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PROTEIN BLOTTING THROUGH A DETERGENT LAYER, A SIMPLE METHOD FOR DETECTING INTEGRAL MEMBRANE PROTEINS SEPARATED BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

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SUMMARY: To selectively detect amphiphilic proteins from a mixture of proteins separated by SDS-polyacrylamide gel electrophoresis, the gel was electro-blotted through another polyacrylamide gel containing a non-ionic detergent (NP40) onto a nylon membrane filter. Most soluble proteins of *E. coli* passed through the detergent-containing gel, whereas a major fraction of the proteins from the cytoplasmic (inner) membrane, including the lactose and melibiose carrier proteins, were trapped in the detergent layer. The major outer membrane proteins, OmpA, OmpF and LamB, partitioned to the detergent layer only when solubilized at low temperature which avoids complete denaturation. This simple procedure, termed "detergent blotting", should have wide application in the study of integral membrane proteins.

Integral membrane proteins usually contain one or more polypeptide segment(s) that are predominantly hydrophobic and which interact with the hydrocarbon part of the lipid bilayer (1,2). They bind a large number of detergent molecules, specifically of the "mild" classes, and detergents are generally required for solubilization of these proteins (3,4). A few relatively simple procedures have been reported for enriching integral membrane proteins over hydrophilic proteins or distinguishing between these two classes. These include phase separation in Triton X-114 solution (5), alkaline extraction of the membrane (6), and charge shift electrophoresis (7). In this work, we adapted the protein blotting technique (ref. 8, for a review) for identification of hydrophobic membrane proteins. Our simple method, termed "detergent blotting", can be applied to proteins separated by SDS-polyacrylamide gel electrophoresis, one of the most-commonly used techniques in biochemistry and molecular biology.

MATERIALS AND METHODS

Bacterial strains and plasmids

Escherichia coli strain MC4100 was described previously (9). Strain KI266, a derivative of CSH26 (10) carrying F'$lacI^q$ O^+ Z^{u118} pro^+ (11), was transformed

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by plasmid pBR322 or pTE18 (11; provided by I. Yamato) to overproduce the lactose carrier protein. Strain N1790 and its derivative carrying pSTY91 (12) were used for identification of the melibiose carrier protein.

Media, labeling and sample preparation

Cells were grown in minimal medium E (13) supplemented with thiamine (2 µg/ml), 18 amino acids (20 µg/ml each, other than methionine and cysteine), and a carbon source (0.4%) as indicated. Exponential-phase cells were labeled with 15 µCi/ml of [35S] methionine (1,100 Ci/mmol, New England Nuclear) for 3 min. Samples for SDS-polyacrylamide gel electrophoresis were prepared by lysozyme-freezing and thawing method described previously (14). Cell fractionation was carried out as described (15) except that cytoplasmic and outer membranes were separated by the method in ref. 16.

Protein blotting through a detergent-containing gel (detergent blotting)

Gel electrophoresis in the presence of SDS was done by a modified Laemmli system with 15% acrylamide and 0.12% N,N'-methylene-bis-acrylamide (14). After electrophoresis, the dye front region was removed from a gel slab (1 mm thick), since radioactive material there occasionally spread and caused artificial spots or streaks. The gel was rinsed briefly (1-5 min) at room temperature with 2.5 mM Tris-19.2 mM glycine buffer (blotting buffer, pH 8.4). The "detergent-containing gel" (1 mm thick), consisting of 10% acrylamide, 0.27% N,N'-methylene-bis-acrylamide, 2% NP40 (Nonidet P40; product of Shell) and the blotting buffer, was placed in contact with the rinsed gel. A nylon membrane filter (Zeta Probe, obtained from Bio-Rad), presoaked in blotting buffer, was then placed on the other side of the detergent-containing gel. The sandwich was supported by a plastic frame in combination with porous plastic sheets and placed in an electro-blotting apparatus (Marysol Industry, Tokyo, Japan) filled with the blotting buffer. Electro-blotting was carried out at 20 v/cm for 20-24 hr at about 4°C. After blotting, the gels and the nylon filter were dried under vacuum and exposed to Fuji X-ray films. Care was taken that the surface of the detergent-containing gel that had been in contact with the original gel was placed on the X-ray film. In this paper, we call this procedure "detergent blotting".

