Biochimica et Biophysica Acta, 464 (1977) 589—601

BBA 77587

OUTER MEMBRANE OF *SALMONELLA TYPHIMURIUM*

IDENTIFICATION OF PROTEINS EXPOSED ON CELL SURFACE *

YOCHIYUKI KAMIO ** and HIROSHI NIKAIDO ***

Department of Bacteriology and Immunology, University of Californa, Berkeley, Calif. 94720 (U.S.A.)

(Received July 2nd, 1976)

Summary

Proteins exposed on the outer surface of the outer membrane of *Salmonella typhimurium* were identified by reacting intact cells with a covalent labeling reagent. Since the outer membrane permitted the free diffusion of small hydrophilic molecules, we used a macromolecular reagent, CNBr-activated dextran, as the non-penetrating labeling agent. We also used a mutant producing a lipopolysaccharide with a very short (i.e. hexasaccharide) carbohydrate chain, in order to avoid steric hindrance by the carbohydrates on membrane surface. Results showed that out of the four "major" proteins of molecular weight around 35 000, three were exposed, and that at least six other proteins were also exposed on cell surface. Only two or three outer membrane proteins consistently did not react with the reagent in intact cells.

Introduction

The envelope of Gram-negative bacteria such as *Escherichia coli* and *Salmonella typhimurium* contains two membranes, the inner, cytoplasmic membrane, and the outer membrane that is located outside the peptidoglycan layer. Both membranes contain proteins and phospholipids. In addition, the outer membrane contains lipopolysaccharides (for review see ref. 1). The outer membrane has a rather simple protein composition, and only about 12—20 proteins have been detected in it [2,3].

We have been interested in determining the distribution of outer membrane

* This is paper X in the series on the outer membrane of bacteria. The preceding paper in this series is ref. 9.

** Present address: Department of Microbiology, Shinshu University School of Medicine, Matsumoto, Japan.

*** To whom inquiries should be addressed.

components in the direction normal to the plane of the membrane. The standard technique for this kind of study is the use of non-penetrating, covalent labeling reagents to identify the components exposed on the outer surface [4—6]. However, the outer membrane appears to allow the penetration of hydrophilic molecules smaller than 600—700 daltons [7—9], and thus presumably of most of the previously described "non-penetrating" reagents. We therefore used a macromolecular labeling reagent, CNBr-activated dextran [10], which was indeed found to be impermeable through the outer membrane [11], and was used successfully for a localization study of phosphatidylethanolamine molecules in the outer membrane [11]. In this study, we tried to identify the proteins exposed on the outer surface of the outer membrane, by treating intact cells of *S. typhimurium* or *E. coli* with this reagent.

Materials and Methods

Bacterial strains and their cultivation. HN366 (*galE503 leu*) is a leucine auxotroph, derived from a UDPgalactose 4-epimerase mutant, HN202, of *S. typhimurium* LT2 [11]. The cells were grown overnight in medium M-9 [12] containing L-leucine (20 μg/ml) and glycerol (0.5%) at 37°C with aeration by shaking. 3 ml of this culture were added to 30 ml of fresh M-9 with 0.5% glycerol and L-[^{3}H]leucine (specific activity: 5.5 Ci/mol) (20 μg/ml) and shaking was continued at 37°C. Cells were harvested by centrifugation when the density of the culture reached 0.2 mg (dry weight)/ml, and were washed twice with ice-cold 0.1 M $NaHCO_3/Na_2CO_3$ buffer, pH 8.5.

Isolation of outer and inner membrane fractions. These fractions were prepared for dextran coupling experiments as follows. HN366 cells were grown as described above, except that the final volume of the culture was increased to 100 ml and the specific radioactivity of L-[^{3}H]leucine was decreased to 1.6 Ci/mol. The cells were passed through a French pressure cell, and the outer membrane-peptidoglycan complex was separated from the inner membrane by centrifugation through a sucrose density gradient [13]. The former fraction was washed once by centrifugation in 5 mM Tris · HCl (pH 8.0), and was resuspended in 3 ml of the same buffer. Egg white lysozyme (150 μg) was then added to digest the peptidoglycan layer, and the suspension was incubated for 4 h at 37°C with brief sonication (5 s with a microtip of a Biosonik IV sonicator) in the middle of the incubation period. The outer membrane was collected by centrifugation, (232 000 × *g* for 30 min) and was washed twice by resuspension and centrifugation (232 000 × *g* for 30 min) in 0.1 M $NaHCO_3/Na_2CO_3$ (pH 8.5).

In order to isolate the outer membrane fraction from dextran-treated cells, a similar procedure involving French pressure cell disruption and sucrose dendity gradient centrifugation was used. The outer membrane-peptidoglycan complex was used directly for analysis, without the removal of the peptidoglycan layer.

Coupling with activated dextran. ^{3}H-Labeled cells (3—5 mg dry weight), prepared as described above, were suspended in 1 ml of 0.1 M $NaHCO_3/Na_2CO_3$ buffer, pH 8.5. A portion (0.25 ml) of this suspension was saved as the untreated control, and the remaining portion was added with stirring to 15 ml

of freshly prepared activated dextran (see below) at 20°C. At times indicated portions of the reaction mixtures were taken out, and were mixed with 0.1 volumes of 1 M ethanolamine (pH 7.4) in order to inactivate the remaining coupling reagent. Cells were recovered by centrifugation, washed twice with 0.1 M ethanolamine, pH 7.4, and cell envelope and/or outer membrane-peptidoglycan complex were prepared according to the published procedure [14] and the method described above, respectively. In many experiments, a smaller portion (0.25—0.5 ml) of the labeled cells was incubated with a smaller amount (2—5 ml) of the activated dextran. Many experiments also contained a control in which the cells were incubated in 0.1 M $NaHCO_3/NA_2CO_3$ buffer, pH 8.5, without the addition of the activated dextran.

