular dichroism of protein H solutions was recorded between 190 to 250 nm at 20°C with a Jobin-Yvon Mark V dichrograph equipped with a thermostatically controlled quartz cell with a path length of 1 mm. The sample contained 0.125 mg of protein per ml of 25 mM sodium phosphate buffer (pH 7.0) with 1% SDS and 0.25 M NaCl. For each analysis, at least three scans were performed and subsequently averaged. Corrections were made for buffer and detergent contribution. $\Delta\varepsilon$ was calculated on the basis of a mean residue mass of 115 Da and expressed in centimeter^{-1} molar concentration^{-1} (58).

Preparation of proteoliposomes and obtention of two-dimensional crystals. Incorporation of protein H into liposomes was achieved by detergent dialysis (19). This procedure was performed at 20°C, using a thermostated three-compartment Teflon cell with two cellulose dialysis membranes (molecular mass cutoff, 5 kDa). The central compartment was filled with 5 ml of 30 mM Hecameg (47) in 50 mM sodium phosphate buffer (pH 7.4) containing 4 mg of egg yolk lecithin, 85 μg of dicetyl phosphate, and 4 mg of purified trimeric protein H. Phosphate buffer (50 mM, pH 7.4) containing 0.02% NaN_3 flowed through the two lateral compartments of the Teflon cell at a rate of 80 ml/h per compartment for 36 h at room temperature. The turbid suspension of proteoliposomes was subsequently sonicated twice for 30 s each time at 0°C with a titanium microtip (70 W) and centrifuged at $285,000 \times g$ for 15 min at 4°C. The pellet was dispersed in 5 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 5 mM $CaCl_2$ and treated for 24 h with 5 μg of phospholipase A_2 (32). The suspension

was further dialyzed for several days at 4°C and then centrifuged at 50,000 × g for 15 min at 4°C. The pellet thus obtained was finally dispersed in 50 mM ammonium acetate buffer (pH 7.0) for electron microscopy.

Negative staining for electron microscopy and image processing. Suspensions of proteoliposomes treated with phospholipase A_2 were deposited onto glow-discharged carbon-coated grids. After 1 min, a drop of 1% uranyl acetate was added. Excess liquid was blotted with filter paper, and grids were air dried. Micrographs were taken under low-dose conditions with a Philips CM12 microscope operating at 80 kV and at a magnification of ×45,000. On-line digital recording of pictures was carried out by using a CF 1500 ELCA high-resolution video camera (Sofretec, Bezons, France) connected to a microcomputer fitted with a digital acquisition card. Images of 512 by 512 pixels were recorded with a sampling of 0.6 nm on the specimen scale.

Correlation averaging (49) was applied to 256- by 256-pixels arrays. Briefly, a reference was obtained by quasi-optical Fourier filtration of a part of a crystalline image. The resulting Fourier average was then used as a reference to compute a correlation average of the entire crystalline field. Cross-correlation maxima were located by a peak search program, and 40-by-40 areas were extracted from the raw image at the exact position of the correlation maxima. An array average was formed by summation of individual subimages. Two array averages corresponding to the addition of 76 and 88 subimages, respectively, were obtained. The averages were next rotationally and translationally aligned (13), weighed, and then added. The resulting image was finally low-pass filtered and symmetrized. All calculations were performed on SUN workstations, using the IBIS program system (12). The resolution limit was estimated by using the phase residual method (13).

Functional assays with planar lipid bilayers. Two levels of membrane permeability analyses, with different ranges of membrane diameter, were used, depending upon the ionic current resolution needed. Both kinds of conductance experiments were performed at room temperature. In macroscopic conductance experiments, the membrane (Montal and Mueller type [38]) was formed by apposing two lipid monolayers over a 125-μm-diameter hole in a Teflon film sandwiched between two glass half-cells containing the electrolyte (1 M KCl in 2.5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES] buffer [pH 7.0]). Bilayer formation was monitored by the membrane capacitance response to a ±10-mV triangular signal. An Ag/AgCl electrode delivered the clamping voltage in the *cis* (positive) side, while the transmembrane current was measured with another Ag/AgCl electrode in the *trans* side and amplified (Keithley 427). Bare or doped membranes were submitted either to a constant holding voltage or to triangular voltage sweeps (duration, 100 to 200 s). To improve resolution at the single-channel level, virtually solvent-free lipid bilayers were formed at the tip of patch clamp pipettes (17), with the pipette interior corresponding to the usual *trans* side. Transmembrane current amplified through a Biologic RK 300 was stored for further analysis in a digital tape recorder (DTR 1200). Amplitude histograms were performed through the Satori software from Intracel (Royston, United Kingdom). In both kinds of conductance experiments, the lipids (Avanti Polar Lipids, Birmingham, Ala.) used for bilayer formation were a 7/3 (wt/wt) mixture of 1-palmitoyl-2-oleylphosphatidylcholine (POPC) and dioleylphosphatidylethanolamine (DOPE) dissolved 1% in hexane for large membranes and 0.1% in hexane in the pipette configuration.

