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Lipopolysaccharide-Free *Escherichia coli* OmpF and *Pseudomonas aeruginosa* Protein P Porins Are Functionally Active in Lipid Bilayer Membranes

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***Escherichia coli* porin OmpF and *Pseudomonas aeruginosa* porin protein P were eluted from sodium dodecyl sulfate-polyacrylamide gels. The resultant porin preparations were found to be devoid of detectable lipopolysaccharide (LPS) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining for LPS, direct enzyme-linked immunosorbent assays with LPS-specific monoclonal antibodies, and 2-keto-3-deoxyoctulosonic acid assays. The average conductances, ionic selectivities and incorporation rates of the electroeluted porins were identical to those of their conventionally purified counterparts. These data suggest that LPS is not required per se for porin function.**

The outer membranes of gram-negative bacteria are known to contain water-filled channels formed by a class of proteins termed porins, which allow the diffusion across these membranes of hydrophilic compounds up to specific molecular size cutoffs (14). Study of these proteins often involves the detergent isolation and purification of the porins, followed by examination of their functional properties under a variety of experimental conditions in liposome or black lipid bilayer systems (14). The information gathered with such techniques has considerably expanded our understanding of porins.

Many porin molecules isolated from outer membranes are tightly associated with lipopolysaccharide (LPS) (5, 14, 16, 18). This association has been suggested as being necessary for the reconstitution of functional pores in planar membrane systems (12, 18). However, we feel that the requirement for LPS in the formation of functional porin molecules has not yet been fully defined.

In an attempt to resolve this problem and to obtain porins in their native trimeric configuration for use as immunogens in monoclonal antibody production, we developed a technique which allows for the purification of functional porin trimers devoid of detectable LPS.

MATERIALS AND METHODS

Strains, growth conditions, and protein isolations. *Escherichia coli* CGSC 6047 (JF733; *proC-24 ompA-252 his-53 ompC-262 purE-41 ilv-277 met-65 lacY-29 xyl-14 rpsL-97 cycA-1 cycB*? *tsx-63* λ^-) (4) and *Pseudomonas aeruginosa* H287 (ATCC 8689; prototroph) (9) were grown as previously described to obtain outer membranes containing protein OmpF and protein P, respectively (9, 11). Cells were harvested, outer membranes were obtained, and proteins were detergent extracted and purified as previously described (9, 11). The purified protein preparations were loaded onto sodium dodecyl sulfate(SDS)-polyacrylamide gels (1.5 mm thickness) containing 14% acrylamide and electrophoresed by a methodology previously described (7). Solubilization of the proteins in gel sample buffer was performed at 20°C in a solubilization mixture without 2-mercaptoethanol (7). A portion of the gel was stained with Coomassie blue to locate the appropriate trimeric bands, and a corresponding region was cut from the unstained, unfixed portion of the gel. The porin-containing gel was crushed with a glass rod to increase its surface area and was then placed in dialysis tubing in phosphate-buffered saline containing 1% SDS. The proteins in the preparations were electroeluted at 50 V for 2 h at 4°C in a Bio-Rad transblotting cell (Bio-Rad Laboratories, Mississauga, Ontario, Canada). The buffer used for electroelution was that previously described by Towbin et al. for electroblotting (19). Alternatively, the proteins were electroeluted in this blotting buffer (19) containing 0.1% SDS for 5 h at 50 V, followed by a change of buffer and an additional 14 h of electroelution at 10 V. After electroelution, the supernatant in the dialysis sac was removed, dialyzed against water containing 0.1% SDS, and concentrated with solid polyethylene glycol 20,000 (Sigma Chemical Co., St. Louis, Mo.) to approximtely 0.5 mg of protein per ml.

ELISAs and antibodies. Enzyme-linked immunosorbent assays (ELISAs) were done as previously described with monoclonal antibodies specific for the LPSs of the strains used (8, 10, 13). The LPS of the *P. aeruginosa* strain was detected with monoclonal antibody MA3-6, which is specific for an antigenic site in the LPS core region, and monoclonal antibody MA1-8, which is specific for an antigenic site in the O-antigen portion of the LPS (10, 13). Detection of LPS by SDS- polyacrylamide gel electrophoresis (PAGE) of OmpF preparations was done with monoclonal antibody MA6-1. Antibody MA6-1 was isolated as previously described (10) but with conventionally purified OmpF protein as the priming antigen. This antibody reacted strongly against conventionally purified OmpF and OmpC preparations and poorly against *E. coli* LPS preparations. On Western blots of LPS or conventionally purified OmpF preparations (Fig. 1A), it reacted only with a low-molecular-weight LPS band. From these data we concluded that MA6-1 interacted with an LPS epitope but that either its reactivity was enhanced when this epitope was presented in the context of the OmpF porins or it reacted specifically with a subfraction of LPS that associated with porins.

