JOURNAL OF BACTERIOLOGY, Dec. 1983, p. 1006–1011
0021-9193/83/121006-06$02.00/0

Vol. 156, No. 3

Purification and Reconstitution in Lipid Bilayer Membranes of an Outer Membrane, Pore-Forming Protein of *Aeromonas salmonicida*

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Received 17 June 1983/Accepted 9 September 1983

We have purified a major outer membrane protein from *Aeromonas salmonicida*. This 42-kilodalton protein shared several physical characteristics with enterobacterial porins in that it was noncovalently associated with the peptidoglycan, it was released from the peptidoglycan in the presence of 0.1 M NaCl and sodium dodecyl sulfate, and its mobility on sodium dodecyl sulfate-polyacrylamide gels was dependent on the solubilization temperature before electrophoresis. When added to the aqueous solution bathing a planar bilayer membrane it caused the conductance of the membrane to increase by several orders of magnitude. At lower protein concentrations, single channels with an average conductance of 1.6 nS in 1 M KCl were incorporated into the membrane in a stepwise fashion. Evidence that the protein formed a large, relatively nonselective, water-filled channel was obtained by performing single-channel experiments at different NaCl concentrations and in a variety of different salts. Current through the channel was a linear function of the applied voltage, and no evidence of voltage gating was observed. In addition, we obtained evidence for a 43-kilodalton channel-forming protein in the outer membrane of *A. hydrophila* with a similar single-channel conductance as the 42-kilodalton protein in 1 M NaCl.

The outer cell envelope of gram-negative bacteria contains three layers, an inner membrane, a peptidoglycan layer, and an outer membrane (20). The outer membrane has been shown to be a barrier for the penetration of a wide variety of compounds (18) while at the same time permitting the size-dependent passage of small molecules (8, 16). This molecular-sieving action of the outer membrane is accomplished by certain proteins with monomer molecular weights in the range of 33,000 to 45,000 which are present in high copy numbers in the outer membrane (2, 8). These proteins, termed porins (20), have been found in several different species of bacteria (8, 20) and appear to form water-filled channels which facilitate the passage of hydrophilic substrates (2, 20). To date, only a limited number of bacteria have been characterized with respect to their porin composition (2, 20).

Within the family *Enterobacteriaceae* the porins that have been examined share the following characteristics: noncovalent association with the peptidoglycan that is maintained in the presence of sodium dodecyl sulfate (SDS); a retention of their native oligomeric (trimer) form in the presence of SDS (12, 20); and release from the peptidoglycan by solutions of high ionic strength containing SDS. Using as methods either leakage of defined sugars from reconstituted vesicles (16), vesicle swelling assays (H. Nikaido, Methods Enzymol., in press), or planar lipid bilayers (2), the channel diameter for this type of porin has been estimated to be between 1.2 and 1.6 nm. Porins from non-enterobacterial species usually do not display the same type of peptidoglycan association as enteric organisms as defined methodologically, although they are clearly peptidoglycan associated (9) as judged by other methods. Furthermore, they often do not form SDS-resistant oligomers (7, 8). In addition, the channels formed from these proteins are often significantly larger (2, 7, 19; R. E. W. Hancock, L. S. Zalman, and H. Nikaido, unpublished data cited in reference 11). For protein F of *Pseudomonas aeruginosa*, the channel diameter has been estimated at 2.2 nm (1), whereas *Neisseria gonorrhoeae* porin has a similar large diameter (9).

As part of our studies on the fish pathogens *Aeromonas salmonicida* and *Aeromonas hydrophila* (11, 13), we isolated and characterized the

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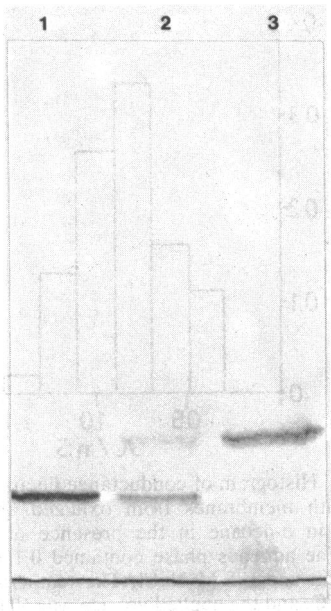