Isolated membrane fractions were coupled to the activated dextran in a similar manner, except that one-third of the membrane fraction obtained was added to 2 ml of the activated dextran solution. The reaction was stopped similarly with ethanolamine, and the membranes were recovered by centrifugation (232 000 × *g* for 1 h).

Acrylamide gel electrophoresis. Cell envelope preparations or membrane fractions were solubilized by heating at 100°C for 2 min in 40—100 μl of "sample buffer" [14] that contained 5% mercaptoethanol and 2% sodium dodecyl sulfate. Portions (usually about 10 μl) of these preparations were applied to the acrylamide slab gels [14] containing 0.1% sodium dodecyl sulfate. In order to make certain that samples in the same series contained the same amounts of proteins, the radioactivity of the solubilized samples was determined and the volumes applied were adjusted so that each sample contained exactly the same amount of radioactivity, which corresponded roughly to 7—10, 15—20, and 15—20 μg proteins for outer membrane, inner membrane, and cell envelope samples, respectively. After electrophoresis the gels were stained with 0.0025% Coomassie brilliant blue in 25% isopropanol/10% acetic acid, and were scanned, while wet, with a "Quick Scan" densitometer (Helena Laboratories) by using a 595 nm filter. The gels were then dried. In some experiments the protein bands of interest were cut out from the dried gels, placed in vials and were heated in 1 ml of 1% sodium dodecyl sulfate in 0.7 M NaOH for 20 min at 100°C. 10 ml of Aquasol (New England Nuclear Corp.) were then added to each vial, and the radioactivity in each protein band was determined with an Amersham-Searle Isocap 300 liquid scintillation spectrometer.

CNBr-activated dextran. This was prepared by a modification of the procedure of Kågedal and Åkerström [10]. Dextran T-10 (Pharmacia, Uppsala) (0.2 g) was dissolved in 10 ml of water, and to this 10 ml of the aqueous solution of CNBr (11 mg/ml) was added with stirring, in two portions at an interval of 1 min. The mixture was stirred vigorously in the hood, and the pH was maintained at 10.7 by the addition of 4 M and then 1 M NaOH. After 30 min at room temperature, the pH of the solution was adjusted to 8.5 with 1 M HCl, and 10 ml of this preparation was applied to a column (2.5 × 22 cm) of Bio-Gel P2, 50-100 mesh (Bio-Rad Laboratories, Richmond, Calif.). The column was immediately eluted with 0.1 M $NaHCO_3/Na_2CO_3$ buffer, pH 8.5, and the void-volume fractions (15 ml) containing the activated dextran were collected. This preparation was used without delay for coupling experiments.

Results

Electrophoretic pattern of envelope proteins

Fig. 1A (samples 4 and 7) shows sodium dodecyl sulfate-acrylamide gel patterns of inner and outer membrane proteins from NH366. These samples were also electrophoresed together with nine proteins of known molecular weights [14]. When the distances of migration of these marker proteins were plotted against the logarithms of their molecular weights, a straight line was obtained so that the apparent molecular weights of the membrane proteins could be obtained from their distances of migration. Following the practice of Ames [14], we refer to each protein band by its apparent molecular weight determined in this manner, expressed by the molecular weight $\times 10^{-3}$ followed by the letter K. It should be emphasized that these numbers are used mainly for identification, and that the molecular weights inferred are often not accurate, especially near both ends of the gel.

As seen in Fig. 1, the outer and inner membranes share few proteins in common; this was also observed by other workers [2,3,15]. The outer membrane pattern is dominated by the "major proteins", i.e. 36K, 35K, 34K, and 33K proteins, and in addition 90K, 84K, 82K, 51K, 48K, 41K, 30K, 23K, 22K, 21K, 12.5K, and 12K bands are always visible. In addition, some, but not all

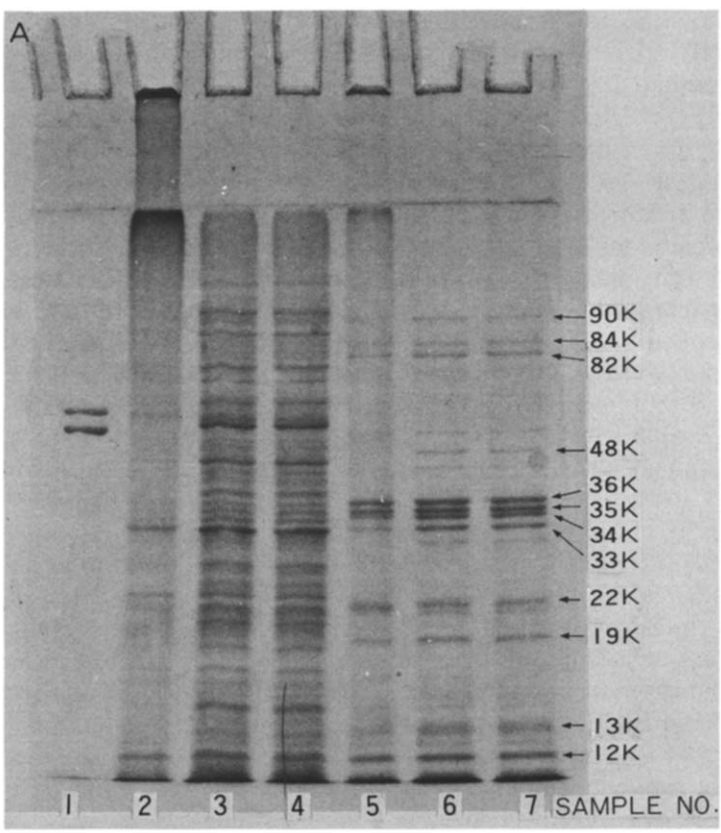

Fig. 1. For legend see opposite page.

