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Existence of a Free Form of a Specific Membrane Lipoprotein in Gram-Negative Bacteria

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The existence of a free form of a specific lipoprotein of molecular weight 7,200 was examined in the envelopes of several gram-negative bacteria. When the envelope proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, distinct peaks were observed in *Salmonella typhimurium*, *Serratia marcescens*, and *Pseudomonas aeruginosa* at the same position as the free form of the lipoprotein of *Escherichia coli*. However, the peak was not observed in *Proteus mirabilis*. The protein at the peak in *S. typhimurium* was shown to contain little or no histidine as expected from the amino acid composition of the lipoprotein. Furthermore, antiserum against the highly purified lipoprotein from *E. coli* was shown to react with the proteins from *S. typhimurium* and *S. marcescens* and to form the specific immunoprecipitates. In contrast, the protein from *P. aeruginosa* did not react with the antiserum at all. Thus, it is concluded that *S. typhimurium* and *S. marcescens* have the free form of the lipoprotein in their envelopes as does *E. coli*. *P. aeruginosa* contains a protein of the same size as the lipoprotein, but it is not certain whether the protein is the same structural protein as the lipoprotein from *E. coli*. *P. mirabilis* may not have any free form of the lipoprotein, may have it in a very small amount, or may have a lipoprotein of different molecular weight serving the same function.

A specific lipoprotein of molecular weight 7,200 has been shown to exist in two forms in the envelope of *Escherichia coli* (14, 21). The bound form of the lipoprotein is covalently attached at its carboxyl terminal end to the peptidoglycan (8, 10). The free form of the lipoprotein exists in twice the abundance of the bound form (14). Both forms exist exclusively in the outer membrane (3, 24), and about 7.5×10^5 molecules of the lipoprotein are assumed to be present in a cell (5). Thus, it is considered to be the most abundant protein in *E. coli*. The entire chemical structure of the lipoprotein has been determined (6, 9), and its mechanism of biosynthesis and assembly has also been investigated (12, 13, 14, 24). In spite of these extensive studies, its function is not yet known. However, recently we have proposed a molecular assembly model of the lipoprotein which suggests that lipoprotein complexes serve as passive diffusion pores through the outer membrane. The model also explains a possible role of the free form as well as that of the bound form (18).

In the course of these investigations on the lipoprotein, it has become very important to examine whether it exists in gram-negative bacteria other than *E. coli* since this may elucidate its role(s) in the cell. Braun and his associates have shown that the bound form of the lipoprotein exists in *Salmonella* and *Serratia* but not in *Pseudomonas* and *Proteus* (7). Furthermore, Ames has reported that the free form could not be detected in *Salmonella* (1).

In the present paper, we have examined the existence of the free form of the lipoprotein in these gram-negative bacteria. We found, contrary to Ames, that the free form exists in the envelope of *Salmonella* in as great a quantity as in the envelope of *E. coli*. The free form was identified immunologically as well as by its unique amino acid composition. *Serratia* was also found to have the free form in its envelope. In the case of *Pseudomonas*, a protein of the same size as the lipoprotein from *E. coli* was found in its envelope but it is not clear whether the protein is the same structural protein as the lipoprotein from *E. coli*. For *Proteus mirabilis* the existence of a free form of the lipoprotein of molecular weight 7,200 remains uncertain. These findings will be discussed in relation to the possible function of the lipoprotein in the cell envelope.

MATERIALS AND METHODS

Bacterial strains. *E. coli* K-12 strain MX74T2 (16), *Salmonella typhimurium* (obtained from M. Freundlich, Stony Brook), *Serratia marcescens*, *Pseu-*

domonas aeruginosa, and *P. mirabilis* (all obtained from G. Tortora, Stony Brook) were used.

Media and growth conditions. For *S. typhimurium*, *S. marcescens*, and *P. aeruginosa*, M9 medium supplemented with glucose (4 mg/ml), and thiamine (2 μg/ml) was used (16). For *E. coli*, the above M9 medium was further supplemented with thymidine (4 μg/ml). For *P. mirabilis*, tryptone broth was used. All cultures were grown at 37 C.

