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LOCALIZATION OF THE LACTOSE PERMEASE PROTEIN(S) IN THE E. COLI ENVELOPE[1]

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SUMMARY

The amino acid double labeling technique was used to identify and localize membrane-bound lactose operon proteins in E. coli. Both the "M" protein, thought to be the y gene product, and a polypeptide of MW ∿15,000 appeared in the membrane following lac operon induction. The amounts of these two proteins were approximately equal.

The inner and outer membrane layers of the cell envelope were separated by sucrose density gradient centrifugation or by selective solubilization of the inner membranes with the detergent Sarkosyl. When gentle lysis conditions were employed to prepare membrane vesicles, both lac induced proteins fractionated with the inner membrane. However, the "M" protein was more easily randomized in the envelope structure by sonication than the 15,000 dalton component or an inner membrane marker enzyme.

INTRODUCTION

Induction of the Escherichia coli lactose operon results in the appearance of proteins that allow the cell to accumulate and metabolize β-galactosides. To understand the mechanism of lactose active transport, the protein product(s) of the y gene must be identified and isolated from the cell envelope. A "lactose permease" was identified as a component of the E. coli envelope by Kennedy and his coworkers (1). The identification was based on specific radioactive labeling procedures using N-ethylmaleimide (NEM) and a amino acid double labeling technique. This intrinsic membrane component (or "M" protein) had a molecular weight of 30,000 estimated by polyacrylamide gel electrophoresis in SDS[2] and gel filtration.

The cell envelope of gram negative bacteria is a multi-layered structure (2). Energy metabolism is localized in the inner membrane. Since active trans-

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2. Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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port requires coupling to an energy source, it seemed likely that the "M" protein would be an inner membrane component. This paper describes the separation of the inner and outer membranes of *E. coli* by several methods and the resulting distribution of membrane-bound lactose operon proteins.

MATERIALS AND METHODS

Chemicals used for electrophoresis were the highest grade available. Acrylamide, bis methylene acrylamide and SDS were obtained from Bio-rad Laboratories. Ammonium persulfate was obtained from Fisher Scientific Co. Analytical grade sucrose was obtained from Mallinckrodt and Sarkosyl from CIBA-Geigy. L-[U-^{14}C]Leucine at 320 mCi/mmol, L-[4,5-^{3}H]leucine at 60 Ci/mmol, L-[^{14}C(U)]Amino Acid Mixture and L-[^{3}H]Amino Acid Mixture were obtained from New England Nuclear.

Bacterial strains and growth conditions. All strains used were derived from *E. coli* K12 and were obtained from Drs. I. Zabin and A. Fowler. Strain A324-4 is *lac* $I^{-}Z^{+}Y^{+}A^{+}$ pro^{-}/F*lac* $I^{+}Z^{+}Y^{+}A^{+}$ pro^{+} and originated in the laboratory of Dr. E. P. Kennedy. The Z deletion strain W4680 (lac Z4680) was isolated and characterized by Dr. J. Beckwith. Cultures were grown at 37C in a rotary shaker in minimal medium 63 (3) containing 1 μg of thiamine per ml and 0.4% (w/v) glycerol or succinate. Cells were induced with isopropylthiogalactoside (Sigma) at 0.5 mM.

Labeling of cultures with radioactive amino acids. In a typical experiment, cultures of the appropriate strain (100 ml each) were grown on the medium described above until the A600 equaled 0.2. One actively growing culture was induced with 0.5 mM IPTG; the other received no inducer. The cultures were grown for approximately one generation before addition of radioactive amino acids. Fifty μc of L-[^{14}C]leucine was added to the induced cells and 125 μc of L-[^{3}H]leucine to the uninduced cells. The two cultures were grown in the presence of the radioactive amino acids for 1.0-1.5 generations (A_{600} ≈0.8). The cells were harvested by centrifugation and washed once with 10 mM Tris pH 7.8. The cell pellets from the two cultures were pooled and suspended in 0.75 M sucrose, 10 mM Tris, 5 mM mercaptoethanol pH 7.8 for preparation of membranes. These experiments were also performed with the labels reversed, with radioactive amino acid mixtures, and for shorter labeling times.

