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Pseudomonas aeruginosa Outer Membrane: Peptidoglycan-Associated Proteins

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The *Pseudomonas aeruginosa* outer membrane was isolated with attached peptidoglycan and fractionated with Triton X-100, ethylenediaminetetraacetate, and lysozyme. The data suggest that major outer membrane proteins F, H2, and I are noncovalently associated with the peptidoglycan.

Recently, Mizuno and Kageyama (7) demonstrated that two of the major outer membrane proteins of *Pseudomonas aeruginosa*, proteins F and H (see reference 2 for nomenclature), remained associated with the peptidoglycan upon treatment of cells at 30°C with sodium dodecyl sulfate (SDS) in the presence of 0.1 M NaCl, suggesting that these were peptidoglycan-associated proteins. However, certain data suggested that this result deserved further examination. We recently demonstrated that protein H is in fact two distinct polypeptides, H1 and H2, and that lipoprotein I of *P. aeruginosa*, equivalent to the Braun lipoprotein of *Escherichia coli* (5, 6), is extremely difficult to visualize in slab gels unless Mg^{2+} is added during or subsequent to sample solubilization (2). In addition, multimers of porin protein F were never seen on SDS-polyacrylamide gels, and the function of protein F as a hydrophilic pore was totally destroyed by SDS treatment (G. Decad, Ph.D. thesis, University of California at Berkeley, Berkeley, 1974), which suggests that, in contrast to results achieved with *E. coli* (8) or *Salmonella typhimurium* porins (12), SDS partially denatures the protein at low temperatures. In this study, the *P. aeruginosa* outer membrane was fractionated with Triton X-100-EDTA, a procedure which allowed porin protein F to retain its function in model membrane systems (4). This enabled an examination of peptidoglycan association under conditions where protein denaturation was limited. Although not presented here, similar results were achieved with toluene-permeabilized whole cells instead of purified outer membranes or by substituting 1% sodium deoxycholate for Triton X-100 in the described experiments.

Exposure of *P. aeruginosa* outer membranes to Triton X-100 solubilized 24% of the lipopolysaccharide (LPS) and 38% of the total protein (Table 1), including a large number of minor polypeptides, some of which seemed to be contaminating cytoplasmic membrane proteins. Of the major outer membrane proteins, only proteins H2 and I were partially solubilized by Triton X-100. If outer membranes were predigested with lysozyme before Triton X-100 treatment, proteins H2 and I were nearly 100% solubilized.

The Triton X-100-insoluble outer membrane was extracted twice with Triton X-100 in the presence of EDTA. The combined Triton-EDTA-soluble fractions contained about two thirds of the LPS and one third of the total outer membrane protein and were highly enriched in major proteins D1 (when induced by growth of cells on glucose [2, 3]), D2, G, and H1, containing 82 to 99% of each of these proteins (Table 1). The addition of 0.2 M Mg^{2+} to the combined Triton-EDTA-soluble fractions caused precipitation of proteins D1, D2, G, and H1 (and the small amount of F present) out of Triton X-100 solution, although many minor proteins remained in solution. When whole sucrose-stabilized cells were extracted with 10 mM EDTA-20 mM Tris-hydrochloride (pH 8.0) in the absence of Triton X-100, the resultant EDTA-soluble fraction (equivalent to the Pr-LPS of Rogers et al. [10]) contained little of any major outer membrane proteins (< of the total protein of Pr-LPS), with the exception of protein E (about 7% of the total). Thus, it may be concluded that the major outer membrane proteins described in Table 1 are integral membrane proteins which do not contribute to the unusually high EDTA susceptibility (10) of *P. aeruginosa*.

The Triton-EDTA-insoluble fraction contained only four major outer membrane proteins, E, F, H2, and I, in substantial amounts (Fig. 1, gel A). Extensive washing with Triton-EDTA did not solubilize these proteins, although it did remove all remaining bound LPS and phospholipids, as judged by the lack of 2-keto-3-deoxy-

TABLE 1. *Solubilization of major outer membrane proteins of P. aeruginosa in Triton X-100*[a]

Protein or LPS	Amount released (%)			
	Triton soluble	Triton-EDTA soluble	Triton-lysozyme soluble	Residue
LPS	24	67	0	9
Protein				
Total proteins	38	32	22	8
D1 (glucose induced)	0	99	0	1
D2	0	82	0	18
E	0	33	2	65
F (porin)	0	7	77	16
G	0	95	0	5
H1	0	92	0	8
H2 (lipoprotein)	30	8	55	7
I (lipoprotein)	49	16	32	3

