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EVIDENCE FOR TWO LAC Y GENE DERIVED PROTEIN PRODUCTS IN THE E. COLI MEMBRANE[1]

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SUMMARY

The membrane protein composition of several lac operon mutant strains has been determined by amino acid double labeling and analysis by SDS-PAGE or gel filtration in SDS. A single component of apparent MW ≃ 30,000 could be identified as the product of the wild type lac Y gene when membrane proteins were dissolved in SDS at room temperature. Heating in SDS in the presence of 50 mM DTT or iodoacetamide allowed complete denaturation and resolution of two protein components with MW's of 33,000 and 15,000. Both polypeptides were altered in strains with mutations in lac Y. The two polypeptides from a wild-type strain fractionated as a single component during extraction and purification in the non-denaturing detergent, Triton X-100. The sum of the MW's of the two proteins is approximately equal to the MW of the protein product predicted from the lac Y gene DNA sequence.

INTRODUCTION

Transport of β-galactosides through the Escherichia coli membrane requires the protein product of the Y gene of the lactose operon (1). This transport protein (lactose permease, lac carrier protein, "M" protein) was identified as an integral membrane component by specific radioactive labeling procedures using N-ethylmaleimide and differential amino acid labeling (2). A molecular weight of 30,000 was estimated by polyacrylamide gel electrophoresis[2] of the labeled protein in SDS and by gel filtration in the presence of SDS (2). The identification of the Y gene product was confirmed by applying these labeling and analytical procedures to a strain carrying lac Y on a plasmid (3). However, no purification method for this integral membrane transport protein has previously been reported.

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[2]Abbreviations used are: PAGE, Polyacrylamide gel electrophoresis; SDS, Sodium dodecyl sulfate: DTT, Dithiothreitol; IPTG, Isopropylthiogalactoside.

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We have used double labeling experiments with radioactive amino acids to identify and localize membrane-bound lac operon proteins. By incorporating [^{14}C]amino acids into lac induced cells and [^{3}H]amino acids into uninduced cells, we also found that a protein of MW ≈ 30,000 appeared to be the major lac induced membrane component when the proteins were dissolved in SDS at room temperature and analyzed by SDS-PAGE. However, heating of the membrane proteins in SDS containing high concentrations of reducing agents allowed resolution of two lac induced proteins, of molecular weights 33,000 and ≈ 15,000. The amounts of the two proteins were approximately equal (4). The double-label technique also identified 3-5% of the β-galactosidase as membrane-bound. This determination was quantitatively corroborated by activity assays. Assays for the product of the A gene, thiogalactoside transacetylase, revealed no detectable activity in the membrane fraction (4).

The recent determination of the complete DNA sequence of the lac Y gene by D.E. Büchel, B. Gronenborn and B. Müller-Hill (Nature, in press) indicates that the Y gene product is a single polypeptide of MW = 46,500. This suggests that the two lac membrane polypeptides we have identified may result from the proteolytic processing of the primary gene product. To clarify the relationship between the genetic information and observed membrane protein components we have analyzed the membrane protein composition of several lac mutants and have partially purified the wild type permease.

MATERIALS AND METHODS

All bacterial strains (Table 1) were derived from E. coli K12 and were kindly provided by Drs. I. Zabin and A. Fowler. NGY328 and NGY707 were originally isolated and characterized by Dr. D. Zipser; (5) A324-4 from the laboratory of Dr. E.P. Kennedy and CSH50 from the Cold Spring Harbor collection.

The composition of radioactive membrane protein samples was analyzed by SDS-PAGE using 12.5% gels and the Laemmli buffer system (6) or by gel filtration on calibrated Bio-Gel P-300 or Sephadex G-200 columns in a buffer containing 10 mM Tris, 50 mM ammonium acetate, 0.5% SDS pH 7.0 (SDS Column Buffer) plus specified reducing agents. Column fractions were mixed with 10 ml of PCS Scintillation Fluid (Amersham-Searle), and radioactivity determined with a Beckman LS230 Liquid Scintillation Counter with appropriate settings to discriminate between ^{3}H and ^{14}C. Calculations of excess ^{3}H (representing membrane protein found in induced, but not uninduced cells) in each fraction were done using the formula [^{3}H cpm-(R x ^{14}C cpm)] where R is the ratio of total ^{3}H recovered from the column to total ^{14}C recovered.

TABLE I

Lac induced membrane polypeptides and lactose operon genotype of E. coli strains

Strain	lac genotype	lactose transport*	Lac induced membrane polypeptides (MW x 10^{-3})
Hfr3000	$i^+z^+y^+a^+$	+	33, 15
A324-4	$i^-z^+y^+a^+/F'$ $i^+z^+y^+a^+$	++	33, 15
W4680	$i^+z^-y^+a^+$	+	33, 15
NGY328	$i^+z^+y^-a^+$	-	--, 5-7
NGY707	$i^+z^+y^-a^+$	-	12-14
CSH50	Δ lac	-	--, --

*Transport activity was measured by uptake of [^{14}C]-lactose.

RESULTS

Identification of the membrane protein product(s) of the Y gene

The membrane protein composition of strains with defined mutations in the lac operon was investigated to establish the relationship between the two lac membrane proteins and Y gene expression. Each strain was amino acid double-labeled with [^{3}H]leucine in the induced culture and [^{14}C]leucine in the uninduced culture. Membranes were prepared as previously described (4), thoroughly washed to remove soluble proteins, dissolved in SDS containing buffers and analyzed by SDS-PAGE gel filtration in SDS. When membranes from Y^+ strains were dissolved in SDS at room temperature, a single lac induced component of MW ≈ 30,000 was detected by SDS-PAGE or gel filtration, as previously reported (2,3). When heated in SDS, this protein tended to form high molecular weight aggregates. Samples analyzed by SDS-PAGE aggregated to a greater degree than identical samples analyzed by gel filtration. The proteins could be fully denatured without the formation of aggregates by including high levels of reducing agents (50 mM DTT) or an alkylating agent such as 10 mM iodoacetamide during heating in SDS. Fig. 1 shows such a separation of membrane proteins from the Y^+ strain A324-4 on Sephadex G-200 following boiling in SDS containing 50 mM dithiothreitol (DTT).