Preparation of membranes and separation on sucrose gradients. Spheroplasts were prepared by lysozyme-EDTA treatment and were lysed by either dilution or sonication (Heat Systems Sonicator Cell Disruptor) as described by Osborn. Sucrose density gradient centrifugation was carried out in a 25-55% (w/w) sucrose gradient in the SW40 rotor (4). Gradients were fractionated by puncturing the bottom of the tube and collecting 50 fractions of 10 drops each.

Enzyme assays. β-Galactosidase was assayed by the hydrolysis of o-nitrophenyl-β-D-galactoside (Sigma) in 0.1 M NaH_2PO_4, 2 mM $MgSO_4$, 0.2 mM $MnSO_4$, pH 7.0 containing 0.05 M β-mercaptoethanol (5). Succinic dehydrogenase was measured by the coupled assay described by Osborn (4). Protein was measured by the Lowry method (6).

SDS-Polyacrylamide gel electrophoresis. Radioactive samples were analyzed on cylindrical gels containing 10% acrylamide utilizing the Laemmli buffer system (7). Gels 14 cm long were cut into 2 mm segments with a Gilson Aliquogel Fractionator. The minced segments were dried, suspended in PCS solubilizer (Amersham/Searle) and radioactivity determined with a Beckman-LS230 Liquid Scintillation counter with appropriate settings to discriminate between ^{3}H and ^{14}C. The ratio of the total amount of ^{3}H on the gel to the total amount of ^{14}C was set equal to 1, and the excess of ^{3}H or ^{14}C calculated for each fraction (1). Alternately, the minced gel segments were dissolved by addition of 0.2 ml of 60% perchloric acid followed by 0.4 ml of 30% hydrogen peroxide and

incubated at 50°C overnight. Ten ml of Aquasol-2 (New England Nuclear) was added and vials counted and data calculated as described above. A third method of protein solubilization from gels involved swelling the minced or sliced gel in 0.5 ml of 90% Protosol (New England Nuclear) overnight followed by addition of 10 ml of Econofluor (New England Nuclear) prior to counting. A gel with six protein standards was used each time for conversion of mobility to molecular weight.

RESULTS

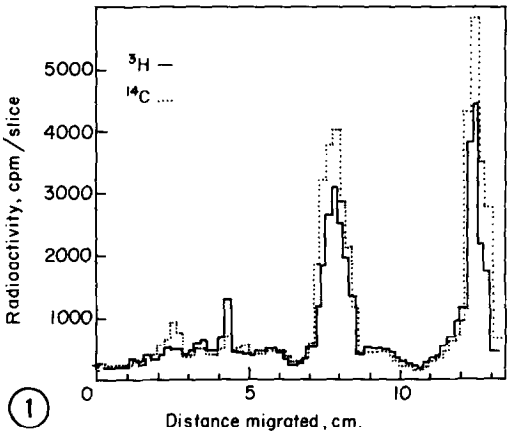
Detection of membrane-bound lac operon proteins

In order to identify all proteins synthesized as a result of lac operon induction, we have used the amino acid double labeling technique. The protein composition of the labeled membrane fractions was then analyzed on SDS-polyacrylamide gels (see METHODS). Figure 1 shows the distribution of radioactivity in the gel fractions from a separation of the total envelope proteins of strain A324-4.

Calculations of excess of ^{14}C (representing membrane protein found in induced, but not in uninduced, cells) in each fraction utilized the formula $[^{14}C-(R \times {}^{3}H)]$ where R is the ratio of total ^{14}C cpm recovered from the gel to the total ^{3}H cpm recovered. This method of data analysis was chosen to allow comparison with previous work (1).

Figure 2A shows such a distribution of excess ^{14}C calculated from the gel separation of the total envelope proteins shown in Figure 1. One protein enriched with ^{14}C co-migrated with a β-galactosidase standard (relative mobility = 0.19±0.02), indicating that some of this soluble enzyme was bound to the membrane fraction. The small amount of β-galactosidase bound was corroborated by assaying the distribution of β-galactosidase activity between membrane and soluble fraction.

The second major peak of radioactivity on the gel in Fig. 2A had a mobility of 0.57±0.04, corresponding to a molecular weight of 33,000. Comparison with earlier work suggests that this is the membrane component designated "M" protein. There was however, an additional ^{14}C enriched membrane component with a mobility of 0.92±0.05 in this system, corresponding to a protein molecular weight of 15,000. We will refer to this small compound as "S" protein.