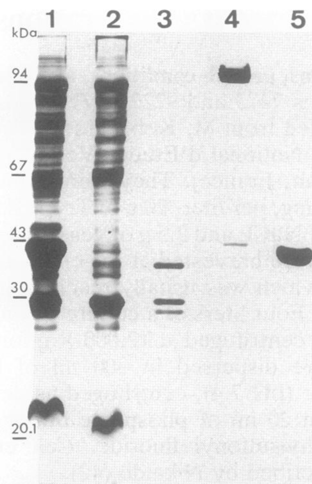

FIG. 1. SDS-PAGE analysis of protein H extraction under the trimeric form. Lanes: 1, SDS extract (100°C) of the cell envelope fraction; 2, first Sarkosyl extract (37°C) of the cell envelope; 3, second Sarkosyl extract of the cell envelope; 4, SDS extract (37°C) in the presence of 0.5 M NaCl of the Sarkosyl residue of the cell envelope; 5, same sample as in lane 4 but heated at 100°C for 10 min. Molecular masses are indicated on the left. Protein bands were stained with Coomassie brilliant blue R250. The temperatures indicated above in parentheses are those at which the extracts were performed.

The protein to be tested, solubilized in 2.5% β-octyl glucoside, was added to the *cis* side (2 ml) of one of the above-described configurations, typically at 10 ng/ml. As a control, we checked that there was no activity from bare membranes or after the addition of detergent alone (at a maximum final volume/volume dilution below 10^{-4}).

RESULTS

Purification of protein H. For the purification of protein H in the trimeric form, different combinations of detergents were tested for the selective and sequential extractions of proteins from murein-protein complexes. The procedure described in details in Materials and Methods gave the best results with respect to yield and purity of protein H. Treatment of isolated envelopes two times with 2% Sarkosyl released a fairly large amount of proteins, but most of protein H remained associated with the insoluble murein fraction. Protein H was then almost quantitatively extracted with 2% SDS in 0.5 M NaCl–5 mM EDTA. Though the solution obtained by this procedure was highly enriched in protein H (Fig. 1), a further step was necessary for protein H to be purified to homogeneity. This was achieved by size exclusion chromatography in the presence of 0.1% SDS and 0.3 M NaCl (Fig. 2). Protein H was thus obtained in the trimeric form (~110 kDa), which could be converted to the denatured monomeric form (37 kDa) upon heating at 100°C (Fig. 1). Average recovery was about 34% of the amount of protein H in isolated cell envelopes, and the purity was >98%, as estimated by scanning densitometry of silver-stained gels.

For the purification of protein H in the monomeric, denatured form, a slightly different procedure was used (see Materials and Methods). Progress in the purification and protein losses through the different steps of the procedure are illustrated in Fig. 3. Average recovery was about 25% of

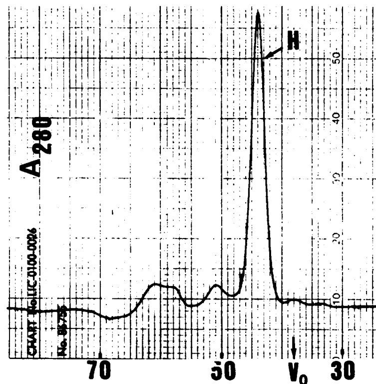

FIG. 2. Purification of trimeric protein H by size exclusion chromatography. The protein H-enriched fraction (~9.5 mg of protein) obtained after selective extraction of the *P. multocida* 9222 cell envelope with Sarkosyl and SDS at 37°C, in the presence of 0.5 M NaCl and 5 mM EDTA, was fractionated at 25°C on an AcA44 column as described in the text. The column was equilibrated with 50 mM sodium phosphate buffer (pH 7.4) containing 0.1% SDS and 0.3 M NaCl. The fractions corresponding to the major peak (H) contained protein H trimers. A_{280} is indicated in arbitrary units. V_0, void volume determined with dextran blue.

protein H in isolated cell envelopes, and the purity was also >98%. As indicated above, it was also possible to obtain monomers of protein H by heat denaturation of trimers in the presence of SDS. However, in that case, lipopolysaccharide (LPS) molecules were still found in the protein preparation. In contrast, protein H purified directly in the monomeric form (first procedure) was virtually LPS free, since KDO, a specific marker of LPS, was no longer detected in the preparations.

Molecular mass and subunit composition of protein H. As often observed in the case of porins, the trimeric form of protein H was resolved by SDS-PAGE in the form of a ladder of several bands (101.6, 106.1, 110.7, 115.4, 127.1, and 140.1 kDa). As in the case of other bacteria (24), these bands probably correspond to the binding of increasing amounts of LPS per trimer. The molecular mass of protein H denatured by heating in the presence of SDS was measured by three different methods of SDS-PAGE. A polypeptide mass of 37 kDa (Fig. 3), very close to the 37.5 kDa reported by Lugtenberg et al. (30), was recorded for the proteins of both strains 9222 and 7473 with the method of Laemmli (gel of constant porosity and discontinuous buffer system). However, higher values were recorded when protein H was analyzed by electrophoresis in a gel gradient and continuous buffer system in the presence of SDS (not shown): 41.8 kDa for protein H of strain 9222 and 41.5 kDa for protein H of strain 7473. When electrophoresis was performed in the presence of SDS and 8 M urea, a single band corresponding to a molecular mass of 41.3 kDa was observed (not shown). It should also be noted that in SDS-PAGE, trimer preparations always contained trace amounts of a 34-kDa component (apparent molecular mass in the Laemmli system) converted to a 37-kDa form upon boiling. This 34-kDa band probably represents undenatured monomers of protein H which, because of their more compact shape, migrate faster than denatured (unfolded) monomers.

