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Interactions of *Escherichia coli* Membrane Lipoproteins with the Murein Sacculus

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Bifunctional cross-linking reagents were used to identify cell envelope proteins that interacted with the murein sacculus. This revealed that a number of [^{3}H]leucine-labeled proteins and [^{3}H]palmitate-labeled lipoproteins were reproducibly cross-linked to the sacculus in plasmolyzed cells. The results suggested that most of the cell envelope lipoproteins, and not only the murein lipoprotein, mediate interactions between the murein sacculus and the inner and/or outer membrane of the cell.

The cell envelope of gram-negative bacteria includes the inner membrane, the outer membrane, and the murein layer that lies within the periplasmic space. The murein sacculus is the shape-determining component of the cell envelope and is responsible for the resistance of the cell to osmotic and mechanical stresses. In standard electron micrographs, it is visible as a dense submembranous layer that underlies the outer membrane both in intact cells and in isolated outer membrane preparations (6, 11, 13). Some of the cellular peptidoglycan is also located between the dense outer layer and the inner membrane (11, 18, 19).

Although the sacculus can be separated from other cell envelope components by treatment with hot sodium dodecyl sulfate (SDS), there are several reasons for believing that in living cells the murein interacts with other cell envelope molecules and that these interactions play an important role in cell envelope organization. This is most clearly shown for murein lipoprotein (MLP), an integral protein of the outer membrane (4). About one-third of the MLP of the cell is covalently bound to murein (12). In mutants that lack the lipoprotein or are defective in forming the covalent attachment to murein, the outer membrane pulls away from the cell to form outer membrane blebs located at cell poles and septation sites (10, 28, 29). Other outer membrane proteins appear to interact noncovalently with the murein sacculus. This conclusion is based on the observations that several outer membrane proteins remain associated with the sacculus unless the sacculus is treated with SDS at high temperatures and that OmpA and the free form of MLP can be chemically cross-linked to murein with bifunctional cross-linking reagents (7, 8, 24, 25). These interactions between outer membrane proteins and murein explain the observation that murein and outer membrane are recovered in a single peak when mechanically disrupted cells are subjected to isopycnic centrifugation (13, 27).

Several pieces of evidence suggest that inner membrane proteins also interact with murein within the cell envelope. First, several penicillin-binding proteins that play a role in murein biosynthesis and metabolism are recovered in the inner membrane fraction in standard membrane fractionation experiments (1, 26). Since the function of these proteins requires that they interact with murein in intact cells, it is reasonable to assume that they represent one class of murein-interactive inner membrane protein. A second line of evidence that suggests that some inner membrane proteins are likely to interact with murein comes from studies of zones of adhesion in plasmolyzed cells (2, 3, 5, 17). In these experiments, cells are exposed to hypertonic solutions of sucrose or other osmotically active solutes that do not enter the cytosol. This leads to the exit of water from the cytosol. The resulting inward pull causes the inner membrane to retract from the rigid murein-outer membrane layer. The adhesion zones represent sites where the inner membrane fails to pull away from the outer cell envelope layers. Since the murein sacculus is the only known structure that appears capable of resisting the inward pull imposed by the plasmolysis procedure, it is likely that the attachment sites reflect direct interactions between the inner membrane and the sacculus. The possibility also exists that the adhesion zones represent direct interactions between the inner membrane and the outer membrane.

In the present article, we have tried to further define membrane-murein interactions by using bifunctional cross-linking reagents to identify proteins that are in close proximity to murein in plasmolyzed cells.

(A preliminary report of a portion of this work has appeared previously [20].)

MATERIALS AND METHODS

Strains and growth conditions. Strain AB2497, a K-12 strain of *Escherichia coli*, was grown in supplemented minimal medium, labeled with [^{3}H]leucine or [^{3}H]palmitate, and harvested in the mid-exponential growth phase as previously described (20). For labeling with [^{14}C]*N*-acetylglucosamine, exponentially growing cells in proteose peptone beef extract medium (Difco) were grown for 20 min in the presence of 10 μM [^{14}C]*N*-acetylglucosamine (58 mCi/mmol).