Label experiments. For the double label experiment, 25 μCi of L-[^{3}H]arginine (8.7 Ci/mmol; Schwarz/Mann) and 5 μCi of L-[^{14}C]histidine (312 mCi/mmol; Schwarz/Mann) were added to a 15-ml culture of *S. typhimurium* at a cell concentration of about 2×10^8 cells/ml. At the same time, nonradioactive L-arginine and L-histidine were added to the culture at final concentrations of 10 and 5 μg/ml, respectively. The culture was then incubated at 37 C for another generation. Single label experiments were carried out in the same way as described above except that L-[^{14}C]histidine and nonradioactive histidine were not added. For *P. mirabilis*, 125 μCi of L-[^{3}H]arginine was added to a 15-ml culture without addition of nonradioactive arginine. The envelope fraction was prepared by differential centrifugation and solubilized in 0.3 ml of 1% sodium dodecyl sulfate as previously described (19, 20).

Immunological assay. One-half of the solubilized envelope fraction (0.15 ml) was subjected to immunoprecipitation and the remaining half was directly subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Immunoprecipitation was carried out according to the method developed in our laboratory using antiserum against *E. coli* lipoprotein serum (Inouye, Takeishi, Hirashima, and Inouye, manuscript in preparation) as follows: to 0.15 ml of the solubilized envelope fraction was added 1.25 ml of 0.12% sodium dodecyl sulfate in 0.12 M sodium phosphate buffer (pH 7.0). To the mixture was added 0.1 ml of antiserum against *E. coli* lipoprotein, which was obtained from a rabbit injected with the highly purified free form of the lipoprotein of *E. coli*. The final mixture was left at room temperature for 30 min and then incubated at 4 C overnight, and the immunoprecipitate thus formed was collected by centrifugation. The precipitate was washed three times with 1 ml of 0.01 M sodium phosphate buffer (pH 7.0) containing 0.9% NaCl, 0.1 mM ethylenediaminetetraacetic acid and 0.01% sodium dodecyl sulfate. After the wash, the precipitate was solubilized in 0.2 ml of the solubilizing solution as mentioned above (19) and then subjected to gel electrophoresis.

Gel electrophoresis. Polyacrylamide gel electrophoresis in 0.5% sodium dodecyl sulfate was carried out on 7.5% acrylamide gels as previously described (19, 20). After gel electrophoresis, the gels were sliced with a razor blade slicer, and the radioactivity of each slice was counted as previously described (20). All of the graphs were drawn by a computer directly from the data (20). All gel electrophoresis were run using internal molecular weight standards made of fluorescent proteins (17).

RESULTS

Lipoprotein in S. typhimurium. The bound form of the lipoprotein from *S. typhimurium* has been shown to contain no histidine residues as in *E. coli* (7). Thus, the envelope proteins of *S. typhimurium* were labeled with [^{3}H]arginine and [^{14}C]histidine and analyzed by polyacrylamide gel electrophoresis as previously done for *E. coli* (17, 21). As shown in Fig. 1, the resulting gel pattern is very similar to the one obtained for *E. coli* (17, 21). Of particular interest is the peak arising between the internal molecular weight standards, e (1-dimethylaminonaphthalene-5-sulfonyl [DANS]-insulin (cytochrome *c*) and f; this is the position of the free form of the lipoprotein from *E. coli*. Furthermore, as can be seen from Fig. 1, the protein at this peak shows a very low histidine content in contrast to the other proteins; this is exactly the same result as in *E. coli* (17, 21). It should also be noted that the peak corresponding to the free form of the lipoprotein in Fig. 1 is a major envelope protein of *S. typhimurium*, accounting for more than 10% of the total [^{3}H]arginine radioactivity incorporated into the envelope fraction.