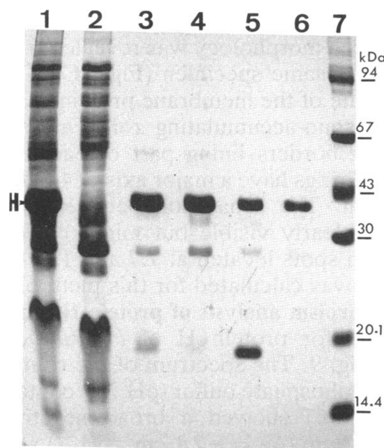

FIG. 3. SDS-PAGE analysis of the purification of protein H under the monomeric form from *P. multocida* 9222. Lanes: 1, SDS extract (100°C) of the cell envelope; 2, Sarkosyl extract (37°C) of the cell envelope; 3, SDS extract (100°C) of the Sarkosyl-insoluble fraction of the cell envelope; 4, SDS extract (100°C) of the Sarkosyl and deoxycholate-insoluble fraction of the cell envelope; 5, SDS extract (100°C) of the envelope fraction unsoluble in SDS–0.5 M NaCl; 6, protein H purified in the form of monomers by size exclusion chromatography (see Materials and Methods for details); 7, protein standards (molecular masses are indicated on the right). Protein bands were silver stained.

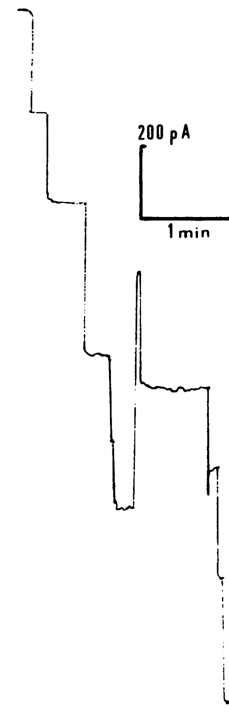

FIG. 4. Stepwise decrease in membrane current after the addition of protein H to the aqueous phase at a final concentration of 10 ng/ml and reduction of applied voltage from 150 to 50 mV. A high voltage was needed to obtain efficient incorporation. The membrane was formed from POPC/DOPE (7/3) planar bilayers.

Ionic conductance induced by protein H in planar lipid bilayers. The addition of 10 ng of protein H per ml to the *cis* side of large planar lipid bilayers (diameter, 125 μm) subjected to 100 to 150 mV led within 10 to 20 min to the development of a large transmembrane current. When the applied voltage was reduced to 50 mV, for example, the

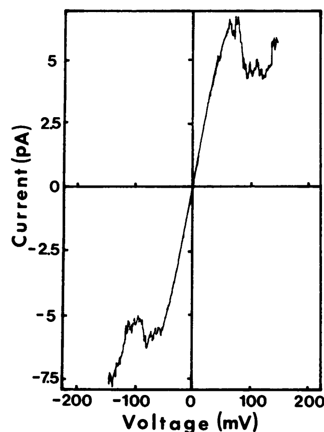

FIG. 5. Current-versus-voltage plot for a protein H-doped membrane submitted to a slow-voltage ramp. The aqueous phase contained 10 ng of protein H per ml and 1 M KCl in 25 mM HEPES buffer (pH 7.0).

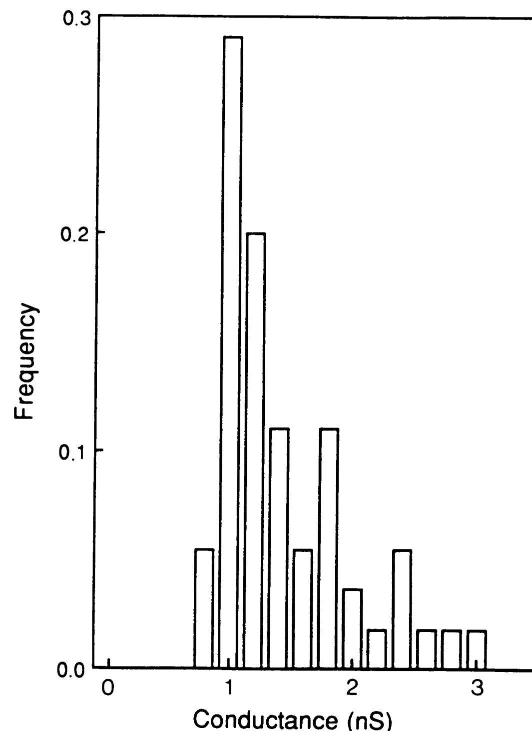

FIG. 7. Histogram of conductance events (example of trace shown in Fig. 6) in 1 M KCl. The total number of steps examined was 60. The average single-trimer conductance was of 1 nS.

current decreased stepwise (Fig. 4), with a staircase pattern typical of porins. These events were not observed when only surfactant (presently β-octyl glucoside) was added at the same concentration.

A useful representation allowing assay of voltage dependence is the macroscopic current versus voltage curve. The doped membrane was thus continuously swept by a triangular voltage ramp at a slow rate (1.5 mV/s) between −150 and +150 mV. After equilibration with the same protein amount as specified above, macroscopic conductance was high and ohmic in the −50- to +50-mV range, while further increasing the absolute voltage led to a current reduction in both quadrants (Fig. 5). Thus, these channels were readily open around 0 mV and tended to close at high voltage.