Cross-linking procedure. Freshly harvested cells were suspended for 3 min at room temperature in 1 ml of 50 mM sodium phosphate (pH 7.4) containing 20% sucrose. A 0.125-ml volume of cross-linking reagent (dithio-bis-succinimidylpropionate [DSP] or dimethyl-3-3′-dithio-bis-propionimidate [DTBP], 80 mg/ml in dimethyl sulfoxide [DMSO]) was then added, and the suspension was allowed to stand for 30 min at room temperature. In all cases, the ratio of cross-linking reagent to protein was approximately 2:1 (wt/wt). When cell envelopes instead of intact cells were cross-linked, the cell suspension was first disrupted by three

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passages through a French pressure cell and a crude cell envelope fraction was collected by centrifugation at 15,000 × *g* for 30 min at 4°C and resuspended in the same phosphate-sucrose solution before addition of the cross-linking reagent. Similar patterns of cross-linked proteins were observed when the cross-linking reagent was suspended in water, in which it is sparingly soluble, instead of DMSO. The intensity of the cross-linked bands was significantly greater when DMSO was used, presumably reflecting the higher solubility of the cross-linking reagent in DMSO. It has been shown that cells are capable of growing in similar concentrations of DMSO and remain viable after long exposures to even higher concentrations, suggesting that the solvent does not significantly interfere with cellular organization (9).

Murein isolation. Murein was isolated by extraction of whole cells or cell envelopes (see above) with 4% SDS at 100°C as previously described (29).

Membrane fractionation. Cells were broken by passage through a French pressure cell, and the cell envelope fraction was collected by centrifugation in a Beckman SW50.1 rotor at 50,000 rpm for 3.5 h onto a cushion of 60% sucrose (13) and then subjected to overnight centrifugation through a sucrose gradient as previously described (13). Pooled samples from the gradient were diluted with 3 volumes of water, and membrane pellets were collected by centrifugation in a TL100 centrifuge at 100,000 × *g* for 2 h at 4°C.

Electrophoresis of proteins and lipoproteins. For analysis of proteins and lipoproteins in cross-linked murein sacculi, [^{3}H]leucine- or [^{3}H]palmitate-labeled samples were suspended in 1% SDS solubilization buffer, heated in a boiling water bath for 3 min, and subjected to SDS gel electrophoresis in 0.4% *N*,*N*-methylenebisacrylamide–15% acrylamide slab gels (15). Unless otherwise stated, β-mercaptoethanol (1.5 M) was present in the solubilization buffer to cleave the chemical cross-links.

Two-dimensional electrophoresis. Two-dimensional electrophoresis was performed on cross-linked sacculi that were prepared from [^{3}H]leucine- or [^{3}H]palmitate-labeled cells. The sample was first treated with lysozyme (0.1 mg/ml in 50 mM Tris-HCl [pH 8]–5 mM EDTA) for 120 min at 37°C. The sample was then suspended in 1% SDS solubilization buffer in the absence of mercaptoethanol, heated for 4 min at 100°C, and electrophoresed as described above. The lane of interest was cut from the slab gel, and the strip was allowed to soak in SDS solubilization buffer containing 1.5 M β-mercaptoethanol for 30 min at room temperature with gentle rocking. The strip was then applied to the top of the stacking gel of a second SDS slab gel and electrophoresed in the second direction. The final gels were dried and exposed to X-ray film for autoradiography. Similar results were obtained when the lysozyme concentration was increased to 1 mg/ml or when Cellosyl muramidase (Boehringer), 0.04 mg/ml, was used instead of lysozyme.

Isolation and quantitation of lipoproteins. The cell envelope fraction from [^{3}H]palmitate-labeled cells (13) was suspended in 0.4 ml of chloroform-methanol (1:1) and allowed to stand for 30 min at room temperature (14). The sample was then centrifuged in a microcentrifuge for 30 min at room temperature, and the pellet was resuspended in fresh solvent and subjected to two additional cycles of centrifugation and solvent extraction before SDS gel electrophoresis. Unless otherwise noted, all lanes shown in the gel figures contained approximately the same amount of labeled sample, expressed as counts per minute. To quantitate the amounts of radioactivity in each gel band, autoradiograms were selected in which the time of exposure was short enough to ensure that the intensity of the band of interest was proportional to the radioactivity (verified independently), and the radiograms were analyzed densitometrically.