Chemical methods and black lipid bilayer system. SDS-PAGE followed by silver staining for LPS was done by the method of Tsai and Frasch (20). The detection limits of the gel system were 1.2 ng (0.24 pmol) for *E. coli* LPS and 10 ng

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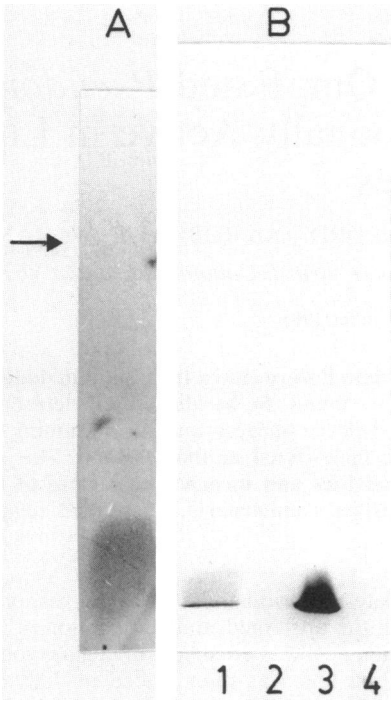

FIG. 1. (A) Demonstration of the specificity of monoclonal antibody MA6-1 for *E. coli* LPS. Conventionally purified OmpF porin (and associated LPS; see Table 1) was separated by SDS-PAGE and transferred to nitrocellulose by the Western blotting procedure (13, 19). The nitrocellulose blot was then incubated with monoclonal antibody MA6-1, followed by an alkaline phosphatase-labeled anti-mouse immunoglobulin antibody and a histochemical substrate for alkaline phosphatase, as previously described (13). The gel system used contained 14% arcylamide in the running gel. The diffuse stained band was identified as LPS on the basis of its mobility in the primary SDS gels and its coelectrophoresis with purified *E. coli* B LPS. The position of the OmpF porin on the blot, revealed by India ink stains of control blots, is shown by the arrow on the left-hand side of the gel. (B) SDS-PAGE of LPS associated with conventionally purified (lanes 1 and 3) and electroeluted (lanes 2 and 4) protein P (lanes 1 and 2) and protein OmpF (lanes 3 and 4). Gels (12% acrylamide) were run and stained by the method of Tsai and Frasch (20). Three micrograms of porin was loaded in each lane. No LPS was detected in the electroeluted porin preparations.

(1.1 pmol) for *P. aeruginosa* LPS. 2-Keto-3-deoxy-octulosonic acid (KDO) was assayed by the method of Osborn (15). This assay was less sensitive than the other assays described here, but could detect as little as 3 nmol of rough LPS (assuming two reactive KDO residues per molecule of LPS). Protein was assayed by the method of Sandermann and Strominger (17). Assay of the function of the conventionally purified and gel-eluted porins was done by using the black lipid bilayer system (2, 3) with 1.5% oxidized cholesterol–*n*-decane as the lipid.

RESULTS

Detection of LPS in the porin preparations. The amount of LPS in the porin preparations that had been electroeluted after SDS-PAGE was compared with that in conventionally purified porin preparations by three methods (Table 1). The data were expressed as molar ratios of LPS to protein. KDO, a constituent of the core regions of both *E. coli* and *P. aeruginosa* LPSs, was not detected in electroeluted porin preparations but was detected at a molar ratio of 0.48:1 (LPS/OmpF protein) in a concentrated, conventionally purified OmpF porin preparation.

TABLE 1. Measurements of LPS associated with conventionally purified and electroeluted porins

Protein	Assay	LPS (mol/mol of protein) associated with:	
		Conventionally purified protein	Electroeluted protein
P	SDS-PAGE	1.7	$<2 \times 10^{-2}$
	ELISA	1.1	$<3.8 \times 10^{-2}$
OmpF	KDO	0.48	ND[a]
	SDS-PAGE	0.54	$<4.3 \times 10^{-3}$
	ELISA		$<7.3 \times 10^{-3}$[b]

[a] ND, Not detectable.

[b] Since monoclonal antibody MA6-1 interacted poorly with isolated LPS but well with LPS which was associated with the OmpF porin, it was not possible to obtain accurate estimates of the actual amount of LPS associated with OmpF. Accurate relative contaminations of the electroeluted and conventionally purified OmpF proteins were obtainable (i.e., 16.5 ng of electroeluted OmpF protein gave an ELISA reading below background, whereas 0.25 ng of conventionally purified OmpF protein gave an ELISA reading 70% above background). Since the amount of LPS, as determined by KDO and SDS-PAGE analyses, in 0.25 ng of conventionally purified OmpF porin was 2.3 ng (0.46 pmol), we considered this to be the detection limit of the system. Thus, the value shown was obtained by assuming that 16.5 ng of electroeluted OmpF porin contained less than 2.3 ng of LPS.

As a more sensitive assay for the presence of LPS, SDS-PAGE of porin preparations followed by periodate oxidation and silver staining was done (Fig. 1B). Both conventionally purified porin preparations had detectable LPS, but none was observed in the electroeluted porin preparations (Fig. 1B). By performing periodate oxidation-silver staining gel analyses on dilutions of the conventionally purified porins and comparing these with similar analyses of pure LPS of known concentration, we were able to estimate the amount of LPS contaminating the porin preparations (Table 1) and the detection limit of this gel system (see Materials and Methods).