The small area at the tip of a patch clamp pipette reduces the membrane impedance and noise and allows the isolation of single events. This method was therefore used to analyze protein H single-channel conductance. Figure 6 shows single-trimer current fluctuations between closed and open states (duration of the order of several hundred milliseconds) induced by the incorporation of protein H into a preformed POPC/DOPE bilayer. The steady-state holding voltage was 50 mV, and the amplitude histogram shows a frequency for the open level of about 20%, with a mean unitary current at 50 pA, i.e., a single-trimer conductance of 1 nS in 1 M KCl (Fig. 7).

Image analysis of two-dimensional crystal arrays of protein H. Membrane vesicles reconstituted from purified protein H and phospholipids do not display any regular arrangement when observed by either negative staining or cryoelectron microscopy. In contrast, treatment with phospholipase A_2 yields well-ordered crystalline arrangements (Fig. 8A). The lattice parameters of the arrays which were computed from Fourier transforms of electron micrographs of negatively stained material gave a hexagonal pattern with a lattice constant of 4.2 nm and faint spots at 7.7 and 12.0 nm (Fig. 8B). The unit cell morphology was revealed from correlation averaging of the same specimen (Fig. 8C). On the picture, the trimeric state of the membrane protein is clearly visible. Indeed, three stain-accumulating zones are surrounded by stain-excluding borders lining part of each "black hole." These elliptical rings have a major axis of 4.0 nm and a minor axis of 3.5 nm. The separation between each trimer is, however, not clearly visible but might correspond to the faint diffraction spots located at 7.7 and 12.0 nm. A resolution of 2.5 nm was calculated for this picture.

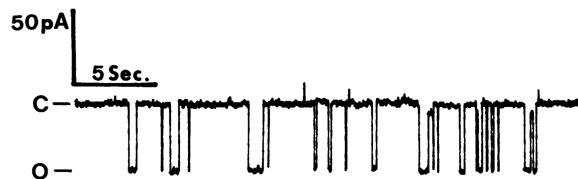

FIG. 6. Single-channel current recording after incorporation of protein H into POPC/DOPE (7/3) planar bilayers. The membranes were formed at the tip of patch clamp pipettes. Protein H was added to a final concentration of 10 ng/ml, and the voltage applied was 50 mV. Both sides contained 1 M KCl. C and O, closed and open conductance levels.

Circular dichroism analysis of protein H. Far-UV circular dichroism data for protein H of *P. multocida* 9222 are presented in Fig. 9. The spectrum of the trimeric protein in 25 mM sodium phosphate buffer (pH 7.0) containing 1% SDS and 0.25 M NaCl showed a broad negative peak with minimum at 217 nm ($\Delta\varepsilon = -2.1$ cm^{-1} M^{-1}) and a crossover at 205 nm. Upon heating of the solution at 100°C for 10 min, a decrease in $\Delta\varepsilon$ (i.e., $\Delta\varepsilon$ still more negative) was observed, and the spectrum exhibited two minima at 205 nm (−4.6 cm^{-1} M^{-1}) and 220 nm (−3.5 cm^{-1} M^{-1}), respectively, with a crossover at 198 nm. Protein H was also analyzed after solubilization with 2.5% β-octyl glucoside. In that case, the spectrum (not shown) was very similar to that recorded for protein H solubilized by SDS without boiling, i.e., a minimum at 218 nm ($\Delta\varepsilon = -3.2$ cm^{-1} M^{-1}) and a crossover at 205 nm.