FIG. 1. Purified 42K protein of *A. salmonicida*. The 42K protein was purified as described in the text and subjected to SDS-polyacrylamide gel electrophoresis. The gel system contained 7.5% acrylamide. The protein preparation (15 μg) was heated at either 100°C (lane 1), 60°C (lane 2), or 37°C (lane 3) for 30 min before electrophoresis. Note that at a solubilization temperature of 60°C both the presumed oligomeric and monomeric forms of the protein are present.

outer membranes (13, 17). Major proteins, with apparent molecular weights ranging between 35,000 and 45,000, were found in the outer membranes of both of these organisms (13, 17). In this report we show that these two *Aeromonas* species contain peptidoglycan-associated proteins with characteristics similar to those found within the family *Enterobacteriaceae*. One of these, a protein with an apparent molecular weight of 42,000 in the outer membrane of *A. salmonicida*, was purified and shown to form water-filled channels in planar bilayer membranes with characteristics similar to those previously described for several enterobacterial porins (2; H. Nikaido, in press).

MATERIALS AND METHODS

Bacterial strains and culture conditions. *A. salmonicida* Ah440 was described previously (13). *A. hydrophila* Ah65 was isolated from rainbow trout (*Salmo gairdneri*) at the University of Victoria. Culture conditions were the same as those used in a previous study (13).

Purification of outer membranes and peptidoglycan-associated proteins. Outer membranes were obtained and analyzed by SDS-polyacrylamide gel electrophoresis as described previously (13). Peptidoglycan-associated proteins were obtained by solubilizing cell envelopes in buffer containing 2% (wt/vol) SDS, 10% (wt/vol) glycerol, and 10 mM Tris-hydrochloride (pH 7.4) at 30 or 37°C for 30 min. The pellet obtained after centrifugation at 100,000 × *g* for 45 min was resuspended in the same buffer containing 0.1 M NaCl. The supernatant obtained after centrifugation as described above was used in the bilayer reconstitution studies.

Black lipid bilayer experiments. The methods used for black lipid bilayer experiments have been described previously in detail (3, 4). The chamber used for bilayer experiments was made of Teflon and consisted of two compartments with a thin Teflon divider perforated by a small hole with an area of 0.1 to 2 mm^2. Membranes were formed by painting a 1 to 2% solution of oxidized cholesterol (kindly provided by R. Benz) in *n*-decane across the aperture separating the compartments. Bilayer formation was indicated by the membrane turning optically black when viewed in incident light. Electrical measurements were conducted by placing Ag-AgCl electrodes into the aqueous solutions on both sides of the membrane and then applying a voltage to the membrane with an Omnical 2000 (W-P Instruments, Inc.) voltage source. The current fluctuations were monitored by a Tektronix 511A storage oscilloscope after being amplified by a Keithley 427 preamplifier and were recorded on a model 8373-10 (Cole-Parmer Instruments, Inc.) recorder.

RESULTS

Purification of the 42K protein from *A. salmonicida*. By performing differential solubilizations of the outer membrane proteins of *A. salmonicida* and *A. hydrophila*, we obtained evidence that there was at least one major protein in both of these organisms that remained with the insoluble peptidoglycan after extraction

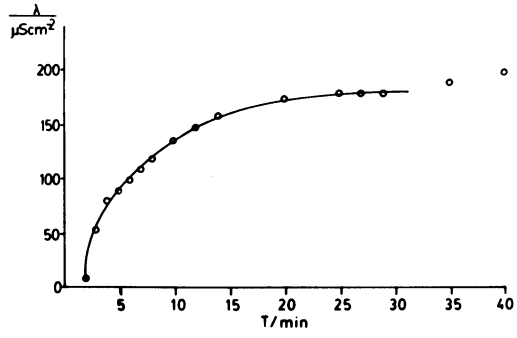

FIG. 2. Specific membrane conductance λ as a function of time t after the formation of the membrane from oxidized cholesterol–*n*-decane. The aqueous phase contained 1 M NaCl (pH 6.5) and 5 ng of isolated 42K protein; the temperature (T) was 25°C; and the applied voltage was 10 mV. The size of the hole separating the two compartments was 2 mm^2. In control experiments where either no protein was added or heat-inactivated protein was added, no significant increase in membrane conductance was observed.