[a] Outer membranes with attached peptidoglycan were prepared as described previously (2). The procedure for fractionation of outer membranes with Triton X-100 was a modification of the method of Schnaitman (11). Outer membranes were suspended at 10 mg of protein per ml (starting volume) in 2% (vol/vol) Triton X-100–20 mM Tris-hydrochloride buffer (pH 8.0), and the samples were sonicated at a setting of 5 with a Biosonik sonicator (Bronwill Scientific Inc., Rochester, N.Y.). The insoluble membrane was separated from the Triton-soluble supernatant by centrifugation at 170,000 × *g* for 60 min in a Beckman 50 Ti rotor. The pellet was suspended at the starting volume in 2% Triton X-100–20 mM Tris-hydrochloride (pH 8.0)–10 mM EDTA by sonication as above and centrifuged to yield the first Triton-EDTA-soluble supernatant. The pooled supernatant after two such extractions was the Triton-EDTA-soluble fraction, and the pellet was the Triton-EDTA-insoluble fraction. The pellet was suspended at half the starting volume in water and treated with 1 mg of egg white lysozyme (Sigma Chemical Co., St. Louis, Mo.) per ml for 30 min at 37°C. Subsequent to this it was diluted to the starting volume to give a final suspension containing 2% Triton X-100, 20 mM Tris-hydrochloride, and 10 mM $MgSO_4$ and then sonicated and centrifuged as above. The pellet was considered the Residue, and the supernatant was considered the Triton-lysozyme-soluble preparation. Results are expressed as the percentage (weight/weight) of the total of each individual protein found in the starting outer membrane fraction or as the percentage (weight/weight) of LPS (estimated as 2-keto-3-deoxyoctanate [2]). The amounts of each major outer membrane protein in the individual fractions were estimated from densitometer traces after Coomassie brilliant blue R250 or G250 staining of SDS-polyacrylamide gel electrophoretograms (2). Control experiments demonstrated that the intensity of staining was linear with protein concentration for the five major outer membrane proteins studied, and we could usually account for greater than 90% of each major protein. The data presented are the means of five experiments, with standard deviations varying from 5 to 15% of the given figures, and should be considered illustrative rather than quantitative.

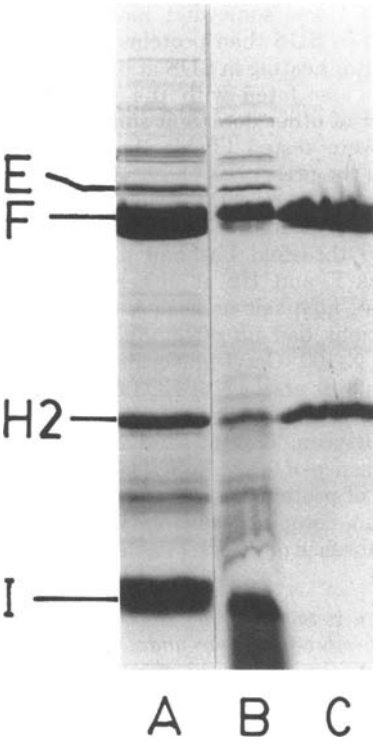

FIG. 1. *SDS-polyacrylamide gel electrophoresis of various peptidoglycan-associated fractions. (A) Triton-EDTA-insoluble outer membrane isolated as described in Table 1, footnote a; (B) SDS-insoluble whole cell preparation isolated by the method of Mizuno and Kageyama (7); (C) SDS-insoluble whole cell preparation extracted three times with 2% (wt/vol) SDS–0.1 M NaCl at 30°C for 30 min.*

octanate and readily extractable lipid phosphorous. In contrast, an SDS-NaCl-insoluble outer membrane fraction (Fig. 1, gel C) contained LPS as its major component (0.90 to 1.35 mg of LPS per mg of protein). Electron microscopy of Triton-EDTA-insoluble outer membrane fragments indicated an ordered matrix on the peptidoglycan (data not shown), although no such structure was seen for SDS-NaCl-insoluble fractions.

Lysozyme digestion of the peptidoglycan of the Triton-EDTA-insoluble fraction followed by Triton X-100 treatment released only three major outer membrane proteins, F, H2, and I, into the supernatant (Table 1), suggesting that they were peptidoglycan-associated proteins. The minor protein species and protein E of the Triton-EDTA-insoluble fraction were found in the residue after lysozyme-Triton treatment and probably represented Triton-insoluble aggregates. To exclude the possibility that any of the proteins in the Triton-EDTA-insoluble fraction were covalently bound to the peptidoglycan, we solubilized this fraction in 2% SDS–10 mM Tris-hydrochloride (pH 8.0) at 100°C before lysozyme treatment. In this case, all of the protein was found in the SDS-soluble fraction. Lysozyme treatment released no further proteins into Triton X-100 solution, demonstrating that F, H2, and I were left bound to the peptidoglycan.