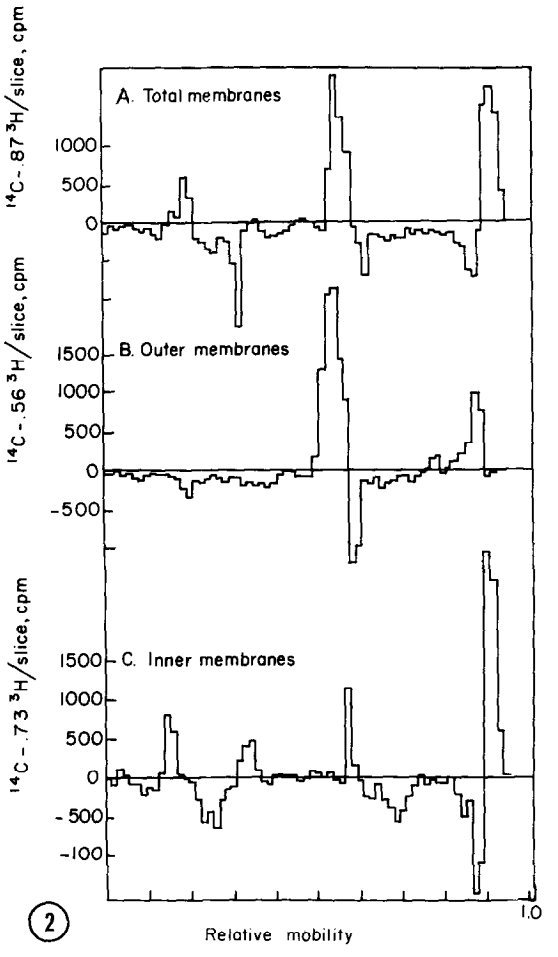


Fig. 1. Distribution of radioactivity incorporated into total membrane proteins of strain A324-4. Proteins were separated on a cylindrical 10% polyacrylamide gel as described in METHODS. Induced cells were grown on leucine-^{14}C and uninduced cells on leucine-^3H. •••• ^{14}C, —— ^3H.

Fig. 2. Distribution of excess ^{14}C in double-labeled membrane proteins of strain A324-4. The excess ^{14}C represents proteins synthesized as a result of lac operon induction, calculated for each fraction as described in the text. Fig. 2A is calculated from the data shown in Fig. 1 for total membrane proteins. Inner and outer membranes were separated by sucrose density gradient centrifugation, fractions pooled and approximately 500 μg protein applied to each gel.

The experiment shown in Fig. 2 is representative of five separate labeling experiments with strain A324-4. The same pattern of labeled polypeptides was obtained from this strain when radioactive amino acid mixtures were substituted

for leucine or when the labels were reversed. To guard against quenching artifacts, three different methods of solubilization from acrylamide gels were tested and each gave the labeling pattern seen in Figure 2 (see METHODS). The same two membrane proteins were also found when the double label procedure was applied to another wild type strain, Hfr 3000, and to a Z deletion strain W4680 (data not shown).

Separation of inner and outer membranes

The membrane preparation analyzed in Fig. 2A was obtained by sonication of spheroplasts. The mixture of inner, outer, and hybrid membrane vesicles was separated by sucrose density gradient centrifugation (4). The two forms of inner membrane vesicles, L1 and L2, had buoyant densities of 1.14 and 1.16 g/cc respectively and the buoyant density of outer membrane vesicles was 1.22 g/cc. A hybrid fraction of intermediate density was also present. These densities are comparable to those previously found with Salmonella typhimurium (4). Inner membranes were also characterized by the presence of oxidative enzyme activities, such as succinic dehydrogenase.

The labeled membrane vesicles obtained from the sucrose density gradients were analyzed by SDS-PAGE on disc gels. Figure 2B shows that a substantial portion of the "M" protein and some of the 15,000 dalton "S" Protein fractionated with the outer membranes in this experiment.

The separation of inner membrane proteins is shown in Fig. 2C. Some β-galactosidase was associated with the inner membranes, as expected from enzyme activity measurements. Little of the "M" protein was found with the inner membrane, and the 15,000 dalton "S" protein was the predominant lac component. The quantitative distribution of each protein between fractions was determined from the area under each peak. Table IA summarizes the distribution of the lac induced polypeptides and an inner membrane marker enzyme, succinic dehydrogenase, in the vesicles obtained by sonication of lysozyme-EDTA treated cells. The data suggest that there is considerable randomization of protein during preparation, despite the distinct density differences of the vesicles.