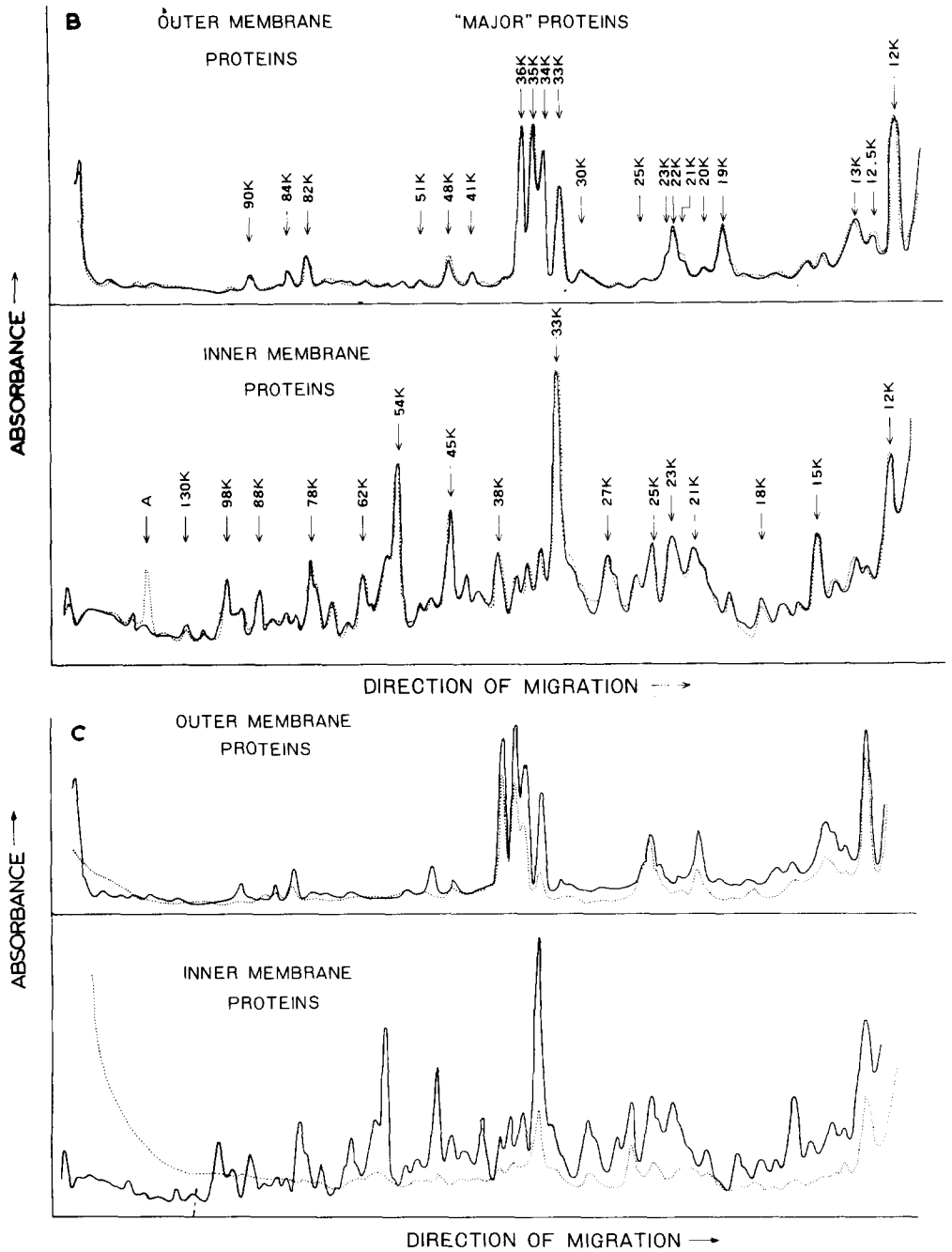

Fig. 1. Sodium dodecyl sulfate-acrylamide gel electrophoresis of outer and inner membrane proteins of *S. typhimurium* HN366. Outer and inner membrane fractions were prepared as described in Materials and Methods, and portions were incubated in 0.1 M $NaHCO_3/Na_2CO_3$ buffer (pH 8.5) with or without activated dextran at 20°C for 1 h. Samples were solubilized, electrophoresed, and scanned as described in Materials and Methods. (A) shows the photograph of the stained gel. Samples 2—4 and 5—7 correspond to inner and outer membranes, respectively. Samples 4 and 7 are controls solubilized without further treatment; samples 3 and 6 were incubated in the buffer alone, and samples 2 and 5 in the buffer containing activated dextran, before solubilization. Sample 1 corresponds to *S. typhimurium* flagellins [14]. (B) shows the scanning of control samples 4 and 7 (solid lines), as well as buffer-incubated samples 3 and 6 (dotted lines). The peak labeled "A" is apparently a scanning artefact, as a band corresponding to this position was not visible in the original gel (see A). (C) shows the scanning of the controls 4 and 7 (solid lines), as well as the dextran-treated samples 2 and 5 (dotted lines).

preparations contain sharp bands at 25K, 20K, and/or 19K. The diffuse band at 13K may contain lysozyme used in the preparation of some of the samples. In contrast, the inner membrane produces at least 40 bands, containing characteristically intense bands at 130K, 98K, 88K, 78K, 62K, 54K, 45K, 38K, 33K, 27K, 25K, 23K, 21K, 18K, 15K, and 12K. These patterns are quite reproducible so that we can identify most of the bands seen in the electropherograms of the whole cell envelope as components of either the outer or the inner membrane (see below).

