An unusual linkage between polysaccharides and some major proteins from the outer membrane of *Escherichia coli*

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A small population of OmpA, a major protein from the outer membrane of *Escherichia coli*, was found covalently associated with either lipopolysaccharide or O-antigen polysaccharide. Radioactive oligosaccharide was elicited linked to OmpA after treatment of the membranes with periodate that hydrolyzed large sugars. Association of saccharides to OmpA could be enhanced by treatment of the outer membrane with $NaBH_4$.

(E. coli) *Outer membrane* *OmpA* *Lipopolysaccharide*

1. INTRODUCTION

Lipopolysaccharide (LPS) is one of the major components of the outer membrane (OM) of Gram-negative bacteria. A number of studies have demonstrated the close physical and physiological links between LPS and some proteins from the OM. Some proteins, like OmpF and OmpC porins, when purified, still retain a small but significant amount of LPS attached to them [1]. In addition, LPS is essential for the biological activity of OM proteins, e.g. LPS is needed for reconstitution of phage receptor activities of OmpA [2], OmpF [2,3] and other porins. Addition of exogenous LPS is also necessary for restoration of biological activity (channelling) of porins in a vesicle-reconstituted system [4]. Early work reported the existence of covalent bonds between LPS and envelope proteins [5]. These conclusions were derived from the fact that a fraction of LPS co-migrated with major OM proteins in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using SDS-solubilized OM. This fact, however, was not sufficient to demonstrate the existence of covalent linkage between LPS and proteins. Further studies showed that LPS could be separated from OM proteins by gel filtration chromatography [6]. It is now believed that the strong interaction between LPS and some OM proteins is due to chemical bonds of a non-covalent nature. For instance, binding studies of LPS and OmpA protein revealed that interaction takes place through the lipid A moiety of LPS, although the polysaccharide region also influences that interaction [7]. Direct interaction of the LPS polysaccharide part and OmpC protein has been reported by Yamada and Mizushima [8]. Here, we present data showing that a small fraction of OmpA protein interacts covalently with either a high M_r or a highly aggregated LPS fraction. This bond can be stabilized by $NaBH_4$. The physiological significance of this finding is discussed.

2. MATERIALS AND METHODS

2.1. *Bacterial strains and culture conditions*

Escherichia coli PL-2 (K12, *thi*-1, *rel* A1, λ−, *spo* T1, *gal* E28) was used throughout. Cells were

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grown in L medium [9] supplemented with 4 g/l D-glucose, 40 mg/l thiamine and 0.10 mM D-galactose, at 37°C under forced aeration. Cells were collected at the mid-log phase (A_{550} 0.6) by centrifugation at low speed after rapid cooling of the culture. For labelling of LPS, cells were allowed to grow up to an A_{550} = 0.6 as described before and then D-[1-^{14}C]galactose (50 Ci/mol, Amersham, Bucks, England) was added at a final concentration of 1 μCi/ml culture. Incubation of the culture was continued for a further 10 min and cells were then collected as described above.

2.2. *Isolation of OM*

OM was isolated by following the method of Osborn et al. [10] after EDTA-lysozyme treatment of the cells. The degree of cross-contamination of the OM, i.e. that coming from the cytoplasmic membrane, was less than 3% as judged by studying membrane markers as fully described in [11]. [^{14}C]Galactose-pelletable material was found 95% associated with the OM.

2.3. *Chemical modification of OM*

(i) 75 μl [^{14}C]galactose-labelled OM suspended in 100 mM TEA buffer, pH 8.0 (5 mg protein/ml), were mixed with 1 mg $NaBH_4$. The mixture was allowed to stand at room temperature for 1 h. The suspension was centrifuged in a Beckman airfuge at about 70000 rpm for 15 min. Pelleted OM was suspended in water. (ii) 100 μl non-radioactive OM suspension in 100 mM TEA buffer, pH 8.0 (5 mg protein/ml), were mixed with 10 mCi NaB[^{3}H]H_4 (5 Ci/mmol, Amersham). The reaction mixture was incubated at 0°C for 3 h and then centrifuged in the airfuge as before. Pelleted OM was resuspended in water. (iii) 100 μl of either borohydride-treated or untreated OM suspension in 200 mM sodium phosphate buffer, pH 7.2 (5 mg protein/ml), were mixed with sodium periodate (50 mM final concentration). The reaction mixture was incubated at room temperature for 1 h and then centrifuged and the pellet resuspended as described above.