Immunoassay of the free form in S. typhimurium. The existence of the free form of the lipoprotein in the envelope of *S. typhimurium*

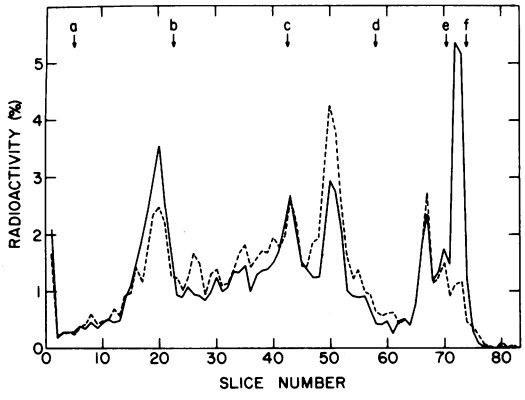

FIG. 1. *Gel electrophoresis of the envelope proteins of S. typhimurium double labeled with* L-[^{3}*H*]*arginine and* L-[^{14}*C*]*histidine. The envelope fraction was prepared as described. The envelope fraction was solubilized and subjected to polyacrylamide gel electrophoresis in 0.5% sodium dodecyl sulfate as described previously (19, 20). After gel electrophoresis, the gel was sliced with a razor blade slicer, and the radioactivity of each slice was counted in a scintillation counter (19, 20). The radioactivity of each slice is expressed as percentage of the total radioactivity.* ——, [^{3}*H*]*arginine;* -----, [^{14}*C*]*histidine. Small arrows with letters indicate the positions of the internal molecular weight standards (17); a, dimer; b, monomer of DANS-bovine serum albumin; c, dimer; d, monomer of DANS-hen egg white lysozyme; e, cytochrome c; f, DANS-insulin.*

was further examined by immunoprecipitation with antiserum against the free form of *E. coli* lipoprotein. The immunoassay developed in our laboratory (Inouye, Takeishi, Hirashima, and Inouye, manuscript in preparation) was highly specific for the free form of the lipoprotein as shown in Fig. 2A and B; of all the envelope proteins (Fig. 2A) only the free form of the lipoprotein was precipitated by antiserum against lipoprotein (Fig. 2B). When the solubilized envelope fraction of *S. typhimurium* was reacted with antiserum against *E. coli* lipoprotein, 14.4% of the total radioactivity was recovered in the immunoprecipitate (Table 1). The precipitate was solubilized and subjected to gel electrophoresis. As shown in Fig. 2D, the peak between the internal molecular weight standards e and f, which corresponds to the free form of the lipoprotein, is the only major peak recovered from the immunoprecipitate, just as for *E. coli* (Fig. 2B). Together with the results above, these results clearly indicate that *S. typhimurium* has the free form of the lipoprotein as a major envelope protein. The amount of the free form calculated from Fig. 2C accounts for 14.2% of the total envelope proteins, which is comparable to 18.0% for *E. coli* (Fig. 2A, Table 1). The recovery of the free form in the immunoprecipitate of *S. typhimurium* was 7.4%, which is also in good agreement with 7.6% for *E. coli* (Table 1).

Existence of the free form in other bacteria. Similar immunoprecipitation experiments were carried out for *S. marcescens*, *P. aeruginosa*, and *P. mirabilis*. The results are presented in Fig. 2 and Table 1. In the case of *S. marcescens*, 8.3% of the total envelope proteins appeared at the position of the free form of the lipoprotein (Fig. 2E), and the protein at that peak reacted with antiserum against *E. coli* lipoprotein to form the immunoprecipitate (Fig. 2F). The recovery of the lipoprotein after gel electrophoresis of the immunoprecipitate was 3.0% (Table 1). These results indicate that *S. marcescens* also has the free form of the lipoprotein in its envelope, although the content of the free form appears to be somewhat smaller than in *E. coli* or *S. typhimurium*.