Protein I was somewhat more susceptible to heating in SDS than proteins F and H2. However, after heating in SDS at 65°C, there was no protein associated with the peptidoglycan. A number of other detergent solubilization conditions were tested (Table 2). Treatment with SDS in the presence of high salt concentrations (0.5 M NaCl) or EDTA at 23°C reduced the amount of protein I on the peptidoglycan by two- to threefold but had a lesser effect on proteins F and H2 at either 23 or 45°C. In contrast, high salt or EDTA (not shown) concentrations had no effect on the solubility in Triton X-100. Octyl glucoside in the presence (Table 2) or absence of EDTA had little effect on the retention of proteins F, H2, and I by the peptidoglycan. Treatment at 23°C with SDS in the presence of 0.1 M $ZnSO_4$ led to the loss of 91 to 97% of proteins F, H2, and I from the peptidoglycan, compared with only a 12 to 28% loss in the absence of $ZnSO_4$ (Table 2). In agreement with this, it was observed that protein H2 did not appear on SDS-polyacrylamide gels after solubilization at low temperatures unless Zn^{2+} was present during solubilization. Based on these data, we suggest that NaCl and $ZnSO_4$ stimulated denaturation of the peptidoglycan-associated proteins by SDS rather than specifically affecting their peptidoglycan associations.

TABLE 2. *Solubility of Triton-EDTA-insoluble outer membrane proteins under various conditions*

Treatment[a]	Temperature (°C)[b]	% Total insoluble protein	% Protein remaining peptidoglycan associated[c]		
			F	H2	I
None		100	100	100	100
SDS	23	62	78	88	72
	35	42	49	56	23
	45	26	27	45	5
	55	10	2	15	0
	65	0	0	0	0
SDS-NaCl	23	48	75	58	24
	45	21	26	55	3
SDS-EDTA	23	51	64	64	29
	45	19	24	64	3
SDS-$ZnSO_4$	23	22	7	9	3
Octyl glucoside-EDTA	23	76	77	90	88
Triton-NaCl	23	89	100	100	100

[a] Concentrations used were as follows: 2% (wt/vol) SDS, 0.5 M NaCl, 10 mM EDTA, 0.1 mM $ZnSO_4$, 2.5% (wt/vol) octyl glucoside, and 2% Triton X-100. All samples contained 10 mM Tris-hydrochloride (pH 8.0) as a buffer and 10% glycerol.

[b] Solubilization was at 23°C for 30 min or 45°C for 15 min.

[c] Percentage precipitable by centrifugation at 180,000 × *g* for 60 min after solubilization as described. This was calculated from densitometer scans of SDS-polyacrylamide gel electrophoretograms of the proteins as discussed in Table 1, footnote *a*.

The above data confirm and extend the observations of Mizuno and Kageyama (7) in showing that proteins F and H2 are noncovalently associated with the peptidoglycan. Another major outer membrane protein, H1, which coruns with protein H2 on the SDS-polyacrylamide gel system of Mizuno and Kageyama (7), was shown here not to be peptidoglycan associated. The importance of this finding is that under specific induction conditions, protein H1 can become the major cellular protein (9). In addition, protein I was also shown to be noncovalently peptidoglycan associated. Whole cells and outer membranes were fractionated by the techniques of Mizuno and Kageyama (7) and analyzed by SDS-polyacrylamide gel techniques designed to visualize lipoprotein I (2). SDS treatment of whole cells left over 60% of protein I peptidoglycan associated (Fig. 1, gel B). A single additional SDS-NaCl treatment reduced peptidoglycan-associated protein I by 30 to 40%, whereas three consecutive SDS-NaCl treatments completely solubilized protein I but left over 50% of proteins F and H2 associated with the peptidoglycan (Fig. 1, gel C). The results support the above conclusion that NaCl promotes denaturation of protein I by SDS. Thus, there appears to be a peptidoglycan-associated form of protein I despite the lack of a covalently bound form of this protein in *P. aeruginosa* strain PAO1 (see above and reference 1).

In conclusion, we have demonstrated that proteins F, H2, and I are peptidoglycan-associated proteins under nondenaturing conditions. The techniques described here allowed purification of functional porin proteins F (4) and D1 (3). Although this study has not revealed the nature of the association between the peptidoglycan and proteins F, H2, and I, it does suggest a rather complex noncovalent association, probably involving more than one type of bond. In particular, the data presented in this paper stress the need for caution when using SDS to demonstrate peptidoglycan association.

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