Proteins were named according to their apparent molecular weights.

Other methods and materials. For digestion with proteinase K, samples were suspended in 10 mM Tris-HCl (pH 8.0)–0.1% SDS–proteinase K (50 μg/ml) and incubated for 30 min at 37°C prior to preparation of SDS gel electropherograms. [^{14}C]*N*-acetylglucosamine-labeled lipopolysaccharide was isolated from a crude cell envelope preparation that was prepared as described above. The lipopolysaccharide was extracted by treatment with 50% aqueous phenol for 5 min at 70°C. The aqueous phase containing the lipopolysaccharide was dialyzed against water and then lyophilized. Cross-linking reagents were obtained from Pierce Chemical Corp.

RESULTS

Cross-linking proteins to murein. To identify proteins that were in close proximity to murein, [^{3}H]leucine-labeled cells or cell envelopes were treated with the bifunctional cross-linking reagent DSP, which forms covalent cross-links between amino groups that lie less than 12 Å (1.2 nm) apart (21). The murein sacculus was then isolated, and proteins that were cross-linked to the sacculus were released by treatment with mercaptoethanol, which cleaves the disulfide bond that holds together the two arms of the bifunctional reagent. The released peptides were then analyzed by SDS gel electrophoresis.

In preliminary studies, we noted that the pattern of proteins that were cross-linked to murein after DSP treatment of isolated cell envelopes was not identical to the pattern obtained after treatment of unbroken cells, raising the possibility that cell breakage might alter associations that were present in whole cells. In the present studies, we therefore applied the cross-linking method to intact cells rather than using isolated cell envelopes or outer membrane fractions as was done in previous studies (7, 8, 24, 26).

This revealed a limited spectrum of [^{3}H]leucine-labeled proteins that were reproducibly cross-linked to the sacculus and could subsequently be released by treatment with mercaptoethanol (Fig. 1, lane a). No proteins were released from the sacculus if DSP was omitted at the cross-linking stage or if mercaptoethanol was omitted at the final stage of the procedure. When another cleavable cross-linking reagent, DTBP (Pierce), was substituted for DSP, the intensity of the cross-linked bands and the reproducibility of the patterns were significantly lower.

The effect of prior plasmolysis on the cross-linking pattern was also examined. There was no qualitative difference between plasmolyzed and unplasmolyzed cells in the gel patterns of cross-linked [^{3}H]leucine-labeled proteins. The intensity of the cross-linked peptide bands was significantly greater in the plasmolyzed preparations, presumably reflecting better access of the cross-linking reagent to the target sites. The studies described in the remainder of the present article were performed with cells that were plasmolyzed by exposure to 20% sucrose.

The cells remained viable under the plasmolysis conditions as shown by essentially 100% recovery of colony-forming ability (4a).

Identification of cross-linked proteins. Of the nine [^{3}H]leucine-labeled peptides that were reproducibly cross-linked to murein (Fig. 1), two were identified as OmpA and MLP. The

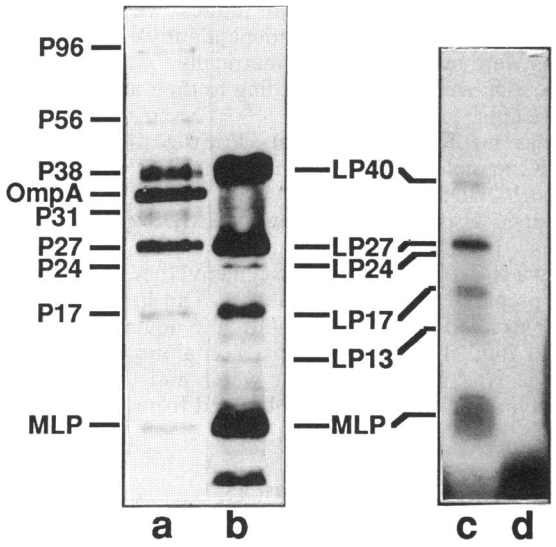

FIG. 1. SDS gel electropherograms of [^{3}H]leucine- and [^{3}H]palmitate-labeled proteins. Cells were labeled with [^{3}H]leucine (lane a) or [^{3}H]palmitate (lanes b to d) and then subjected to DSP treatment, murein isolation, treatment with mercaptoethanol, and SDS gel electrophoresis as described in Materials and Methods (adapted from reference 20). The sample shown in lane d was treated with proteinase K prior to mercaptoethanol treatment and SDS gel electrophoresis.