The protein patterns were also compared semiquantitatively by scanning the gels with a recording photometer (see Fig. 1B). In order to assess the reproducibility of the entire operation including the isolation of envelopes, their solubilization, electrophoresis, staining and scanning, a suspension of HN366 cells was divided in two portions, and cell envelope fraction was prepared from each of them and was applied for electrophoresis. As seen in Fig. 2, the patterns obtained agree with each other down to minor details, a result indicating a very high reproducibility of the entire procedure.

Preliminary coupling experiments with activated dextran

CNBr-activated dextran forms covalent linkages with substances containing amino groups [10]. Thus if a part of an outer membrane protein is exposed to the outside medium, and if this portion contains amino groups, incubation of intact cells with the activated dextran is expected to lead to the formation of covalent dextran-protein complexes. Since the "activation" of dextran leads to its polymerization [10,11], and under our conditions produces molecules with a weight-average molecular weight in excess of 50 000 as judged by gel filtration through Bio-Gel P-100 [11], coupling of this large dextran molecule

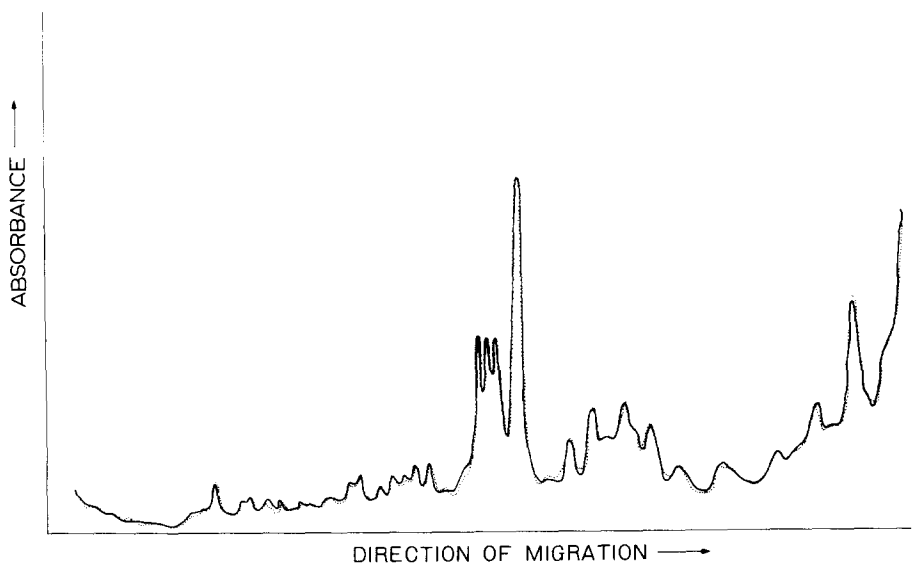

Fig. 2. Reproducibility of the analytical procedure. A suspension of HN366 cells was divided in two, and each portion was individually processed by sonication, centrifugation, solubilization in sodium dodecyl sulfate, electrophoresis, staining, and scanning as described in Materials and Methods. The absorbance profiles for these samples are shown by solid and dotted lines.

to several, or even to one molecule of protein would make it difficult for the complex to enter the sodium dodecyl sulfate-acrylamide gel. Thus when the membrane samples or intact cells are reacted with activated dextran, solubilized with sodium dodecyl sulfate, and then analyzed by sodium dodecyl sulfate-acrylamide electrophoresis, the coupling of dextran to any protein in the membrane should result in a decrease in the intensity of that particular protein band in the electropherogram.

Intact cells of *S. typhimurium* HN366 were treated with the activated dextran for 30, 60, and 90 min at 20°C as described in Materials and Methods, and the cell envelope fractions were isolated from them and analyzed by sodium dodecyl sulfate-acrylamide gel electrophoresis. Inspection of the stained gel as well as the scanning of the gel indicated that some of the protein bands were indeed diminished in intensity in comparison with the untreated control. The reaction seemed almost complete by 30 min: for example, the peak intensity for the 34K band decreased to 62% of the control already at 30 min, and thereafter showed only a very slow decrease to 59% at 60 min. On the basis of these results we treated all subsequent samples for 60 min at 20°C, in order to make sure that a near-maximal reaction would take place.

Coupling of proteins in isolated membrane vesicles

Isolated outer and inner membrane fractions from HN366 (Materials and Methods) were reacted with activated dextran, and then the proteins were analyzed by sodium dodecyl sulfate-acrylamide gel electrophoresis. As seen in samples 2 and 5 of Fig. 1A, the intensity of most bands was decreased in comparison with controls (samples 4 and 7). That this decrease is indeed the result of coupling with dextran is seen from the following. (i) There was a thick smearing of substances that were stained by Coomassie blue (probably protein-dextran complexes) at both the top of the stacking gel and the top of the separating gel; this is particularly evident with the inner membrane sample (sample 2), where samples containing a large amount of protein had to be applied because of the large number of protein bands present. (ii) Incubation in the same $NaHCO_3/Na_2CO_3$ buffer (pH 8.5) at 20°C for 1 h without activated dextran did not decrease the intensity of any of the bands (compare samples 3 and 6 in Fig. 1A with samples 4 and 7; see also Fig. 1B), suggesting that incubation under our conditions did not produce any measurable degradation of membrane proteins by endogenous proteases.