TABLE I

Distribution of Lac Proteins and Succinic Dehydrogenase in the E. coli Envelope Fractionated by Several Methods

	Membrane Fraction		
	Outer (H)	Hybrid (M)	Inner (L1 + L2)
A. Sonicated Vesicles			
"M" Protein (MW 33,000)	58	27	15
"S" Protein (MW 15,000)	20	33	47
Succinic dehydrogenase	12	13	75
B. Osmotically Lysed Vesicles			
"M" Protein (MW 33,000)	>1	8	92
"S" Protein (MW 15,000)	6	23	71
Succinic dehydrogenase	5	32	63
C. Sarkosyl Extraction of Sonicated Membranes*			
"M" Protein (MW 33,000)	44		56
"S" protein (MW 15,000)	7		93
Succinic dehydrogenase	-		+**

*Sarkosyl solubilized material is shown as "inner membrane" and Sarkosyl insoluble as "outer membrane". **There was a 60% loss of total succinic dehydrogenase activity during Sarkosyl extraction. All detectable activity was in the Sarkosyl soluble fraction.

The data shown in Table IA are the results of a typical experiment. In several experiments, the proportion of the "M" protein (MW 33,000) that fractonated with the outer membrane varied between 40 and 60%. Longer sonication times resulted in more of this component becoming outer membrane bound.

Spheroplasts prepared by lysozyme-EDTA treatment can be osmotically lysed by rapid dilution to form vesicles functional in active transport of lactose (8). Amino acid double labeled vesicles were prepared by osmotic lysis and separated on sucrose density gradients. The proteins from each fraction were separated both by SDS-PAGE as described above and by gel filtration on Sephadex G-200 in SDS. Table IB summarizes the distribution of lactose operon-induced membrane proteins and succinic dehydrogenase in the envelope structure when vesicles are formed by the osmotic lysis of spheroplasts. These data show that

both the 33,000 and 15,000 dalton polypeptides are situated in the inner membrane.

Another method of fractionation of the E. coli envelope is the selective solubilization of the inner membrane by the detergent sodium laurel sarcosinate (Sarkosyl). Sarkosyl has been shown to completely solubilize inner membrane vesicles while leaving outer membranes intact (9). Double-labeled sonicated membrane fragments were extracted with 2% Sarkosyl and analyzed in SDS on acrylamide gels and Sephadex G-200. The results in Table IC show that only half of the MW 33,00 protein was extracted, while virtually all of the MW 15,000 protein and succinic dehydrogenase activity were solubilized by Sarkosyl.

DISCUSSION

The amino acid double labeling technique was used to identify and localize membrane-bound proteins resulting from lac operon induction. In agreement with the pioneering work of Kennedy et al. (1), we have identified a protein of 33,000 MW (the "M" protein) in the cell envelope. The "M" protein fractionated with the inner membrane when gentle lysis conditions were employed, but was easily randomized in the envelope structure by sonication. An additional protein, the "S" protein, (of MW ≈15,000) also appeared in the envelope in approximately equivalent amounts after induction. This polypeptide fractionated with the inner membrane also but was less susceptible to randomization by sonication. Kennedy et al. using the double-label procedure, did not detect the high mobility (15,000 dalton) component (1). This discrepancy between their results and ours may be due to differences in solubilization procedures prior to analysis on SDS gels. They suspended cell envelope fragments in SDS at room temperature and removed undissolved material by centrifugation. We have boiled our samples in SDS, a technique more likely to solubilize all membrane components (10).

The relationship of the 15,000 dalton polypeptide to transport function or y gene expression is not yet clear. The two membrane proteins might arise by proteolytic cleavage of a primary translation product of the y gene, or the smaller peptide might result from a degradative process. Preliminary experi-

ments with y gene termination mutants suggest that both polypeptides are absent in these strains. The A gene of the lac operon produces the soluble enzyme thiogalactoside transacetylase, MW = 31,000 (11). We have found no detectable thiogalactoside transacetylase activity in any membrane fraction.

The localization of the lac carrier protein, the "M" protein, in the energized inner membrane is the expected result. However, the data indicate that some proteins are more susceptible than others to randomization in the envelope structure. This can be an important consideration in the isolation of membrane proteins. Further work in our laboratory is focused on the purification of these proteins to clarify in structural terms their relation to each other, to the y gene and to lactose transport.

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