TABLE 1. Membrane distribution of [^{3}H]palmitate-labeled lipoproteins[a]

Lipoprotein	Lipoprotein in membrane fraction[b]					Total lipoprotein[c] (10^2)	Fraction of lipoprotein cross-linked to murein[d]
	a	b	c	d	e		
LP40	0	0	0.002	0.08	0.92	2.8	0.23
LP35	0.48	0.27	0.25	0	0	0.3	0
LP29	0.6	0.14	0.26	0	0	0.1	0
LP27	0	0.001	0.017	0.16	0.83	9.0	1.38
LP24	0.41	0.16	0.42	0	0	0.1	1.9
LP17	0	0	0.07	0.28	0.64	1.7	0.17
LP13.5/22[e]	0.62	0.15	0.24			1.6	0
LP13	0	0.016	0.06	0.36	0.56	1.3	0.1
MLP	0.005	0.023	0.11	0.37	0.49	83.2	0.03

[a] Quantitation of individual [^{3}H]palmitate-labeled bands was performed with the samples shown in Fig. 3b.

[b] Expressed as counts per minute in the indicated membrane fraction divided by counts per minute in all membrane fractions, determined from gels of un-cross-linked samples.

[c] Expressed as counts per minute of the indicated lipoprotein divided by counts per minute of all lipoproteins, determined from gels of un-cross-linked samples.

[d] Fraction of the indicated lipoprotein that was cross-linked to murein, based on analysis of autoradiograms in which the time of exposure was short enough to ensure that the intensity of the band of interest was proportional to radioactivity. For LP27 and MLP, autoradiograms were exposed for 3 weeks; for other bands, exposures were for 10 weeks. The value for LP24 is probably an overestimate because of a variable loss of LP24 during preparation of the un-cross-linked sample.

[e] LP13.5/22 refers to the ladder of bands with apparent molecular masses of 13.5 to 22 kDa that was visible in membrane fractions a to c. No value is shown for membrane fractions d and e because of the failure to clearly visualize the ladder in these fractions.

ability to cross-link these two outer membrane proteins to murein was reported previously by others (7, 8, 24, 26). The approximately 33-kDa band in the cross-linked samples was identified as OmpA by staining with anti-OmpA antibody in immunoblots. The band was absent when the cross-linking was performed with cells from an *ompA* mutant strain that lacks detectable OmpA protein. The band with an apparent molecular mass of 7.5 kDa was identified as MLP on the bases of its electrophoretic mobility and its ability to be labeled with [^{3}H]palmitate (see below).

Three of the remaining [^{3}H]leucine-labeled cross-linkable peptides (Fig. 1, P27, P24, and P17) corresponded in gel mobility to cross-linked proteins that could also be labeled with [^{3}H]palmitate, as described further below.

Cross-linking of lipoproteins to murein. Evidence that several of the cross-linked proteins were lipoproteins came from studies in which the cross-linking procedure was applied to cells that were labeled with [^{3}H]palmitate. As shown in Fig. 1 and lane XL of Fig. 3b, this procedure identified six [^{3}H]palmitate-labeled peptides that were reproducibly cross-linked to the sacculus.

The cross-linked bands were identified as lipoproteins on the basis of their sensitivity to treatment with proteinase K (Fig. 1, lanes c and d) and on the basis of the solubility of the released ^{3}H-labeled moiety in organic solvents after complete acid hydrolysis of the samples. None of the [^{3}H]palmitate-labeled bands comigrated with purified [^{14}C]*N*-acetylglucosamine-labeled lipopolysaccharide, which ran near the dye front under these conditions.