The ELISA provided a sensitive assay for the presence or absence of LPS in the porin samples. Using monoclonal antibodies specific for the LPS of each strain, we were able to detect LPS in the conventionally purified protein P and OmpF samples. The calculated LPS/protein P molar ratio was 1.1:1, a result consistent with the gel analyses described above. There was no reaction above background for either of the electroeluted porin preparations. The detection limit of the ELISA was 50 ng (5.5 pmol) for *P. aeruginosa* LPS. Owing to the specificity of monoclonal antibody MA6-1, we were unable to calculate a true detection limit for *E. coli* LPS; however, an extrapolated detection limit (Table 1, footnote *b*) of 2.3 ng (0.46 pmol) was calculated. Overall, the data summarized in Table 1 suggested that the electroeluted porin preparations contained less than 0.5 to 2 mol% LPS.

Lipid bilayer experiments. To assay the function of the electroeluted porins, the planar lipid membrane system was used (2, 3). This system offers the unique opportunity to examine individual molecular events, registering the stepwise incorporation of single porin molecules into the lipid bilayer. Table 2 shows the data accumulated with 1 M KCl as the bathing salt solution. The average single-channel conductances Λ of protein P were 235 pS and 234 pS for conventionally purified and electroeluted porins, respectively. Conventionally purified OmpF porin had an average single-channel conductance of 2.2 nS, whereas the average

TABLE 2. Function of coventionally purified and electroeluted porins reconstituted into lipid bilayer membranes

Porin	Purification procedure	Avg single-channel conductance in 1 M KCl (nS)	No. of single-channel events measured	Selectivity (ratio of K^+ permeability to Cl^- permeability)
OmpF	Conventional	2.2	314	5.89 ± 0.06
	Electroelution	2.1	695	5.50 ± 0.62
Protein P	Conventional	0.235	317	
	Electroelution	0.234	224	

single-channel conductance of electroeluted OmpF porin was 2.1 nS. These data indicated that there were no significant differences between electroeluted and conventionally purified porins with respect to their pore sizes. In addition, the average single-channel conductances obtained for both electroeluted and conventionally purified preparations of both porins were equal to those previously reported (2, 3, 9). The large number of individual channels examined (224 to 695) provided great confidence in the numbers obtained.

The incorporation rates (i.e., 11 ± 4.9 channels reconstituted per min per 10 ng of protein for a membrane 0.1 mm^2 in diameter) were equal for given quantities of the respective porins, whether they were conventionally purified or electroeluted. Thus, it was extremely unlikely that contamination of our electroeluted preparations with, e.g., 0.1% LPS-associated porin (i.e., below the detection limits of our assay systems) could explain the single-channel data obtained.

The ion selectivity of the OmpF porin was also examined (Table 2). There was no significant difference between electroeluted and conventionally purified porin preparations, and the data were quite similar to those obtained previously for preparations of the *E. coli* B OmpF porin (3).

DISCUSSION

In this paper we provide evidence that LPS is not essential for the function of *P. aeruginosa* and *E. coli* porin proteins. Identical average conductance measurements, incorporation rates, and ionic selectivities were seen in electroeluted and conventionally purified porin preparations. The electroeluted preparations of *P. aeruginosa* protein P and *E. coli* OmpF porins were not contaminated with detectable LPS, whereas the conventionally purified preparations were. The low detection limits found with the various methods permitted the conclusion that the electroeluted porins were contaminated with less than 1 mol of LPS per 100 mol of porin protein. This observation, combined with equal incorporation rates for conventionally purified and electroeluted porin preparations, demonstrated that LPS is not essential for pore formation or incorporation into the planar bilayer system. Furthermore, the LPS associated with conventionally purified porins did not apparently influence the channel size or selectivity of these porins.

In 1978, Schindler and Rosenbusch suggested that LPS was essential for the reconstitution of porin function in planar bilayers (18). Their data have become accepted and have been cited in major reviews (12, 14). These findings may be dependent on the reconstitution method used and, on the basis of the results reported here, do not reflect an absolute requirement for LPS. In addition, we previously suggested that the LPS of *P. aeruginosa* may alter porin function (1). Godfrey et al. also presented data suggesting that LPS influenced porin function in *P. aeruginosa* (6). In light of the data presented in this paper, it may be useful to reexamine the conclusions drawn from these studies.

The production of monoclonal antibodies to OmpF porin in our laboratory has been hindered by the high immunogenicity of the LPS associated with this porin. The electroelution technique has enabled us to make an LPS-free OmpF antigen for monoclonal antibody production. We hope that this will substantially increase our yield of clones directed at the OmpF protein. The data accumulated in this paper showing identical functions for electroeluted and conventionally purified porin preparations assure us that the electroeluted OmpF antigen is very similar to the conventionally purified OmpF antigen.

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