SDS-SDS two-dimensional gel electrophoresis

The details of this procedure have been described elsewhere (17). Briefly, electrophoresis in the presence of SDS was carried out successively through two different concentrations of polyacrylamide gels (first dimension, 15% acrylamide-0.12% N,N'-methylene-bis-acrylamide; second dimension, 20% acrylamide-0.53% N.N'-methylene-bis-acrylamide).

RESULTS AND DISCUSSION

Behavior of soluble and cytoplasmic membrane proteins of E. coli in detergent blotting

Cells of *E. coli* strain MC4100 were labeled with [^{35}S] methionine, and fractionated into the periplasmic, cytoplasmic, cytoplasmic membrane, and outer membrane fractions. Since some integral membrane proteins aggregate upon heating in SDS (14) while certain other proteins require boiling in SDS for complete denaturation (18), the samples were treated in SDS sample buffer either at 37°C or 100°C. After SDS-polyacrylamide gel electrophoresis, an another gel containing NP40 was sandwiched between the SDS gel and a nylon membrane filter for detergent blotting (see MATERIALS AND METHODS). The majority of soluble

proteins from the periplasmic space and the cytoplasm, were effectively transferred to the membrane filter through the detergent-containing gel (Fig. 1; compare A and B for lanes 1,2,5, and 6). In contrast, a major fraction of proteins from the cytoplasmic membrane were not transferred to the membrane filter but found associated with the intermediary gel containing NP40 (Fig. 1A, lanes 3 and 7). The inclusion of NP40 was essential for this trapping (data not shown for a gel without NP40).

The behavior of some well-defined integral membrane proteins, the lactose-carrier protein and the melibiose-carrier protein, was then studied using the

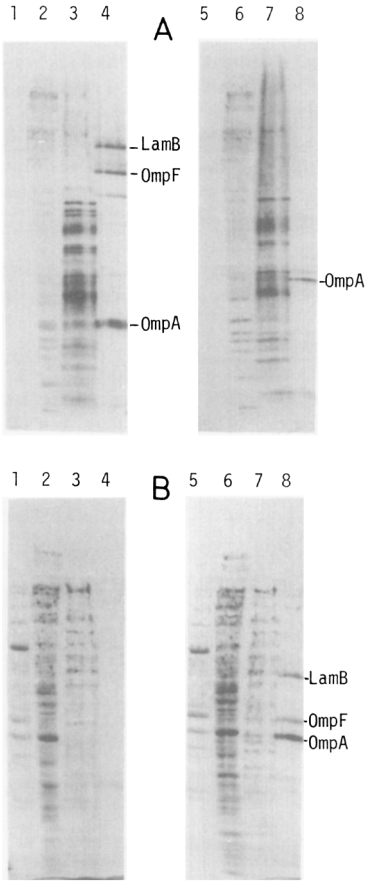

Fig. 1. Detergent blotting of fractionated E. coli proteins. Cells of MC4100 were grown with maltose at 30°C, labeled with [35S]methionine, and fractionated. The amounts loaded onto the gel did not quantitatively reflect the relative abundance of each fraction. **A**, NP40-containing gel; **B**, Nylon membrane filter. Lanes **1** and **5**, periplasmic fraction; lanes **2** and **6**, cytoplasmic fraction; lanes **3** and **7**, cytoplasmic membrane fraction; lanes **4** and **8**, outer membrane fraction. Samples for lanes **1-4** were treated at 37°C whereas those for lanes **5-8** were heated at 100°C before SDS-polyacrylamide gel electrophoresis. Only faint bands were seen with the original gel after blotting (not shown).

strains overproducing these proteins. These integral membrane proteins were trapped in the detergent layer (Fig 2B. lanes 1 and 3). The overproduced carrier proteins in the whole cell lysate could be identified clearly only after detergent blotting (Fig. 2; compare A and B for lanes 1 and 3). Hanatani et al. (12) had to use a reconstitution system to visualize the overproduced melibiose carrier.