Four of the cross-linked lipoproteins (LP27, LP24, LP17, and MLP) corresponded in apparent molecular weight to four of the cross-linked [^{3}H]leucine-labeled bands described above (P27, P24, P17, and MLP). The [^{3}H]palmitate-labeled band LP40 ran slightly slower in gels than did the [^{3}H]leucine-labeled band P38, and the two bands are therefore considered to represent different species. A band corresponding to LP40 was not visible in the [^{3}H]leucine-labeled samples, presumably indicating that the amount of LP40 was relatively small, despite the significant labeling with [^{3}H]palmitate, or that the leucine content of LP40 was relatively low. The recoveries of LP40 and LP24 in the cross-linked fraction varied considerably from experiment to experiment. LP17 is likely to correspond to lipoprotein PAL (molecular mass, 19 kDa) (16, 23).

The efficiencies of cross-linking of the various lipoproteins ranged from 3% for the free form of MLP to essentially 100% for LP24 and LP27, when expressed relative to the total amount of the individual proteins in the cell envelope (Table 1). The percentage of cross-linking of individual proteins to the peptidoglycan is likely in most cases to be an underestimate of the extent of the protein-peptidoglycan interaction in the cell envelope because of the inherent inefficiency of the cross-linking reaction and its dependence on the juxtaposition of cross-linkable amino groups at the time of exposure to the reagent. The same factors presumably explain the differences between individual proteins in their efficiency of cross-linking.

Direct versus indirect protein-murein cross-links. The experiments described above do not indicate whether the peptides released by mercaptoethanol treatment were directly cross-linked to murein or whether one or more may have been cross-linked to murein via another protein.

To distinguish between directly and indirectly cross-linked peptides, a two-dimensional gel analysis was performed. In the first step, the murein sacculus containing the cross-linked proteins was digested with muramidase, liberating small peptidoglycan fragments still linked to the cross-linked

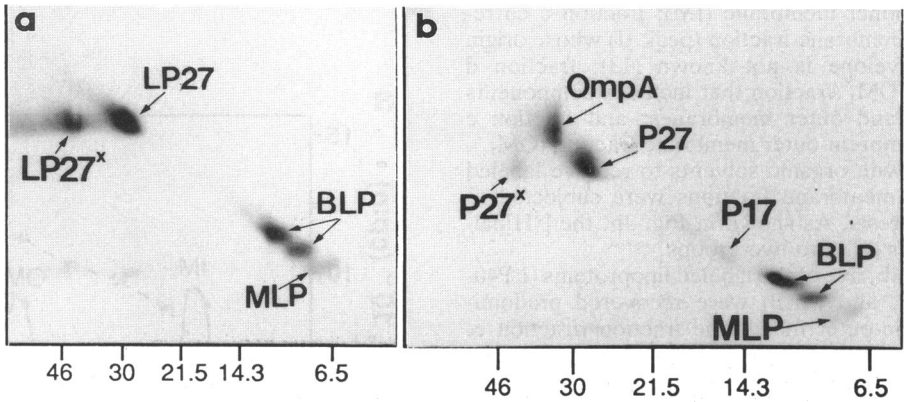

FIG. 2. Two-dimensional gel analysis of lysozyme digests. Murein was prepared from [^{3}H]palmitate-labeled and [^{3}H]leucine-labeled cells that had been treated with DSP. The samples were then analyzed by two-dimensional SDS gel electrophoresis as described in Materials and Methods. The first dimension, oriented along the horizontal axis in the figure (electrophoresis from left to right), represents the lysozyme-treated sample electrophoresed in the absence of mercaptoethanol. The location of molecular weight markers (indicated along the lower edge of the gel) was determined by running the marker proteins in a separate lane in the same gel. The second dimension (oriented along the vertical axis in the figure) represents electrophoresis of the first-dimension gel strip after treatment with mercaptoethanol. Abbreviation: BLP, bound form of MLP (12).

proteins. The sample was then electrophoresed in the absence of a reducing agent, thereby preserving the cross-links. If one or more of the cross-linked proteins was attached to murein via an intermediary protein, the gel migration of the composite structure should reflect the total molecular weight of the cross-linked peptides plus the small contribution of the peptidoglycan digestion fragment.