2.4. *Removal of LPS*

LPS was removed from OM following the method of Westphal and Jann [12] developed for isolation of LPS. Briefly, OM (about 0.5 mg protein) suspended in 5 ml H_2O, was mixed with 5 ml aqueous phenol and incubated for 20 min at 70°C. The top aqueous phase was sucked off and discarded. The phenol phase was mixed with 5 ml H_2O and the mixture incubated as before. The extraction procedure was repeated 3 times. The final phenol phase was diluted with water and extensively dialyzed against water. The dialysate was finally lyophilized and the lyophilisate resuspended in a small amount of water.

2.5. *SDS-PAGE*

OM proteins were fractionated in an SDS-PAGE system following the method of Lugtenberg et al. [13]. After electrophoresis, gels were stained and destained [14] and prepared for fluorography as described by Bonner and Laskey [15]. Treated gels were exposed on pre-fogged Kodak X-Omat X-ray films.

3. RESULTS

Fig.1 shows a fluorograph of an electrophoretic pattern of D-[^{14}C]galactose-lablelled OM. Lane a contains untreated OM and, as can be observed, most radioactivity migrated as low-molecular mass components whilst part of the labelled LPS remained as either high-molecular-mass species or large aggregates near the top of the gels. It should be noted that the type of gel used (11%, w/v, initial acrylamide concentration) did not allow the resolution of most LPS species. Lane b of fig.1 shows the pattern of labelled LPS containing OM after treatment with periodate. As can be seen, this treatment produced the rupture and subsequent detachment of nearly all the labelled LPS from the OM. Particularly, molecular-mass-labelled material disappeared while, strikingly, 2 radioactive bands appeared by the middle of the electrophoretic run. The lower band corresponded to OmpA protein since it could be superimposed on Coomassie blue-stained OmpA (not shown).

Fig.2 displays the results obtained with phenol-treated OM. This OM was also obtained from D-[^{14}C]galactose-labelled cells and treatment of OM with phenol was performed to remove most LPS molecules. The fluorograph in lane a of fig.2 shows the results of the phenol treatment. As can be observed, the low-molecular-mass species of LPS were removed by the treatment. Subsequent treatment of phenol-treated OM with periodate

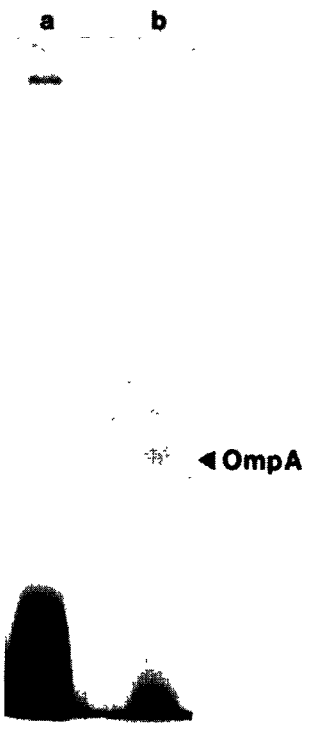

Fig.1. Fluorography of an SDS-PAGE gel of D-[^{14}C]galactose-labelled OM. Lanes: a, untreated OM; b, periodate-treated OM.

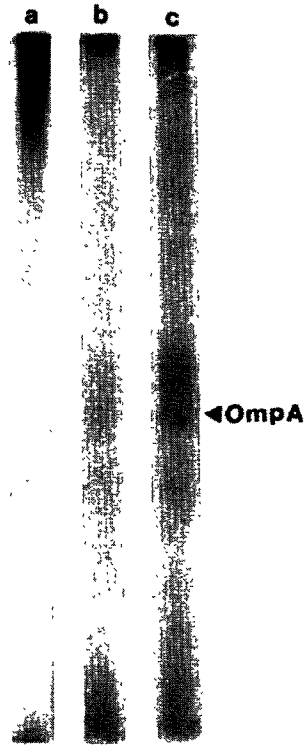

Fig.2. Fluorography of an SDS-PAGE gel D-[^{14}C]galactose-labelled OM. After preparation, OM was treated with phenol to remove LPS. Lanes: a, phenol-treated OM; b, periodate-treated OM; c, phenolized OM was first treated with $NaBH_4$, then washed extensively so that removal of the reducing agent was effected and finally treated with periodate.

removed most radioactivity from the top part of the gel (fig.2, lane b) while, as in fig.1, 2 diffuse bands appeared at the middle of the gel. The lower band corresponds to OmpA protein. Treatment of phenol-treated OM with $NaBH_4$ and then with periodate brought about a significant increase of the radioactivity associated to OmpA. It should be pointed out that, in our hands at least, treatment of OM with phenol produced a poor resolution of OM components in our gel electrophoresis system.