with buffer containing SDS. For *A. salmonicida*, resuspension of the insoluble peptidoglycan pellet in buffer containing 0.1 M NaCl and 2% SDS followed by centrifugation to pellet the peptidoglycan resulted in release of a single major protein with an apparent molecular weight of 42,000 (42K protein) into the supernatant (Fig. 1). Although there may be several minor protein contaminants, if a single contaminant was present at a level of 10% or greater it would have been easily visualized. The apparent molecular weight of the 42K protein was related to the temperature of solubilization before polyacrylamide gel electrophoresis (Fig. 1) in a manner similar to that demonstrated previously for other porins (18).

Macroscopic conductance experiments. To determine whether the 42K protein was capable of increasing the membrane conductance in a manner similar to that observed for other porins, we performed macroscopic conductance experiments. In control experiments, when membranes were formed and no protein was added to the salt solutions bathing the membrane (the aqueous phase), the membrane conductance did not increase significantly over a period of 30 min. In addition, after heating the 42K protein at 100°C for 5 min in the presence of SDS to denature it to its monomer form (Fig. 1, lane 1) before addition of the protein to the aqueous phase, no major change in membrane conductance over time resulted. However, when the undenatured (Fig. 1, lane 3) 42K protein was added to the aqueous phase, the conductance of the lipid bilayer membrane increased by many orders of magnitude. A typical experiment is shown in Fig. 2. Membrane conductance increased rapidly during the first 20 min and then more gradually until membrane breakage occurred. The conductance recorded 20 min after the addition of the 42K protein to the aqueous phase increased when increasing amounts of the 42K protein were added. The addition to the chamber of SDS alone at 100-fold-higher concentrations caused no increase in conductance, indicating that the SDS in the protein solution (0.04%) did not account for the conductance changes.

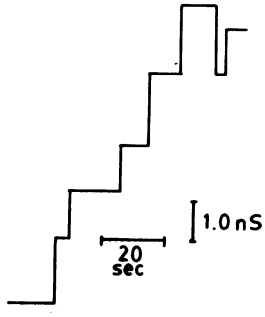

FIG. 3. Conductance steps caused by the addition of 0.1 ng of 42K protein to the aqueous phase bathing a membrane of oxidized cholesterol in *n*-decane. The aqueous solution contained, in addition to the 42K protein, 1 M KCl (pH 6.5). The hole between the two compartments was 0.1 mm^2, the temperature was kept at 25°C, and 50 mV was applied across the membrane.

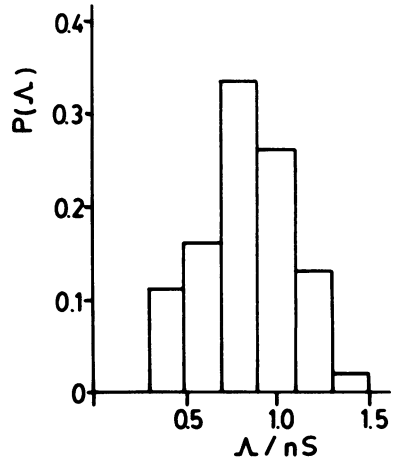

FIG. 4. Histogram of conductance fluctuations observed with membranes from oxidized cholesterol dissolved in *n*-decane in the presence of the 42K protein. The aqueous phase contained 0.1 ng of the 42K protein and 0.5 M NaCl. The temperature was kept at 25°C, and the applied voltage was 50 mV. $P(\Lambda)$ is the probability of a given conductance increment (Λ) taken from recorder traces such as those shown in Fig. 3. An average conductance increment (Λ) of 0.82 nS was obtained by measuring 148 conductance increments.