The sample was then electrophoresed in the second dimension after being exposed to mercaptoethanol. The mercaptoethanol treatment should cleave all DSP-mediated cross-links, thereby liberating the individual proteins to migrate according to their monomeric molecular weights.

Therefore, spots corresponding to labeled proteins that were cross-linked directly to murein should fall along a diagonal line that reflects the similar electrophoretic mobilities of the cross-linked and the un-cross-linked species. The short peptidoglycan fragments that remain attached to the peptides during the first stage (predicted molecular masses, less than 3 kDa [12, 22]) are expected to have minor effects on the mobility of the cross-linked complexes.

In contrast, labeled proteins that are indirectly cross-linked to murein should migrate more slowly in the first than in the second dimension because of their presence in a cross-linked multiprotein unit. Therefore, spots corresponding to these peptides should fall to the left of the diagonal in the second dimension. The difference in mobility between the first- and second-dimension gels will reflect the sizes of the other cross-linked components of the complex. Similarly, if more than one protein was directly attached to the same peptidoglycan fragment, the spots corresponding to these peptides also would fall to the left of the diagonal, even if the peptides were not cross-linked to each other.

As shown in Fig. 2, one of the [^{3}H]palmitate-labeled spots (LP27*) migrated significantly more slowly in the first dimension than in the second, as shown by its position to the left of the diagonal. This indicates that LP27* was present in a higher-molecular-weight complex prior to cleavage of the cross-links. A similar species (P27*) was present in the [^{3}H]leucine-labeled sample. The original complex that included LP27* had an apparent molecular mass of 43 kDa on the basis of a comparison of its migration in the first dimension (Fig. 2, left to right) with the migration of standard proteins. The 43-kDa complex therefore consisted of LP27* plus one or more additional components whose total apparent molecular mass was 15 kDa. The nature of the additional component(s) remains to be defined since a band in the 15-kDa region was not seen when samples labeled with [^{3}H]leucine, [^{3}H]palmitate, or [^{3}H]*N*-acetylglucosamine were examined in the two-dimensional gel system. Because the less-abundant cross-linked lipoproteins, such as LP17, were not clearly visualized in the two-dimensional gels, it remains possible that one or more of these might also be indirectly linked to murein.

Because of their similar gel migrations, it seems likely that LP27* (P27*) and LP27 (P27) represent the same protein. If this assumption is correct, about 20 to 30% of the total LP27 in the original muramidase digest was present in the higher-molecular-weight complex prior to mercaptoethanol treatment, on the basis of analysis of the [^{3}H]palmitate-labeled sample (Fig. 2a). The remaining two-thirds of LP27 fell on the diagonal and therefore appeared to be directly cross-linked to the peptidoglycan.

The two-dimensional gels of the muramidase-treated samples also showed several lower-molecular-weight components that were not present in samples that had not been treated with muramidase. These had apparent molecular masses of 8.6 and 10 kDa in both dimensions (Fig. 2, BLP). The BLP spots were not present in preparations that were not treated with muramidase, even if the samples were treated with mercaptoethanol before being applied to the gels. Therefore, the BLP spots identify lipoproteins that were covalently attached to murein prior to the cross-linking procedure. All of these properties suggest that the 8.6- and 10-kDa BLP spots represent the bound form of the MLP, covalently linked to the tetrasaccharide and hexasaccharide peptidoglycan fragments that are produced by the muramidase treatment (4).

Location of lipoproteins within the native cell envelope. To determine the cellular location of the lipoproteins prior to the cross-linking procedure, [^{3}H]palmitate-labeled cells that had not been cross-linked were disrupted and subjected to membrane fractionation (Fig. 3a). The membrane peaks and protein SDS gel patterns resembled those described previously (13). As indicated in Fig. 3a, fractions a and b

correspond to the inner membrane (IM); fraction c corresponds to a minor membrane fraction (peak II) whose origin within the cell envelope is not known (13); fraction d corresponds to the OM_L fraction that includes components of both the inner and outer membranes; and fraction e corresponds to the murein-outer membrane fraction (OM_H).

After extraction with organic solvents to remove labeled phospholipids, the membrane fractions were subjected to SDS gel electrophoresis. As shown in Fig. 3b, the [^{3}H]palmitate-labeled bands fell into two groups.