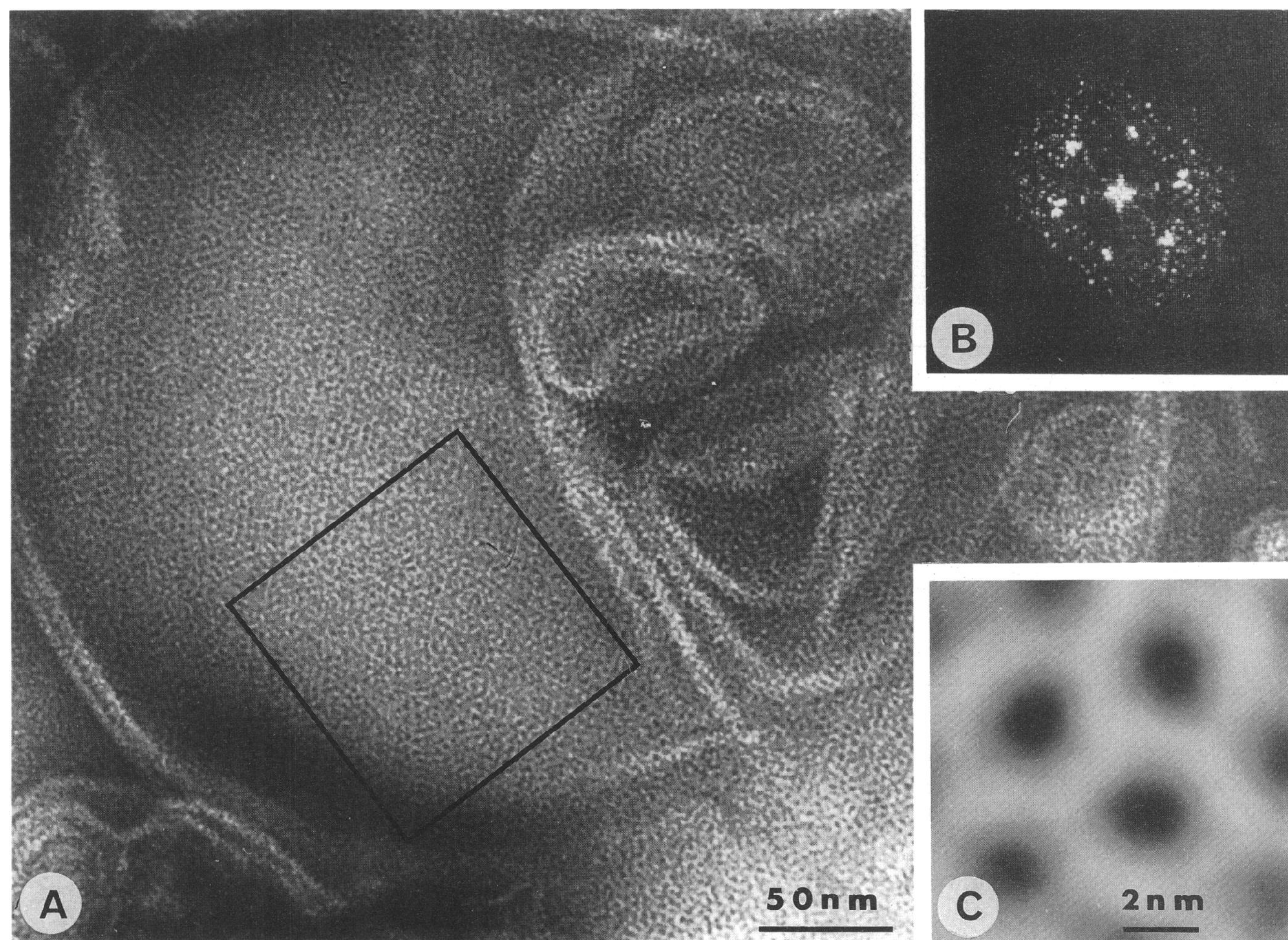

FIG. 8. Electron microscope analysis of two-dimensional crystals of protein H. (A) Electron micrograph of a two-dimensional protein array obtained after phospholipase A_2 treatment of reconstituted proteoliposomes and negative staining with uranyl acetate. Magnification, ×360,000. (B) Computed Fourier transforms obtained from a selected area of panel A. The geometry of the reciprocal lattice is hexagonal, with diffraction peaks extending to 4.2 nm. These reflections appear as doublets very probably due to a superposition of almost-in-register underlying subunits. (C) Two-dimensional projection map of a membrane protein trimer calculated from the average of 164 images and threefold symmetrized. Three stained (dark) zones are surrounded by a stain-excluding (light grey) border which corresponds to the membrane protein.

These data indicate that protein H solubilized in the form of trimers by SDS or β-octyl glucoside contains a large amount of β sheets. Boiling in SDS, which leads to the dissociation of the trimers (see above), resulted in a loss of β structure with a correlative increase in α helix and irregular structure.

Amino acid composition of protein H. The amino acid compositions of proteins H purified from *P. multocida* 7473 and 9222 (Table 1) were compared with the compositions of 15 bacterial porins (Table 2). Protein H exhibits several properties shared by all of the porins listed in Table 2: low proline content, high glycine content, absence of cysteine, and a negative hydropathy index indicating a hydrophilic overall composition. Two striking differences are noteworthy: (i) both proteins H contain, similar to porin P2 of *Haemophilus influenzae* and porin class 2 of *Neisseria meningitidis* but in contrast to the other porins listed in Table 2, a high content of lysine; and (ii) both proteins H have a much lower content of carboxylic amino acids (Asx and Glx) than do the enterobacterial porins OmpC, OmpF, and PhoE.

The use of the Marchalonis and Weltman index (8, 33) revealed that protein H of strain 7473 is, as expected, very closely related to protein H of strain 9222 ($S\Delta Q$ = 10.6). Furthermore, protein H is more closely related to the porins of *H. influenzae* and *Neisseria* spp. ($34.1 < S\Delta Q_{av} < 45.7$; Table 2) than to the enterobacterial porins ($71.0 < S\Delta Q_{av} < 114.3$). The 40-kDa porin of *Bordetella pertussis*, with an $S\Delta Q_{av}$ value of 77.2, occupies an intermediate position between the two groups defined above.

N-terminal amino acid sequence of protein H. Proteins H purified from *P. multocida* 7473 and 9222 were sequenced over 36 residues from their N termini by the Edman method. As expected, the sequences of the two proteins (Fig. 10) were very similar, differing by only two residues located at positions 24 (Leu versus Ile) and 61 (Met versus Val) in the alignment diagram. A striking feature of the N-terminal sequence of protein H in comparison with those of 15 porins is that it contains the residue stretch 1 through 9 shared by the general porins of enterobacteria and porin P2 of *H. influenzae* but missing in *Neisseria* porins. In the 40-kDa