The extent of coupling can be seen more quantitatively in Fig. 1C, and we can conclude that most of proteins in the outer and inner membranes are at least partially exposed to the aqueous medium. It should also be emphasized that the extent of coupling is very high especially with the inner membrane, and that the peak intensity decreased to less than 30% of the control in many bands. There is a possibility that the reactivity of the outer membrane proteins was caused by the lipolysis and/or proteolysis that may have occurred during the 4-h incubation with lysozyme at 37°C (Materials and Methods). However, this seems rather unlikely as the proteins of the inner membrane, which was never exposed to temperatures higher than 4°C during its preparation, still reacted extensively with the activated dextran (Fig. 1C).

Coupling with intact cells

Intact cells of *S. typhimurium* HN366 were treated with the activated

dextran, dextran was then inactivated by the addition of ethanolamine, and outer and inner membranes were prepared from these cells as described in Materials and Methods. The outer membrane band thus isolated had a higher density than the outer membrane from untreated cells, a result suggesting the attachment of dextran molecules to the membrane. There was no alteration in

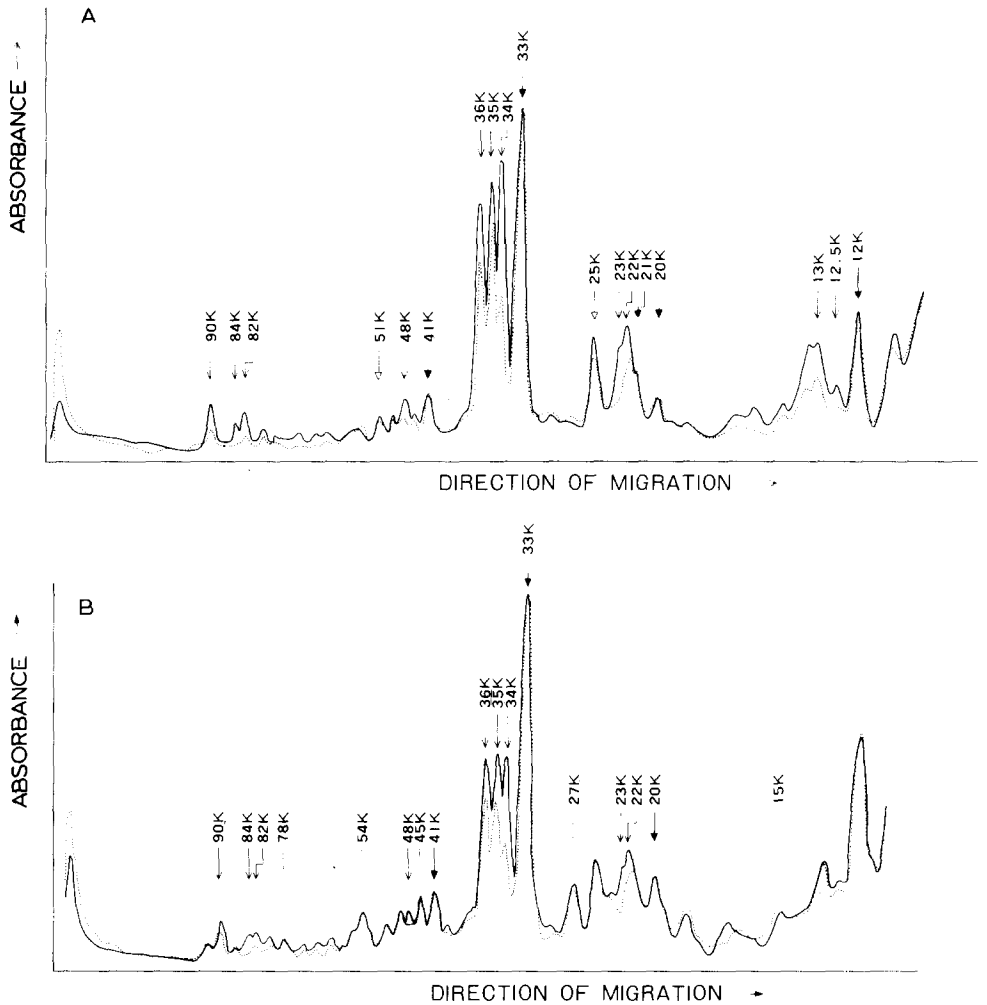

Fig. 3. Treatment of intact cells of HN366 with activated dextran. A suspension of HN366 was divided into three equal portions. The first portion served as a control. The second portion was incubated in 0.1 M $NaHCO_3/Na_2CO_3$ (pH 8.5) at 20°C for 1 h. The third portion was incubated in the same buffer containing activated dextran. To all suspensions 0.1 volumes of 1 M ethanolamine were added, and the cells were recovered by centrifugation. A portion of each sample was then used for the isolation of outer membrane-peptidoglycan fraction, and the remaining portion for the preparation of cell envelopes (see Materials and Methods). These samples were then solubilized, electrophoresed, stained, and scanned as described in Materials and Methods. (A) and (B) show the analysis of the outer membrane fractions and the cell envelope fractions, respectively. In both, the solid and dotted lines correspond to samples obtained from control and dextran-treated cells, respectively. The cells incubated in buffer alone produced patterns almost completely identical to the control samples (not shown). Symbols: →. Outer membrane protein significantly decreased in activated-dextran-treated cells; ⇢, outer membrane protein probably decreased in activated-dextran-treated cells; ⇢, outer membrane protein unaffected by activated-dextran treatment; and —, inner membrane protein.

the sedimentation behavior of the inner membrane band.