As shown in Fig. 3b, five of the labeled lipoproteins (LP40, LP27, LP17, LP13, and MLP) were recovered predominantly in the murein-outer membrane fraction (fraction e) and in the OM_L fraction (fraction d). All of these outer membrane lipoproteins were capable of being cross-linked to murein, as described above. (The prominent MLP band in gels of fractions b and c represents only a small percentage of the total free lipoprotein of the cell since fractions b and c were minor components of the cell envelope.) The other lipoproteins (LP35, LP29, LP24, and the ladder of labeled bands in the 13.5- to 22-kDa region of the gel [LP13.5-22]) were recovered predominantly in the inner membrane fractions (fractions a and b). Peak II (fraction c) included contributions from both groups.

All of the [^{3}H]palmitate-labeled bands, with the exception of LP13.5-22, disappeared when the purified membrane fractions were treated with proteinase K in 2.5% SDS at 37°C. Further work will be needed to establish whether or not the LP13.5-22 bands are lipoproteins.

Because they included the most abundant of the cellular lipoproteins, the lipoproteins recovered in the outer membrane fraction accounted for >95% of the total [^{3}H]palmitate-labeled proteins of the cell.

Ishihara et al. also found that five lipoproteins were recovered in the outer membrane fraction of *E. coli* B (14). Exact comparisons of individual lipoproteins in the two studies are difficult because the migrations of the lipoprotein bands relative to the molecular weight standards were not identical in the two studies. This may reflect strain differences or differences in the gel systems that were used.

Membrane locations of cross-linkable lipoproteins. As noted above, five of the six cross-linkable lipoproteins (LP40, LP27, LP17, LP13, and MLP) were recovered predominantly in the murein-outer membrane and OM_L fractions.

One of the lipoproteins that was cross-linked to murein when cells were treated with DSP (LP24) was recovered in the inner membrane fraction recovered from un-cross-linked cells (Fig. 3b). This was not invariant, however, since in some experiments LP24 was recovered in the murein-outer membrane fraction of un-cross-linked cells. This may indicate that LP24 is an inner membrane protein whose membrane attachment is easily disrupted during cell breakage and may explain the observation that recovery of LP24 in the membrane fractionation procedure was quite variable from experiment to experiment.

DISCUSSION

In the present study, proteins were cross-linked to the murein sacculus by treatment of unbroken cells with a bifunctional cross-linking reagent that can interact with amino groups that are <12 Å (1.2 nm) apart. The short span of the cross-linking reagent and the relatively small number of species that were cross-linked suggests that the proteins