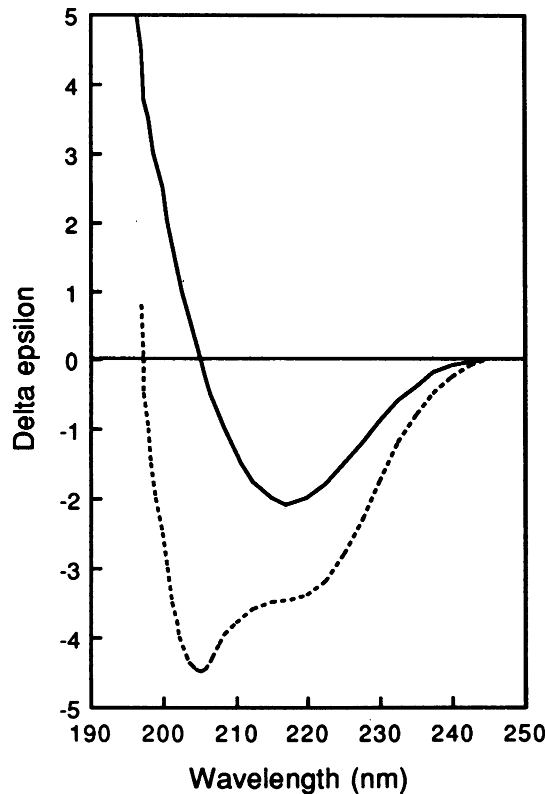

FIG. 9. Circular dichroism spectra of protein H. Protein H (0.125 mg/ml) was dissolved in 25 mM sodium phosphate buffer (pH 7.0) containing 1% SDS and 0.25 M NaCl. Dissociation of the trimers and denaturation of protein H were achieved by boiling the solution for 10 min. Shown are data for native (——) and denatured (.....) protein.

porin of *B. pertussis*, the missing part corresponds to residues 1 to 7.

Specifically, in terms of identity (percentage of sequence similarity calculated by taking into account only identical amino acids) and in terms of homology (percentage of sequence similarity based upon both identical and homologous residues), the N-terminal sequence of protein H is more closely related to those of the general porins of the enterobacteria/*Haemophilus* cluster than to the porins of the *Neisseria*/*Bordetella* cluster.

DISCUSSION

Since protein H of *P. multocida* shares several properties with porins, notably noncovalent attachment to murein (31), we attempted to purify the protein by methods similar to those described for other bacterial porins (42). Two different procedures that allow protein H to be obtained in the form of trimers or as fully denatured monomers were developed. In both cases, purification was achieved to homogeneity (purity of >98%) by size exclusion chromatography after selective extraction with detergents. For purification of the trimeric form, it proved of utmost importance to perform the extraction with SDS at room temperature in the presence of 0.5 M NaCl. Indeed, as for many bacterial porins (22), the quaternary structure of protein H was stabilized by a high ionic strength. Thus, to avoid the spontaneous dissociation of the trimers, the chromatography must also be performed in the presence of salt. In contrast, for purification of the monomers, extraction was performed at 100°C in the presence of EDTA, which enhanced the denaturation of the protein by SDS. Circular dichroism spectra of protein H showed that, similarly to porins, the conversion of trimers into monomers by heat denaturation in SDS resulted in the loss of β-sheet structure. It is noteworthy that monomers obtained by direct purification (in contrast to those obtained by denaturation of purified trimers) were virtually free of LPS, which might

TABLE 1. Comparison of the amino acid compositions of proteins H of *P. multocida* 7473 and 9222 with the compositions of porins P2 of *H. influenzae* and OmpC of *E. coli*

Amino acid	Composition (mol%)[a]			
	P. multocida protein H		*H. influenzae* P2	*E. coli* OmpC
	7473	9222		
Asp	12.57	10.86	12.32	18.20
Thr	4.69	6.08	7.04	6.94
Ser	5.60	4.71	4.99	4.62
Glu	7.68	8.96	11.14	9.25
Pro	1.51	0.43	0.88	0.87
Gly	12.18	11.97	11.73	13.87
Ala	8.21	9.21	7.04	6.94
Cys	0	0	0	0
Val	9.23	9.32	7.04	6.07
Met	0.67	0.80	0.29	0.87
Ile	3.09	2.47	4.40	2.89
Leu	7.90	7.92	7.04	6.36
Tyr	4.55	5.09	6.74	8.38
Phe	5.01	4.58	3.81	5.49
His	1.30	1.21	2.05	0.29
Lys	8.95	9.21	8.80	4.33
Arg	4.04	4.46	4.69	3.76
Trp	2.82	2.72	0	0.87

[a] The compositions of protein H from strain 9222, porin P2, and porin OmpC are taken from references 7, 18, and 36, respectively.

TABLE 2. Quantitative comparison of the amino acid compositions of a series of 15 bacterial general porins with the compositions of proteins H of *P. multocida* 7473 and 9222

Porin	Bacterial species	Reference	Subdivision	HI[a]	$S\Delta Q_{av}$[b]
P2	*Haemophilus influenzae*	18	γ-3	−0.69	42.4
OmpF	*Escherichia coli* K-12	21	γ-3	−0.55	79.2
OmpC	*E. coli* K-12	36	γ-3	−0.66	107.8
PhoE	*E. coli* K-12	44	γ-3	−0.68	92.8
NmpC	*E. coli* K-12	4	γ-3	−0.55	71.0
OmpC	*Salmonella typhi*	48	γ-3	−0.65	114.3
PhoE	*Klebsiella pneumoniae*	52	γ-3	−0.65	65.6
PhoE	*Enterobacter cloacae*	52	γ-3	−0.65	80.8
40 kDa	*Bordetella pertussis*	28	β-2	−0.30	77.2
PIA	*Neisseria gonorrhoeae*	6	β-3	−0.61	37.8
PIB	*N. gonorrhoeae*	14	β-3	−0.45	44.6
Class 1	*Neisseria meningitidis*	1	β-3	−0.49	41.5
Class 2	*N. meningitidis*	41	β-3	−0.54	34.1
Class 3	*N. meningitidis*	56	β-3	−0.49	34.5
PorA	*N. meningitidis*	23	β-3	−0.49	45.7

[a] HI, hydropathy index as defined by Kyte and Doolittle (25); negative values indicate hydrophilic compositions.