Fig.3 shows the effect of the treatment of isolated OM with NaB[^{3}H]H_4. Most radioactivity migrated near the front and might correspond to hydrolyzed products containing reducible ends. Apart from this broad band, the main band seen in the gel was associated with the OmpA protein (lane a). Treatment of NaB[^{3}H]H_4-treated OM with periodate dramatically increased the amount of radioactivity associated with OmpA (lane b). Other minor bands of an unknown nature that appear in the gel were not affected by periodate treatment.

4. DISCUSSION

Extraction of Gram-negative bacteria with either aqueous 1-butanol or trichloroacetic acid yielded LPS that contained proteinaceous material earlier called endotoxin protein [16]. Endotoxin protein was found to consist of at leat 13 OM proteins. Some protein material can be released by further phenol treatment while other material is partially released by proteolytic treatment of the LPS-protein complex [16]. The strong binding of proteins to LPS led earlier to the suggestion that such binding was of a covalent nature [5]. However,

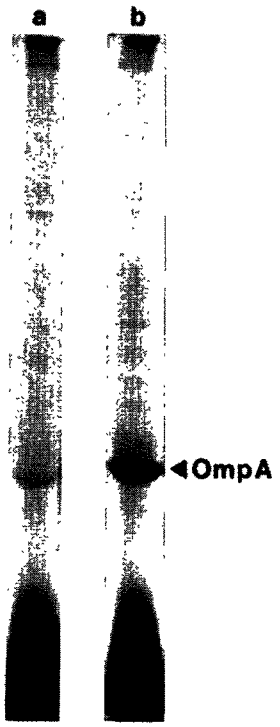

Fig.3. Fluorography of an SDS-PAGE gel of OM. Lanes: a, OM reduced by NaB[^{3}H]H$_4$; b, OM after reduction was extensively washed to remove the excess reagent and then treated with periodate.

further work showed that other shorts of bonds were implicated in LPS-protein association [6–8]. Clearly, co-migration of labelled LPS with proteins in SDS-PAGE gels does not prove that there are covalent bonds between LPS and proteins [6]. Nevertheless, the present results strongly suggest that a small amount of OmpA molecules was covalently bound either to high-molecular-mass species or to highly aggregated forms of either LPS, or O-antigen polysaccharide not covalently bound to lipid A [17,18]. When the OM polysaccharide was broken by periodate, an OmpA-associated radioactive band was elicitied (fig.1). Treatment of OM with phenol did not totally remove OmpA-associated radioactivity which was, again, elicited after periodate treatment. Further treatment with NaBH$_4$ substantially increased the amount of OmpA-associated radioactivity found in the gels (fig.2). The experiment shown in fig.3 clearly shows that the amount of radioactivity associated with OmpA greatly increased when NaB[^{3}H]H$_4$-treated OM was further treated with periodate.

These data raise some questions that are at present difficult to answer. The first concerns the nature of the chemical groups involved in the bond and the class of bond itself. The stabilization of the bonds by NaBH$_4$ suggests that Schiff bases, connecting both OmpA and polysaccharides, might be involved. If so, it is difficult to tell which element contains a reactive carbonyl group that could be involved in bond formation. Diedrich and Schnaitman [19] reported the existence of allysine residues in OM proteins. Allysine, derived from lysine by oxidative deamination, could serve to cross-link the protein to other structures containing amino groups. However, these data were not confirmed by Chen et al. [20], who sequenced OmpF protein and did not find allysine residues, although these authors speculated about the possibility that modified lysine could be present in such small amounts that it escaped detection. Some molecules of OmpA protein might contain reactive modified groups. We must point out that OmpA molecules which reacted with polysaccharide account for a small amount of the total OmpA as we did not see an increase of OmpA content after treatment of OM with periodate in Coomassie blue-stained gels (not shown). Finally, we believe that the formation of covalent but reversible bonds might contribute to the cohesion of OM structures and explain how protein material is always present in LPS preparations.

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