To demonstrate that the retention of the membrane proteins by the NP40-containing gel was quantitative, the lactose-carrier protein was separated from other proteins by SDS-gel electrophoresis in two-dimension. As reported elsewhere (17), many membrane proteins form spots off and above the main diagonal line when electrophoresed successively through gels of low (first dimension) and high (second dimension) polyacrylamide concentrations; the two-dimensional system of O'Farrell (19) does not work for certain membrane proteins, due presumably to their strong interaction with NP40 (17). As shown in Fig. 3, the overproduced lactose-carrier, which migrated as a spot off the diagonal line, was trapped completely in the NP40-containing gel (compare A and C). The secY gene product (14), a membrane component of the bacterial protein export machinery, also partitioned exclusively into the detergent layer (17).

The results that a minor fraction of proteins from the cytoplasmic preparation were trapped in the detergent-containing gel (Fig. 1A, lanes 2 and 6) could be explained, at least partly, by a contamination by small membrane fragments produced by sonication, since a soluble fraction prepared by a milder method resulted in less trapping (data not shown). On the other hand, some quarter of the proteins from the membrane fraction were able to pass through the detergent-containing gel (Fig 1B, lanes 3 and 7). They may represent peripheral membrane proteins. This is consistent with the statement that 70-80% of proteins found in biological membranes are integral (1).

The above results have shown that soluble proteins are able to pass through the detergent-containing gel whereas many membrane proteins are retained by the detergent-containing gel during their migration toward the anode. This is presumably due to the amphiphilic nature of the membrane proteins. The

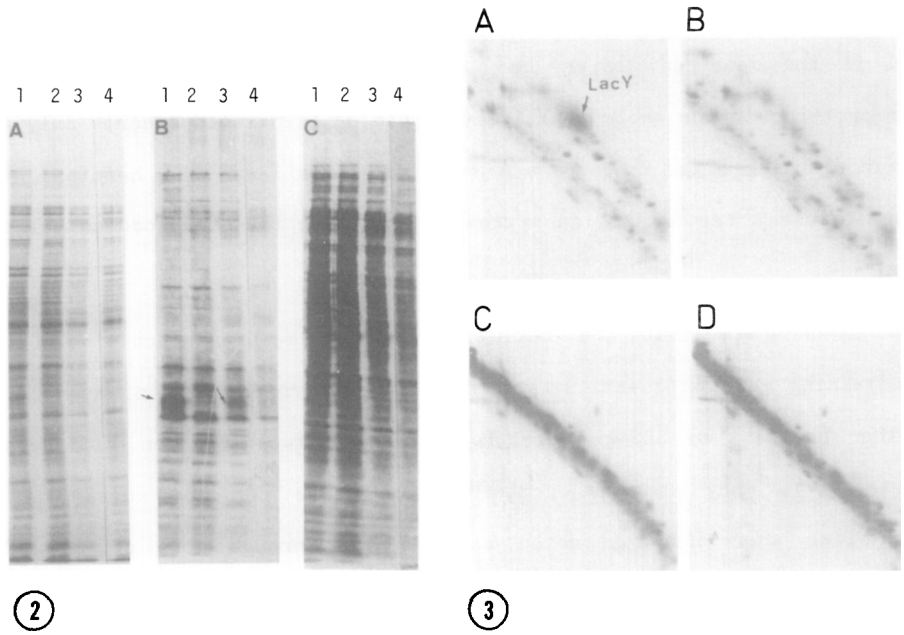

Fig. 2. Detection of the overproduced lactose-carrier protein and the melibiose-carrier protein by detergent blotting. Cells of KI266/pTE18 (lane **1**) and KI266/pBR322 (lane **2**) were grown with glycerol at 37°C and induced by 1 mM isopropyl-ß-D-thiogalactoside for 5 min before labeling with [35S]methionine. Cells of N1790/pSTY91 (lane **3**) and N1790 (lane **4**) were grown with glucose at 37°C and labeled. The whole cell lysates were electrophoresed in SDS followed by detergent blotting. **A.** SDS-gel without blotting; **B**, NP40-containing gel after blotting; **C.** Nylon membrane filter after blotting. The exposure time was shorter for **A** than for **B** and **C**. The arrows in **B** indicate the lactose-carrier protein (lane **1**) and the melibiose-carrier protein (lane **3**).