[b] $S\Delta Q_{av} = 0.5\ (S\Delta Q_{9222} + S\Delta Q_{7473})$. $S\Delta Q_{7473}$ and $S\Delta Q_{9222}$ are the $S\Delta Q$ values obtained by comparing the amino acid compositions of the different proteins listed with, respectively, protein H of *P. multocida* 7473 and protein H of *P. multocida* 9222. It should be noted that the extent of relatedness is in inverse ratio to $S\Delta Q$. See the text for definition of $S\Delta Q$ and comments.

```
Porins
         1                                       2 0                            3 6
P2       A V V Y N  N E G T N V E L - G G - - R L S - - I I A E Q S N - - - - - -
H        A T V Y N  Q D G T K V D G N G S V - R L - - - I L K K E K D - - - - - -
H        A T V Y N  Q D G T K V D G N G S V - R L - - - L L K K E K D - - - - - -
OmpC     A E I Y N  K D G N K L D L F G K V D G L H - - Y F S D D K G - - - - - -
OmpC     A E V Y N  K D G N K L D L Y G K V D G L H - - Y F S D N K D - - - - - -
PhoE     A E I Y N  K D G N K L D V Y G K V K A M H - - Y M S D N A S - - - - - -
PhoE     A E V Y N  K N A N K L D V Y G K I K A M H - - Y F S D Y D S - - - - - -
PhoE     A E V Y N  K N G N K L D V Y G K V K A M H - - Y I S D D D T - - - - - -
NmpC     A E I Y N  K D S N K L D L Y G K V N A K H - - Y F S S N N A - - - - - -
OmpF     A E I Y N  K D G N K V D L Y G K A V G L H - - Y F S K G N G - - - - - -
PIA      - - - - - - - - - D V T L Y G T I K A G V E T S R S V A H H - - - - - -
PIB      - - - - - - - - - D V T L Y G A I K A G V Q T Y R S V E H T - - - - - -
cl. 3    - - - - - - - - - D V S L Y G E I K A G V E T S R S V F H Q - - - - - -
cl. 2    - - - - - - - - - D V T L Y G T I K A G V E V S R - V K D AGTY - - -
cl. 1    - - - - - - - - - D V S L Y G E I K A G V E G - R N I Q A QLTEQPQ
PorA     - - - - - - - - - D V S L Y G E I K A G V E G - R N F Q L QLTEPP-
40 kDa   - - - - - - - E T S V T L Y G I I D T G I - G Y N D V D F K - - - - - -

         3 7    4 0                                    6 0                  7 2
P2       - - - - S T V D N Q K Q - - - - - - - - - Q A G A L R N Q G S RFHI KAT
H        - - - - - - - - - - - - - - - - - - - - - K R G D L V D N G S
H        - - - - - - - - - - - - - - - - - - - - - K R G D L M D N G S
OmpC     - - - - - - - - - - - - - - - - - - - - - S D G D Q T Y M - - RI GFKGE
OmpC     - - - - - - - - - - - - - - - - - - - - - V D G D Q T Y M - - RLGFKGE
PhoE     - - - - - - - - - - - - - - - - - - - - - K D G D Q S Y I - - RFGFKGE
PhoE     - - - - - - - - - - - - - - - - - - - - - K D G D Q T Y V - - RFGI KGE
PhoE     - - - - - - - - - - - - - - - - - - - - - K D G D Q T Y V - - RFGFKGE
NmpC     - - - - - - - - - - - - - - - - - - - - - D D G D T T Y A - - RLGFKGE
OmpF     - - - - - - E N S Y G - - - - - - - - - - G N G D M T Y A - - RLGFKGE
PIA      - - - GA Q A D R V K - - - - - - - - - - T A T E I A D L G S KI GFKGQ
PIB      - - - DG K V S K V E - - - - - - - - - - T G S E I A D F G S KI GFKGQ
cl. 3    - - - NG Q V T E V T - - - - - - - - - - T A T G I V D L G S KI GFKGQ
cl. 2    - - - KA Q G G K S K - - - - - - - - - - T A T Q I A D F G S KI GFKGQ
cl. 1    VTNGV Q G N Q V K V T K A K S R - I R T K I S D F G S FI GFKGS
PorA     - SKS - Q P - Q V K V T K A K S R - I R T K I S D F G S FI GFKGS
40 kDa   - VKGA N A D D S D F K Y N H S R F G M I N G V Q N G S RWGLRGT
```

FIG. 10. Comparison and alignment of the N-terminal sequence of protein H with the N-terminal sequences of a series of bacterial porins. Regions of identity with protein H of *P. multocida* 9222 are blocked together. The proteins used for this comparison (see Table 1) are, from top to bottom, porin P2 (*H. influenzae*), protein H (*P. multocida* 9222), protein H (*P. multocida* 7473), OmpC (*Salmonella typhi*), OmpC (*E. coli*), PhoE (*E. coli*), PhoE (*Klebsiella pneumoniae*), PhoE (*Enterobacter cloacae*), NmpC (*E. coli*), OmpF (*E. coli*), PIA (*N. gonorrhoeae*), PIB (*N. gonorrhoeae*), class 3 (*N. meningitidis*), class 2 (*N. meningitidis*), class 1 (*N. meningitidis*), porin A (*N. meningitidis*), and 40-kDa protein (*B. pertussis*).

prove useful in experiments in which LPS interference must be avoided (e.g., immunological studies).