For *P. aeruginosa*, 7.6% of the total radioactivity was recovered as a peak at the position corresponding to the free form of the lipoprotein between the internal molecular weight standards e and f in Fig. 2G. However, in contrast to the cases of *Salmonella* and *Serratia*, the protein at the peak did not react at all with antiserum against *E. coli* lipoprotein; after gel electrophoresis of the immunoprecipitate, no peak was observed at the position of the free form of the lipoprotein (Fig. 2H). Although

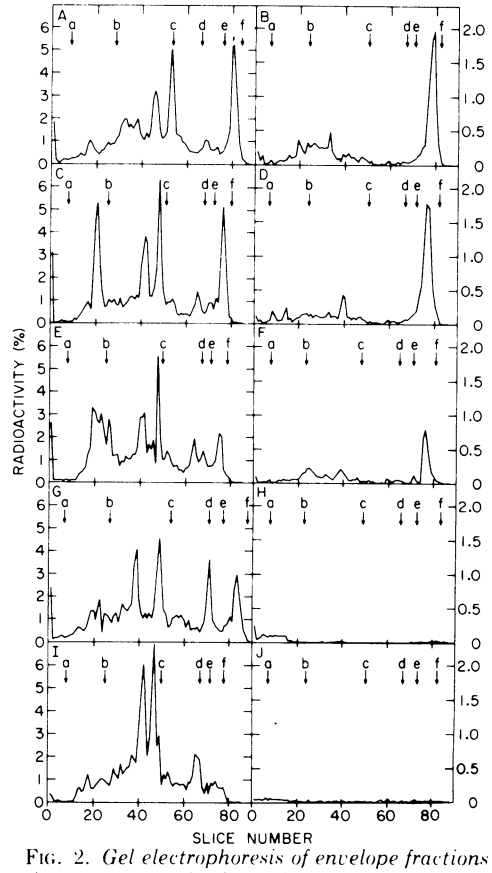

FIG. 2. *Gel electrophoresis of envelope fractions of various gram-negative bacteria, and of their immunoprecipitates with antiserum against E. coli lipoprotein. Envelope fractions labeled with* L-[^{3}H]*arginine were prepared as described. One-half of each envelope fraction was directly subjected to gel electrophoresis (figures at the left-hand side) and the other half was treated with anti-E. coli lipoprotein serum as described. The immunoprecipitate thus formed was then subjected to gel electrophoresis (figures at the right-hand side). The radioactivity of each slice is expressed as a percentage of total radioactivity in the original envelope fraction. A, Total envelope proteins; B, the immunoprecipitate from E. coli; C, total proteins; D, the immunoprecipitate from S. typhimurium; E, total envelope proteins; F, the immunoprecipitate from S. marcescens; G, total envelope proteins; H, the immunoprecipitate from P. aeruginosa; I, total envelope proteins; J, the immunoprecipitate from P. mirabilis. The small arrows with letters indicate the positions of the internal molecular weight standards (17): a, dimer; b, monomer of DANS-bovine serum albumin; c, dimer; d, monomer of DANS-hen egg white lysozyme; e, cytochrome c; f, DANS-insulin.*

Pseudomonas has no bound form (7), the bacterium may still have the free form of the lipoprotein. If this is the case, the free form may be structurally different from that of *E. coli* so that

the former cannot react with antiserum against *E. coli* lipoprotein.

For *P. mirabilis*, no significant peak was observed at the position corresponding to the free form of the lipoprotein as shown in Fig. 2I. Thus, no peak was observed in gel electrophoresis of the immunoprecipitate either (Fig. 2J).