Fig. 3. Separation of the lactose-carrier protein by SDS-SDS two-dimensional gel electrophoresis and detergent blotting. The labeled whole cell proteins from KI266/pTE18 (**A** and **C**) and KI266/pBR322 (**B** and **D**) were subjected to SDS-SDS two-dimensional gel electrophoresis (see MATERIALS AND METHODS) followed by detergent blotting. **A** and **B**, NP40-containing gel; **C** and **D**, nylon membrane filter.

hydrophobic segments of these polypeptides may interact strongly with the non-ionic detergent, forming mixed micelles that are large enough to retard the electrophoretic movement in polyacrylamide gel. During this retardation, a fraction of the SDS molecules bound to the polypeptide might be removed electrophoretically, decreasing the negative charge of the complex. This would in turn cause further retardation. In concert with this notion, when a lower acrylamide concentration was used for the detergent-containing gel, a leakage of membrane proteins onto the nylon membrane filter was observed (data not shown). It is not known how the denatured and extended polypeptides change their conformation during the blotting procedure.

Behavior of major outer membrane proteins in detergent blotting

The major outer membrane proteins exhibited more complex behaviors. The OmpA, OmpF and LamB proteins were trapped in the detergent layer when solubilized at 37°C (Fig. 1A, lane 4), whereas the same proteins passed through the detergent gel when solubilized at 100°C (Fig. 1B, lane 8). These proteins require high temperature for complete denaturation in SDS (18). The OmpA protein is "heat modifiable" and its apparent molecular weight in SDS-gel electrophoresis is about 28,000 or 33,000, when solubilized at 37°C or 100°C, respectively. The porin proteins (OmpF and LamB) exist in the membrane as trimeric pores whose structure is preserved in SDS solution at low temperatures (18).

The OmpF and LamB proteins lack long stretches of hydrophobic amino acids (20, 21). Also, the OmpA protein does not contain a hydrophobic segment that is big enough to traverse the membrane in the α-helix configuration (22). Thus, the mode of interaction of these major outer membrane proteins with the lipid bilayer may be different from that of the more typical integral membrane proteins. Probably, some ordered structures, like amphiphilic helices (23) or the extensively hydrogen-bonded β-sheets (24), are required for interaction with the lipid bilayer. The present results that the denatured forms of the major outer membrane proteins do not partition to the detergent phase, suggest that such an ordered structure is also required for effective interaction with the detergent micelles. A detergent-binding zone was indeed reported for the trimeric form of the porin protein (25).

Potential use of detergent blotting technique

The most salient feature of the present technique is that the proteins are first separated by SDS into denatured individual polypeptides before being tested for their amphiphilicity. This is a distinct feature from the Triton X-114 phase separation procedure (5), which mostly preserves protein native conformations as well as subunit structures, if any. Detergent blotting should especially be effective for detecting those proteins which are anchored in the membrane by virtue of direct interactions between the lipid bilayer and the

specific segments of the polypeptide. Less predictable may be the behaviors of the other types of membrane proteins which are associated with the membrane in more complex manners, as illustrated here by the major outer membrane proteins. In the Triton X-114 phase separation system, some integral membrane proteins have been reported to behave anomalously (26, 27).

The procedure of detergent blotting does not involve any centrifugation or phase separation steps and, hence, is particulary suited for microscale experiments. It is an easy extension of SDS-polyacrylamide gel electrophoresis and it can deal with complex biological materials because of the high resolution of SDS-polyacrylamide gel. Its usefulness as a method for identifying a cloned gene product has been demonstrated in this paper for the two carrier proteins of E. coli. In addition, the two-dimensional separation before blotting will greatly improve the resolution. These new techniques may prove generally useful in the study of membrane proteins.

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