The electrophoretic pattern of the proteins of the isolated outer membrane is seen in Fig. 3A. Among the 18 outer membrane protein bands, 11 (90K, 84K, 82K, 48K, 36K, 35K, 34K, 23K, 22K, 13K, and 12.5K) showed significant decrease in intensity, 2 (51K and 25K) seemed to decrease somewhat, and 5 (41K, 33K, 21K, 20K, and 12K) did not show any decrease.

Since the separation of the outer from inner membrane requires lengthy equilibrium centrifugation which increased the possibility of artefactual alterations in membrane composition, we also analyzed the cell envelope fraction of activated-dextran-treated cells directly by sodium dodecyl sulfate-acrylamide gel electrophoresis. The results (Fig. 3B) show clearly the decrease of some outer membrane proteins, such as 90K, 84K, 82K, 48K, 36K, 35K, 34K, 23K, and 22K. The decrease in 13K and 12.5K proteins, seen in the isolated outer membrane, was less apparent presumably because this region of the gel contains much inner membrane proteins (see Fig. 1). It should be emphasized that

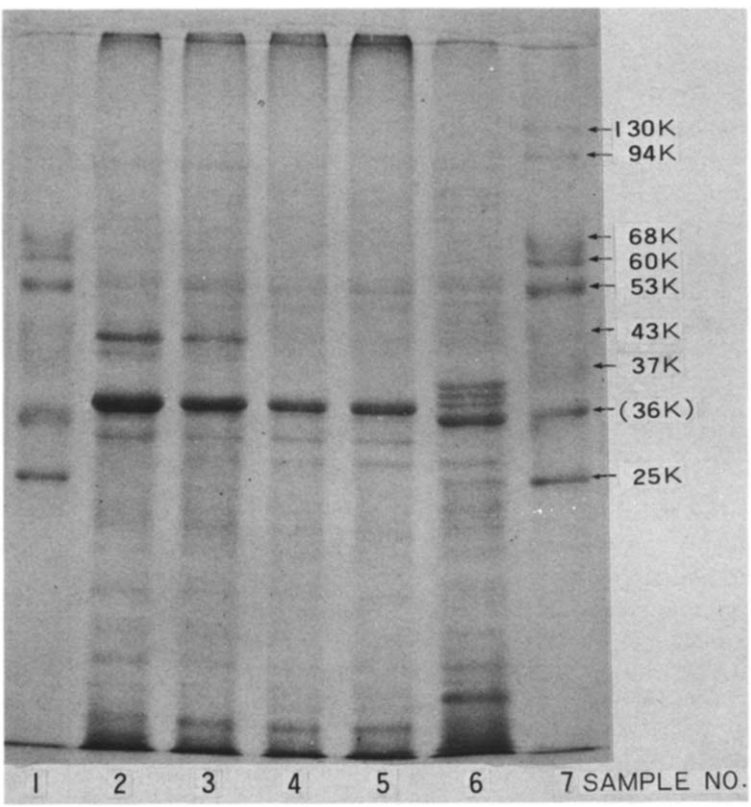

Fig. 4. Treatment of intact cells of *E. coli* B with activated dextran. The experiment was performed as described in Materials and Methods, except that *E. coli* B was used. Columns 1 and 7 are molecular weight standards described earlier [14]; the 36 000 dalton protein, however, has a mobility higher than expected from its molecular weight [14]. Columns 2—5 are cell envelope fractions isolated from cells treated with the activated dextran for 0, 30, 60, and 90 min, respectively. Column 6 is a cell envelope fraction from untreated *S. typhimurium* HN366 cells, electrophoresed for comparison. In this experiment the cells were not labeled with radioactivity. Consequently the amounts of protein added to each trough are not exactly equal.

none of the inner membrane proteins showed clear signs of decrease: this is seen for example in 78K, 54K, 45K, 27K, and 15K bands of Fig. 3B.

These alterations produced by coupling with the activated dextran were quite reproducible. Altogether we have repeated twelve times the activated-dextran coupling of intact HN366 cells, and the results were analyzed by sodium dodecyl sulfate-acrylamide gel electrophoresis of the total cell envelope fraction. In eleven experiments we could confirm the main results, i.e., (i) the decrease of 90K, 84K, 82K, 48K, 36K, 35K, 34K, 23K, and 22K bands, and (ii) the lack of decrease in 41K and 33K bands. Some of the other alterations found in Fig. 3 were found in many experiments, but not in some, presumably because the change occurred in faint bands or got obscured by inner membrane proteins.

In some experiments the decrease in some of the bands was confirmed also by cutting out the bands from the dried gel, and measuring the radioactivity in each band as described in Materials and Methods. In one experiment, we found in this manner that 50, 70, 20, 32, and 65% decrease occurred in the 90K, 84K + 82K, 36K, 35K, and 34K bands, in contrast to only 5% decrease in the 33K band.

Experiments with E. coli B

E. coli B is of interest to us because it has an outer membrane with a rather simple protein composition. Above all, it contains only two protein bands in the "major protein" region, one easily extractable with sodium dodecyl sulfate at 60°C, and only one protein which is not extracted under the same conditions [16,17] instead of three such proteins (36K, 35K, and 34K) found in *Salmonella* [3]. Electrophoresis of cell envelope fraction from *E. coli* revealed three prominent protein bands (Fig. 4), all of which were found to be components of the outer membrane in a separate experiment (not shown). When intact cells of *E. coli* B were treated with the activated dextran, all three proteins became coupled to dextran (Fig. 4).