Single-channel experiments. When a low amount (0.1 ng) of the 42K protein was added to the aqueous phase bathing the membrane bilayer, the membrane conductance increased in a stepwise fashion (Fig. 3). These discrete conductance increments did not occur when SDS was added to the chamber in the absence of the 42K protein or if heat-inactivated 42K protein was used. In the latter case however, there was an increase in the noise level of the background conductance, possibly due to denatured protein associating with the lipid bilayer. Most of the single steps observed in experiments with the native 42K protein occurred in an upward direction, indicating the incorporation of a channel into the bilayer. Downward-directed steps corresponding to a decrease in the membrane conductance (possibly due to inactivation or loss from the membrane of a pore) were uncommon, but when observed appeared to be of the same magnitude as the upward steps. The rise time of

TABLE 1. Average conductance increment (Λ) measured on membranes made from oxidized cholesterol—*n*-decane in the presence of 42K protein[a]

Salt	Conc (M)	Avg single-channel conductance (Λ) (nS)	Specific conductance (σ) of the given salt solution ($mS \cdot cm^{-1}$)	Λ/σ (10^{-8} cm)	No. of conductance steps recorded
NaCl	0.1	0.19	9.4	2.0	71
	1.0	1.3	84	1.5	68
KCl	1.0	1.6	110	1.4	201
$MgCl_2$	0.5	0.78	64	1.2	100
$Tris^+Cl^-$	0.5	0.40	30	1.3	134
Na^+HEPES^-(pH 9)[b]	0.5	0.35	18	1.9	147

[a] The pH of the salt solutions was between 6 and 7 if not otherwise indicated; the temperature was 25°C; the applied voltage was 50 mV. Λ was determined by averaging a large number of individual conductance steps.
[b] HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

the single conductance step was always faster than the time resolution of the preamplifier and was smooth without any indication of smaller intermediate steps. As observed for other porins (3, 4), the single-channel conductance steps were not uniform in size, but distributed over a certain range. A histogram of the conductance steps observed for the 42K protein in the presence of 0.5 M NaCl is presented in Fig. 4. The average conductance increment (Λ) was determined for five different NaCl concentrations between 0.1 and 1.5 M. It was found to be a linear function of the concentration of NaCl in the aqueous phase (data not shown), a result expected for a large water-filled pore. Further evidence that the conductance pathway was an aqueous channel was obtained by determining the average conductance increment of the 42K protein in the presence of anions and cations of different size and charge. The data listed in Table 1 show that although the bulk conductance of the salts (σ) in the absence of a membrane varied by a factor of about 8, the Λ/σ ratio varied by less than 2. These data would indicate that the single-channel conductance of the 42K protein followed reasonably well the conductivity of the salt solutions bathing the membrane, suggesting that these solutions are also filling the pore.

Further evidence that the 42K protein was able to form large aqueous channels was obtained from current-voltage experiments. The current-voltage characteristics of an large water-filled hole should be ohmic, i.e., the increase in current should be directly proportional to the applied voltage. This behavior was observed for the 42K protein for applied voltages up to 120 mV (Fig. 5).

In addition to the above studies with the isolated 42K protein from *A. salmonicida*, we examined a similar SDS-NaCl extract of peptidoglycan-associated proteins obtained from *A. hydrophila*. A major protein in this extract (molecular weight, 43,000) displayed physical properties similar to those of the 42K protein; i.e., apparent peptidoglycan association, release from the peptidoglycan by high salt and SDS concentrations, and lower mobility on SDS-polyacrylamide gel electrophoresis after solubilization at low temperatures. A histogram of single-channel conductance increases observed after the addition of this extract to the 1 M NaCl solution bathing a lipid bilayer membrane is shown in Fig. 6. The results revealed that a pore with an average conductance of 1.33 nS was present. It seemed likely that the major protein

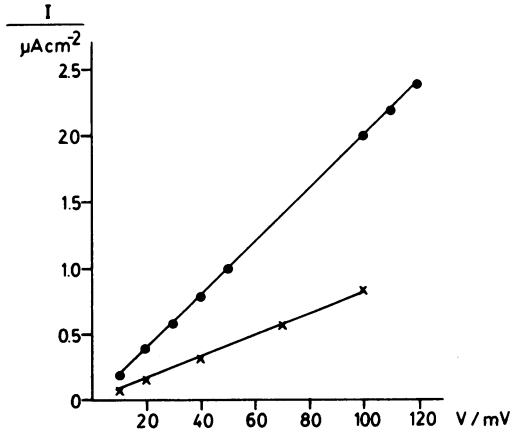

FIG. 5. Current versus voltage characteristics of a membrane formed from oxidized cholesterol in *n*-decane with 0.1 ng of 42K protein added to an aqueous phase containing 1 M NaCl. The temperature was kept at 25°C. The results from two different membranes are shown.

with an apparent molecular weight of 43,000 was the porin; however, since this preparation contained other minor protein contaminants, we did not perform extensive characterizations.