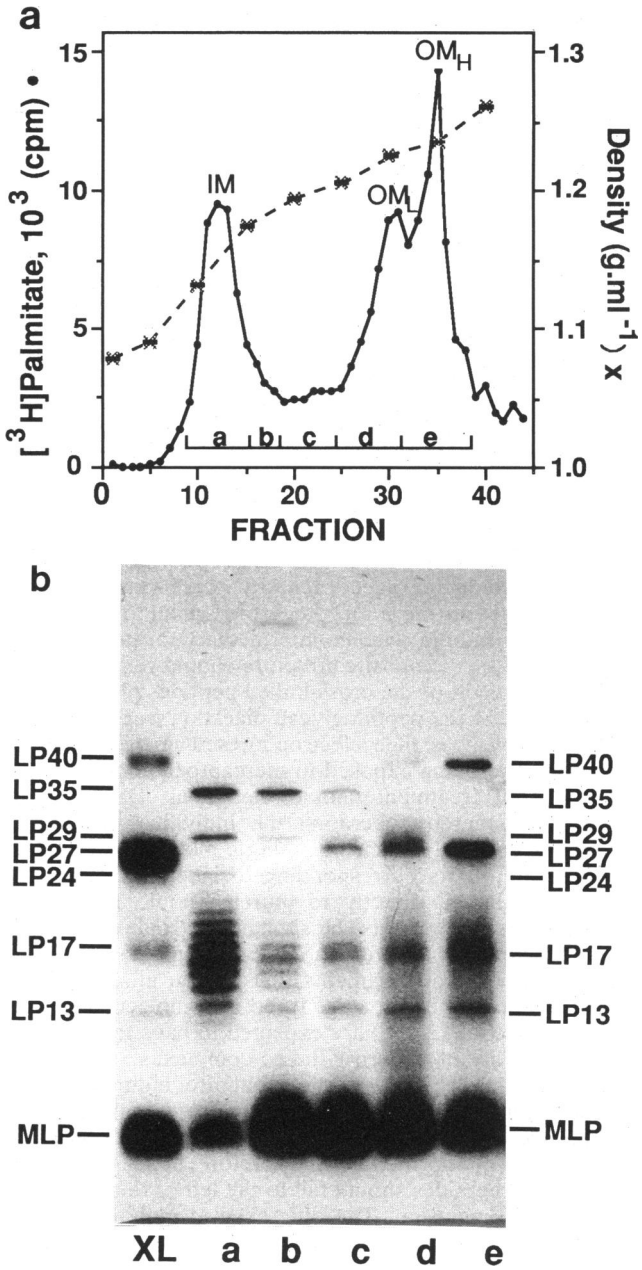

FIG. 3. Membrane fractionation of [^{3}H]palmitate-labeled lipoproteins from un-cross-linked cells. (a) Membrane fractionation was performed on un-cross-linked [^{3}H]palmitate-labeled cells. The indicated fractions (a to e) were pooled and examined as described for panel b. The nomenclature of the peaks (IM, OM_L, and OM_H) is as described by Ishidate et al. (13). (b) A portion of the [^{3}H]palmitate-labeled cells was treated with DSP, murein was isolated, and proteins that were cross-linked to murein were prepared and electrophoresed as described in Materials and Methods (lane XL). Membrane fractions a to e (see panel a) were subjected to SDS gel electrophoresis after extraction with chloroform-methanol (lanes a to e). The autoradiograms were exposed for 10 weeks.

were closely associated with the sacculus within the cell envelope.

An unexpected result of the present study was the finding that most of the labeled proteins that were cross-linked to the murein sacculus were lipoproteins (Fig. 1). On the basis of the assumption that cellular lipoproteins are anchored in membranes by their nonpolar lipid moieties, this suggests that most of the cell envelope lipoproteins, and not only the MLP, mediate interactions between the murein sacculus and the membranous components of the cell envelope.

All but one of the [^{3}H]palmitate-labeled cross-linkable lipoproteins were recovered in the murein-outer membrane fraction. They are therefore tentatively assigned an outer membrane location, although it is possible that one or more could be inner membrane proteins whose association with the membrane was broken during the cell disruption procedure. One of the low-abundance cross-linkable lipoproteins (LP24) was assigned an inner membrane location on the basis of its recovery in the inner membrane fraction isolated from un-cross-linked cells.

Of the cross-linkable proteins that were not labeled with [^{3}H]palmitate, OmpA and MLP are proven outer membrane proteins. The cellular locations of the other nonlipoprotein species have not been established.

All but one of the major cross-linkable proteins appeared to be directly cross-linked to the sacculus rather than cross-linked via an intermediary protein, as shown by two-dimensional gel studies of muramidase-treated sacculi. These results also exclude the possibility that the proteins were cross-linked to the sacculus and then acted as sites for cross-linking of other murein-associated proteins.

One lipoprotein, LP27, appeared to exist in two forms in the cross-linked samples. Two-dimensional gel analysis showed that about two-thirds of the labeled lipoprotein was directly associated with the sacculus, without other associated cross-linked proteins. The remainder was present in a complex with an apparent molecular mass of 43 kDa. It should be noted that the formal assignment of 15 kDa to the additional component of the cross-linked complex speaks only to behavior in SDS gels and may not indicate its true molecular mass. At least a portion of the additional 15 kDa can be accounted for by the peptidoglycan fragment(s) present in the cross-linked complex that was run in the first dimension. The tetra- and hexasaccharide units that are the major products of muramidase digestion have molecular masses of less than 3 kDa (12, 22). Therefore, they are unlikely to account for all of the additional material unless several peptidoglycan fragments are cross-linked to the same LP27* peptide. Further work will be needed to confirm the existence and nature of the missing 15-kDa component of the 43-kDa complex.

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