Discussion

Our covalent labeling study showed clearly that, when activated dextran was added to intact cells of *S. typhimurium* or *E. coli*, many of the outer membrane proteins became coupled to dextran. Most probably these proteins are exposed on the outside surface of the outer membrane. Although we used a *S. typhimurium* mutant that produces lipopolysaccharides with short carbohydrate chains, the mutant does not belong to the "deep rough" class [3] that often produces "leaky" outer membrane, and indeed the outer membrane of this mutant does not allow the penetration of the activated dextran [11]. An alternative interpretation is that the coupling of dextran damages the outer membrane so that it eventually allows the penetration of the activated dextran, which then becomes coupled to proteins on the inner surface of the outer membrane. This, however, is unlikely since none of the inner membrane proteins were labeled as judged by the gel electrophoresis (Fig. 3B) as well as by the unaltered buoyant density of the inner membrane, results suggesting that the activated dextran did not penetrate through the outer membrane. Although we cannot completely exclude the possibility that coupling of dextran molecules

exposes initially hidden proteins through the production of conformational changes in the readily coupled proteins, this seems rather unlikely as the amidine group produced by imidoester coupling has the same positive charge as the amino group of the starting material [18].

Among 18—20 protein bands detected on the electropherograms of *S. typhimurium* outer membrane, between 11 and 13 bands become coupled to dextran added to the intact cells, and thus are presumably exposed on the outer surface of the outer membrane. Furthermore, three out of the four "major" proteins behave in this manner. We can thus conclude that a major portion of the proteins of the outer membrane is exposed on the outside, and therefore is located at least partly in the outer half of the outer membrane. These results are consistent with other lines of evidence. (i) Results of labeling with activated dextran, as well as of phospholipase C treatment, suggest that most of the phospholipid molecules in the outer membrane are located in the inner half of the membrane [11]. Since phospholipids are present in an amount sufficient to cover about 90% of the inner surface of the membrane [13], the inner half of the membrane does not have much room left for proteins, most of which therefore are expected to be located in the outer half of the membrane. (ii) Freeze-fracture electron microscopy of the outer membrane showed that most protein "particles" are found on the concave fracture face, suggesting that the major portion of these proteins is located in the outer half of the outer membrane [13]. (iii) Many outer membrane proteins, including the two "major" proteins (36K and 34K) [19] as well as the "binding proteins" for vitamin B_{12} [20], ferrichrome [21,22], and enterobactin [23], are known to be used as receptors by phages and colicins, and must therefore be exposed on surface. (iv) Reconstitution studies [9] indicate that a complex containing 36K, 35K, and 34K proteins produces aqueous channels in lipopolysaccharide-phospholipid bilayer, presumably by penetrating through the thickness of the reconstituted membrane. Thus at least one of these proteins must be at least partially exposed on the outer surface.

It should be emphasized that several of the proteins expected to be exposed, as described above, were indeed labeled by the activated dextran. These include, first of all, the 36K, 35K, and 34K "major" proteins. Secondly, the Fe^{3+}-ferrichrome "receptor" of *E. coli* has a molecular weight of 85 000 [24], and a doublet of bands in this region was found to be repressed strongly by growth in the presence of Fe^{3+} [25]. The 82K-84K doublet in *S. typhimurium* may thus correspond to the "receptors" or "binding proteins" for Fe^{3+}-chelator complexes, and these proteins were indeed coupled to the activated dextran (Fig. 3).

Our gel system did not resolve peptides smaller than 12 000 daltons, and we therefore cannot tell whether the Braun lipoprotein [26] (about 7000 daltons) is exposed on surface. Results suggesting its exposure have recently appeared [27].

None of the bands disappeared completely when intact cells were reacted with the activated dextran. It is possible that the attachment of the macromolecular labeling reagent to a neighboring protein molecule could sometimes produce steric hindrance so that even exposed proteins might not have become fully labeled.

One major protein of *S. typhimurium* (33K) and four minor proteins were not coupled to dextran when the labeling was done with intact cells. This could be due either to their location or to the absence of reactivity. The latter explanation, however, is unlikely since most membrane proteins are expected to have free amino groups in their hydrophilic portion, and since these proteins were coupled to dextran when isolated outer membrane vesicles were used for coupling (Fig. 1C). The unlabeled proteins thus probably are inaccessible for the labeling reagent either because they are not exposed on cell surface, or because they are hidden by the saccharide chains of lipopolysaccharide. We favor the former interpretation in view of the following. (i) The strain used produces an incomplete, Rc-type lipopolysaccharide, the saccharide chain of which contains only 6 sugar residues in contrast to 50 or more found in the wild type [1]. It seems unlikely that such short oligosaccharide chains completely cover any protein in the outer membrane. (ii) The 33K protein reacts extensively with activated dextran, when isolated outer membranes were used (Fig. 1C). French pressure cell treatment of *E. coli* cells produces inside-out vesicles of the cytoplasmic membrane [28], and this is consistent with the almost complete labeling of proteins in the isolated inner membrane vesicles (Fig. 1C), as most intrinsic proteins of the inner membrane are located in the inner half of the membrane [11]. The extensive reaction of the 33K protein is thus most likely explained by assuming that this protein is normally located at the inner surface of the outer membrane, which however gets inverted during the preparation of isolated membrane vesicles. These results are difficult to explain by the steric hindrance hypothesis, as few lipopolysaccharide molecules are lost during the isolation of outer membranes [11]. (iii) In contrast to the exposed proteins including 36K and 34K, phage or colicin using the 33K protein as receptor has not been found [19].