DISCUSSION

Since the early work of Nakae (15) and Nikaido (20), it has become evident that the outer membrane of gram-negative bacteria contains proteins which facilitate the passage of small hydrophilic substrates. These proteins, appropriately termed porins, have been found in several different species of bacteria (2; H. Nikaido, in press) and all appear to be aqueous channels (1, 2). Within the family *Enterobacteriaceae*, the porin proteins that have been studied share several distinctive characteristics (1, 7, 20). For example, the channels of enterobacterial porins appear to be narrower than the porin channels of other bacteria (1, 19) (with one exception, the 0.6-nm protein P channel of *P. aeruginosa* [9]). This has led to the proposal that these narrow channels may be located exclusively within the family *Enterobacteriaceae* (19).

Aeromonas spp. are known to share several characteristics with members of the family *Enterobacteriaceae* (5). We found that there were proteins present in the outer membrane of *A. salmonicida* and *A. hydrophila* that were similar to the *Escherichia coli* porins with respect to their behavior on SDS-polyacrylamide gel electrophoresis, apparent molecular weights, and mode of peptidoglycan association. Since similar properties have been reported for the major porin proteins of family *Enterobacteriaceae* (12, 14), we examined the major peptidoglycan-associated 42K protein of *A. salmonicida* for its pore-forming ability.

The studies presented in this paper provide evidence that the 42K protein from *A. salmonicida* is a porin which forms aqueous channels in lipid bilayers with an average conductance of 1.6 nS in 1 M KCl. If it is assumed that the pore is a cylinder with spherical cross sections and is filled with an aqueous solution of the same conductance as the external bulk phase, according to the formula $\Lambda = \sigma \pi r^2/l$ the average effective pore diameter d (=2r) may be estimated for a pore length l (which can be assumed to be functionally equal to the width of the membrane or ca. 7.5 nm [6]). From the data in Table 1, the average effective channel diameter for the 42K protein would be around 1.2 nm. This is in close agreement with channel diameters that have been calculated for the porins of *E. coli* from studies by using either the vesicle swelling assay (19; H. Nikaido, in press) or black lipid bilayers (2, 3).

It appears, therefore, that porins like those of the *Enterobacteriaceae* can exist outside of this family. The occurrence of this type of pore in *Aeromonas* spp. may be a further indicator of their close relationship to members the family *Enterobacteriaceae* (5).

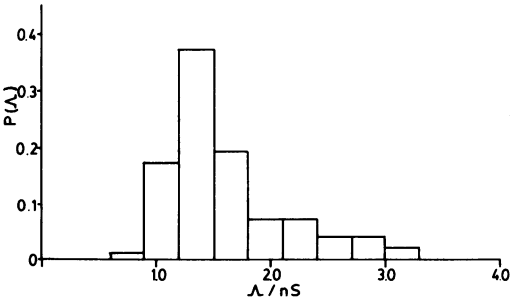

FIG. 6. Histogram of conductance fluctuations observed with membranes from oxidized cholesterol dissolved in *n*-decane in the presence of the 43K protein obtained from *A. hydrophila*. The aqueous phase contained 0.1 ng of the 43K protein and 1.0 M NaCl (pH 6.5). The temperature was kept at 25°C, and the applied voltage was 50 mV. $P(\Lambda)$ is the probability of a given conductance increment (Λ) taken from recorder traces similar to those presented in Fig. 3. An average conductance increment (Λ) of 1.33 nS was obtained by measuring 134 conductance increments.

ACKNOWLEDGMENTS

We would like to thank Roland Benz for kindly providing us with his expertise in setting up a planar bilayer apparatus in the laboratory of R.E.W.H. and for the donation of a Faraday chamber.

This work was supported by separate grants from the Natural Sciences and Engineering Research Council of Canada to R.E.W.H. and J.T.B.

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