DISCUSSION

The present paper revealed that in both *S. typhimurium* and *S. marcescens* the free form of the lipoprotein is a major envelope protein as in *E. coli*. The free form of the lipoprotein from both of these bacteria reacted with antiserum against *E. coli* lipoprotein to form immunoprecipitates. Furthermore, the existence of the free form in *S. typhimurium* was supported by the extremely low content of histidine in the position of the peak corresponding to the free form of the lipoprotein. It has already been shown that these bacteria have the bound form (7). Thus, it can be concluded that the envelopes of these bacteria contain both the bound form and the free form of the lipoprotein, just like *E. coli* (14, 21). On the other hand, in the gel pattern of the envelope proteins of *P. aeruginosa*, a major peak appears at the position of the free form of the lipoprotein (Fig. 2G), which does not form an immunoprecipitate with antiserum against *E. coli* lipoprotein (Fig. 2H). This bacterium is assumed to have no bound form of the lipoprotein, since the bound form has not been found in *Pseudomonas fluorescens* (7). Thus *P. aeruginosa* may only have a free form of the lipoprotein, structurally different from that of *E. coli* since it does not react with antiserum against *E. coli* lipoprotein. However, further investigation should be done to elucidate whether the protein at the peak corresponding to the lipoprotein is actually the free form of the lipoprotein. *P. mirabilis*, which has been shown to have no bound form of the lipoprotein (7), may not have free form either since no significant peak was observed at the position corresponding to the free form of the lipoprotein (Fig. 2I), and no specific envelope proteins reacted with antiserum against *E. coli* lipoprotein (Fig. 2J). However, it is also possible that the free form of the lipoprotein exists in a very small amount.

Recently we have presented a three-dimensional molecular assembly model of the lipoprotein, which may form a complex that serves as a passive diffusion pore through the outer membrane of *E. coli* (18). In this regard, it is of great interest that *P. mirabilis* appears not to have the bound form or the free form of the lipoprotein in contrast to the other gram-negative bacteria tested in this paper. It may have a similar protein of different molecular weight or, alternatively, the outer membrane may be arranged differently from that of *E. coli*, in such a way that the function of the lipoprotein is not required.

TABLE 1. *The occurrence of the free form of the lipoprotein in various bacteria, and the recovery of the protein after immunoprecipitation with antiserum against E. coli lipoprotein*

Bacteria	Lipoprotein in the envelope (%)[a]	Total radioactivity in the immunoprecipitate (%)[b]	Lipoprotein recovered from the immunoprecipitate (%)[c]
E. coli	18.0	16.0	7.6
S. typhimurium	14.2	14.4	7.4
S. marcescens	8.3	7.7	3.0
P. aeruginosa	7.6	2.0	0
P. mirabilis	(3)[d]	1.8	0

[a] Calculated from Fig. 2; radioactivity at the position of the free form of the lipoprotein.

[b] Immunoprecipitation was carried out as described. Values are expressed as percentage of the total radioactivity in the envelope fraction.

[c] The immunoprecipitate obtained above was subjected to gel electrophoresis. The radioactivity recovered at the position of the free form of the lipoprotein was expressed as percentage of the total radioactivity in the original envelope fraction.

[d] Radioactivity at the position of the free form of the lipoprotein.

There are many reports on gel electrophoresis of the envelope proteins of *E. coli* and *S. typhimurium* (1, 2, 4, 11, 15, 23, 25–29). Most of them overlooked the existence of the free form of the lipoprotein. Recently Ames suggested, using slab gel electrophoresis, that *S. typhimurium* either has the free form of the lipoprotein of molecular weight 7,200 in smaller amount than *E. coli*, may not have it at all, or the lipoprotein does not occur in the free form or the free form has a different molecular weight (1). However, the existence of the free form of the lipoprotein has been explicitly demonstrated not only in *E. coli* (14, 21) but also in *S. typhimurium* and *S. marcescens* in the present paper. Recently, Henning et al. have confirmed the existence of the free form of the lipoprotein in the envelope of *E. coli* (11). Some possible reasons that the free form was overlooked are: (i) many authors ignored the region of lower molecular weight in gel electrophoresis. (ii) Proper radioactive amino acids were not used to detect the free form of the lipoprotein. Since the protein has a very unique amino acid composition (8), some amino acids are incorporated into the lipoprotein in a very small amount or not at all. (iii) The free form of the lipoprotein is

soluble even in 10% trichloroacetic acid in the presence of sodium dodecyl sulfate (14). Therefore, if the proper staining procedure was not applied, the free form of the lipoprotein might not be detected in gels by staining. We have also demonstrated the existence of the free form of the lipoprotein by staining (19, 22).

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