The 33K protein of *S. typhimurium* seems to share some similarities with the protein 3 [16] (or protein II* [29]) of *E. coli* outer membrane in that both are fully extracted by sodium dodecyl sulfate at temperatures below 60°C, and that their mobilities in sodium dodecyl sulfate-acrylamide gels get reduced when they are boiled in sodium dodecyl sulfate; yet the former differs from the *E. coli* protein which is reported to act as the receptor for a colicin [30] and a phage [31]. We also found that the 31K protein of *E. coli* B, presumably corresponding to protein 3, was exposed on cell surface (Fig. 4).

Kabir [32], by using lactoperoxidase labeling technique, found that only one outer membrane protein (12-13K) was labeled in intact cells of a rough mutant of *S. typhimurium*. It is not clear why other proteins were not labeled. Possibly the other exposed proteins do not possess tyrosine residues at easily labeled positions. It is also possible that the labeling of many minor proteins was not detected because of the poor resolution of his gels. More recently, the lactoperoxidase technique was found to result in a poor labeling of the smooth (wild type) strain of *Proteus mirabilis*, presumably because the long polysaccharide chains of lipopolysaccharide formed a thick, difficult-to-penetrate layer on the cell surface [33]. It should be stressed that our experiments were performed with an Rc mutant that produces lipopolysaccharide with very short carbohydrate chains, precisely to avoid complications of this type.

We have shown previously that CNBr-activated dextran is very useful as a

non-penetrating reagent for labeling phosphatidylethanolamine molecules in membranes [11]. This study further shows its usefulness in labeling membrane proteins and in determining their locations. Since the reagent is easily prepared, reacts with usually quite abundant groups on proteins under nearly physiological conditions, and does not suffer from problems of slow penetration, it is expected to be useful for studies of many other biological membranes.

Acknowledgements

This work was sopported by research grants from the United States Public Health Service (AI-09644) and from American Cancer Society (BC-20).

References

1 Nikaido, H. (1973) in Bacterial Membranes and Walls (Leive, L., ed.), pp. 131—208, Marcel Dekker, New York
2 Schnaitman, C.A. (1970) J. Bacteriol. 104, 890—901
3 Ames, G.F., Spudich, E.N. and Nikaido, H. (1974) J. Bacteriol. 117, 406—416
4 Berg, H.C. (1969) Biochim. Biophys. Acta 183, 65—78
5 Bretscher, M.S. (1972) Nat. New Biol. 236, 11—12
6 Phillips, D.R. and Morrison, M. (1970) Biochem. Biophys. Res. Commun. 40, 284—289
7 Decad, G., Nakae, T. and Nikaido, H. (1974) Fed. Proc. 33, 1140
8 Nakae, T. and Nikaido, H. (1975) J. Biol. Chem. 250, 3359—3365
9 Nakae, T. (1976) J. Biol. Chem. 251, 2176—2178
10 Kågedal, L. and Åkerström, S. (1971) Acta Chem. Scand. 25, 1855—1859
11 Kamio, Y. and Nikaido, H. (1976) Biochemistry 15, 2561—2570
12 Anderson, E.H. (1946) Proc. Natl. Acad. Sci. U.S. 32, 120—125
13 Smit, J., Kamio, Y. and Nikaido, H. (1975) J. Bacteriol. 124, 942—958
14 Ames, G.F. (1974) J. Biol. Chem. 249, 634—644
15 Schnaitman, C.A. (1971) J. Bacteriol. 108, 545—552
16 Schnaitman, C.A. (1973) J. Bacteriol. 118, 454—464
17 Rosenbusch, J.P. (1974) J. Biol. Chem. 249, 8019—8029
18 Ludwig, M.L. and Hunter, M.J. (1972) Meth. Enzymol. 25, 585—596
19 Nurminen, M., Lounatmaa, K., Sarvas, M., Mäkelä, P.H. and Nakae, T. (1976) J. Bacteriol. 127, 941—955
20 Di Masi, D.R., White, J.C., Schnaitman, C.A. and Bradbeer, C. (1973) J. Bacteriol. 115, 506—513
21 Wayne, R. and Neilands, J.B. (1975) J. Bacteriol. 121, 497—503
22 Hantke, K. and Braun, V. (1975) FEBS Lett. 49, 301—305
23 Wayne, R., Frick, F. and Neilands, J.B. (1976) J. Bacteriol. 126, 7—12
24 Braun, V. and Wolff, H. (1973) FEBS Lett. 34, 77—80
25 Uemura, J. and Mizushima, S. (1975) Biochim. Biophys. Acta 413, 163—176
26 Braun, V. (1975) Biochim. Biophys. Acta 415, 335—377
27 Braun, V., Bosch, V., Klumpp, E.R., Neff, I., Mayer, H. and Schlecht, S. (1976) Eur. J. Biochem. 62, 555—566
28 Altendorf, K.H. and Staehelin, L.A. (1974) J. Bacteriol. 117, 888—899
29 Hindennach, I. and Henning, U. (1975) Eur. J. Biochem. 59, 207—213
30 Chai, T. and Foulds, J. (1974) J. Mol. Biol. 85, 465—474
31 Henning, U. and Haller, I. (1975) FEBS Lett. 55, 161—164
32 Kabir, S. (1975) Can. J. Microbiol. 21, 1131—1136
33 Hasin, M., Razin, S. and Rottem, S. (1976) Biochim. Biophys. Acta 433, 229—239