Since protein H is tightly bound to murein and may be purified by procedures similar to those used for bacterial porins, it was tempting to determine whether this protein was able to form transmembrane aqueous channels. The use of the planar lipid bilayer technique revealed that incorporation of protein H into the membrane increased the permeability stepwise. In Fig. 4 we show a stepwise decrease in membrane current following a reduction of the applied voltage because a high voltage was first needed to obtain efficient incorporation of the protein into the membrane. The observed staircase pattern is typical of porins (15) and indicates that protein H forms water-filled transmembrane channels. An apparent single-channel conductance of 1 nS, slightly smaller than those reported for enterobacterial porins (see reference 15 for a review), was recorded by using the patch clamp technique. However, taking into account the trimeric arrangement of protein H with one channel per monomer (see below), the actual single-channel conductance of porin H is in fact one-third of the recorded value, i.e., 0.33 nS. In the case of porins, pore diameters have been extrapolated from single-channel measurements (3). Since this method often underestimates the true diameters of the channels (22), it was not used in this study.

The current through protein H pores was directly proportional to the applied voltage in the −50- to +50-mV range but was partially reduced beyond these values. This behavior (negative resistance) is similar to that described for OmpF porin of *Escherichia coli* (27), but only with bilayers prepared according to the method of Montal and Mueller (38), whereas those prepared according to the method of Mueller et al. (40) (decane instead of hexane for solubilizing lipids) failed to demonstrate any voltage dependence for this porin (27). Regulation of porin activity in bacteria is still a controversial problem (see reference 15 for a review), though recent reports suggest that pores of the outer membrane might be regulated by physiological stimuli (5). The work described here supports the conclusion that protein H behaves in planar lipid bilayers in the same way as do other bacterial nonspecific porins and thus is probably not voltage regulated in vivo.

Essentially three shapes have been proposed for porin channels (see reference 16 for a review): (i) each porin trimer

FIG. 11. Hydropathy (A and B) and β-sheet amphiphilicity (C and D) of the N-terminal sequence of protein H. The 36-residue N-terminal sequences of proteins H of *P. multocida* 7473 (A and C) and 9222 (B and D) were scanned with an 11-residue-wide window. See the text for comments.

FIG. 12. Tentative model for the topology and the transmembrane folding of the N-terminal sequence of protein H of *P. multocida*. Protein H of strain 9222 (3) was compared with porins PhoE (1) and OmpF (2) of *E. coli*. The N termini of the three proteins are localized in the periplasmic space.

contains three distinct channels (i.e., one channel per monomer), (ii) there is a single channel per trimer, and (iii) there are three channels per trimer which are open on the outer face of the membrane and coalesce into a single channel opening on the periplasmic face of the membrane (11). However, determination of the three-dimensional structure of the porin of *R. capsulatus* (53) and generalization to other nonspecific bacterial porins (45) indicate that probably only the first shape (i.e., one channel per monomer) is correct. Image analysis of negatively stained two-dimensional crystal arrays of protein H revealed only trimers containing one pore per monomer. In naturally occurring porine crystals, the separation between adjacent trimers is usually visualized as a stain-accumulating zone. However, such a boundary was not observed in the case of protein H, likely because of a very tight packing of the trimers resulting from phospholipid degradation in the proteoliposomes by phospholipase A_2 treatment. It should be stressed that in this study no LPS was added to the protein H reconstitution system. Therefore, because of the contribution of LPS in reconstitution of lipid-porin membranes (20), it should prove useful to perform similar experiments with *P. multocida* LPS. Thus, at the present stage of our study on protein H structure, the data obtained appear to be in agreement with current concepts of porin structure. An improved description of the protein will require the use of more sophisticated electron microscope methods or the obtention of three-dimensional crystals suitable for X-ray diffraction analysis.

The amino acid composition of protein H is typical of nonspecific bacterial porins: a highly negative hydropathy index, high glycine content, low proline content, and lack of cysteine. It was confirmed by quantitative comparisons of amino acid compositions that protein H is related to bacterial general porins, and notably to the porins of the *Neisseria* spp. and porin P2 of *H. influenzae* (Table 2). However, comparison of N-terminal sequences indicated that in terms both of identity and homology, protein H is more closely related to the enterobacterial general porins and less related to the porins of the neisseriae. Though the similarities of N-terminal sequences do not necessarily reflect similarities of whole sequences, the latter data are in better agreement with the taxonomy of the purple bacteria, since *P. multocida* belongs together with the enterobacteria and *H. influenzae* to the γ-3 subgroup, whereas the neisseriae are classified within subgroup β-3 (9, 55). Taken together, amino acid composition and sequence data strongly suggest that among the porins thus far described in the literature, protein P2 of *H. influenzae* is the porin to which protein H is the most closely related. The N-terminal sequence of protein H does not contain any significantly hydrophobic stretch of amino acids (Fig. 11A and B). However, the nine-residue segment Val-11 to Leu-19 exhibits the characteristics of a potential amphipathic β sheet (Fig. 11C and D). Thus, by analogy to porins of known topography (23, 45, 53), this segment might represent the first transmembrane β strand of protein H (Fig. 12).

In conclusion, data concerning the structure and function of protein H show that this protein is capable of forming water-filled transmembrane channels. Furthermore, comparison of amino acid compositions and N-terminal sequences reveals that this protein is related to the superfamily of nonspecific bacterial porins.

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