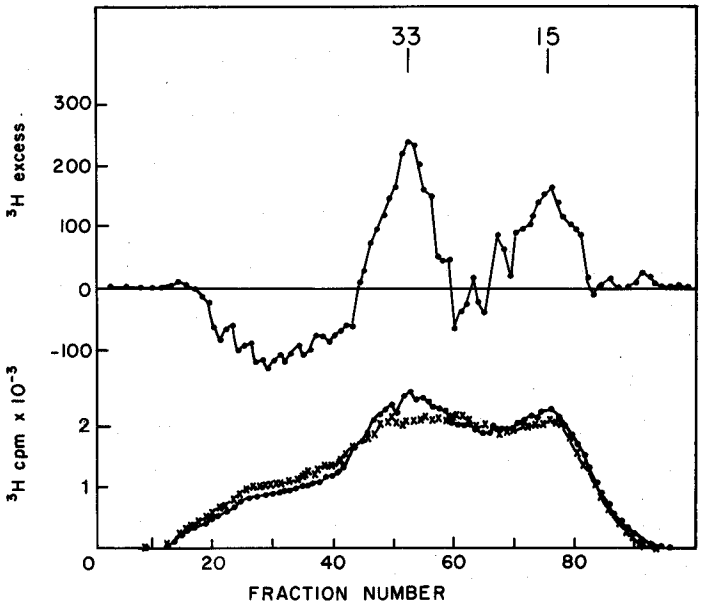

Figure 1. Separation of Membrane Proteins on Sephadex G-200 in SDS. Amino acid double-labeled membrane proteins were separated on a 1.5 x 85 cm column of Sephadex G-200 in SDS Column Buffer. The total radioactivity of each fraction is plotted in the lower portion of the figure ●—●, [^{3}H]leucine; X - X, [^{14}C]leucine. The tritium excess in each fraction, representing proteins present in the lac induced culture and absent in the uninduced culture, is shown above. The MW x 10^{-3} of each peak of tritium excess is shown above the peak.

An estimation of molecular weight for the two peaks of tritium excess was made by calibrating the columnn with soluble protein standards boiled in SDS. The MW estimates of 33,000 for the "M" protein and 15,000 for the other peak of tritium excess are in agreement with results obtained from this strain by SDS-PAGE (4). These same two membrane polypeptides were found in two other strains with wild-type Y genes, Hfr3000 and W4680 (Table 1). Strain W4680 carriers a deletion mutation in lac Z, and is also deficient in the melibiose transport system,an operon known to be partially induced by IPTG (7,8). Strain W4680 contains wild-type levels of both membrane proteins (Table 1) which suggests that neither the Z gene nor a related operon have coded for their synthesis. In strains NGY328 and NGY707 which contain termination mutations in the lac Y gene both protein products are apparently altered. In a lac deletion strain, CSH50, no membrane proteins are induced by IPTG (Table 1).

When strains with a single wild-type Y gene such as Hfr3000 and W4680 are grown in succinate medium, the total amount of lac induced membrane proteins accounts for approximately 1% of the plasma membrane protein. In strain A324-4, lac operon expression of succinate grown cells is 4-5 times greater than wild-type as measured by β-galactosidase activity (9). Under these conditions the level of the sum of the contributions of the two lac proteins is proportionately increased to 4-5%. In the two termination mutant strains, the amount of lac induced membrane protein is less than 50% of wild type levels.

Partial Purification of the Transport Protein from Wild-Type Cells

Belaich et al. have shown that the lac transport protein retains some substrate binding affinity when solubilized in Triton X-100 containing buffers (10). Extraction and purification in Triton X-100 should, therefore, aid in the characterization of the native protein. Isotopically labeled membranes from strain A324-4 were extracted with 2% Triton X-100, 5 mM EDTA by the method of Schnaitman (11). The solubilized extract was dialyzed extensively against 15 mM Tris, 5 mM EDTA, 0.05% Triton X-100, 5 mM β-mercaptoethanol pH 7.0 prior to application to DEAE-cellulose. The column was washed with a linear 0 - 0.3 M NaCl gradient in the column buffer followed by a 1 M NaCl step. Fig. 2 shows that the permease protein peak of tritium excess is well-separated from the small amount of membrane bound β-galactosidase on this column.

The fractions within the main peak of tritium excess from the DEAE-cellulose column were pooled and concentrated with a Millipore CX-10 Immersible Ultrafiltration Unit, acetone precipitated (9/1, vol/vol) and redissolved in 10 mM Tris, 50 mM ammonium acetate, 0.05% Triton X-100, 5 mM β-mercaptoethanol pH 7.0. The protein mixture was applied to a Bio-Gel A 1.5 M column (1.5 x 87 cm) equilibrated with the same buffer and fractions sampled for determination of ^{14}C and ^{3}H content. The V_e/V_o for the transport protein peak was 1.5. The protein within the peak was subjected to gel filtration on a 1.5 x 85 cm column of Sephadex G-150 in SDS column buffer. The elution profile from Sephadex G-150 showed that the material that appeared as a single component in

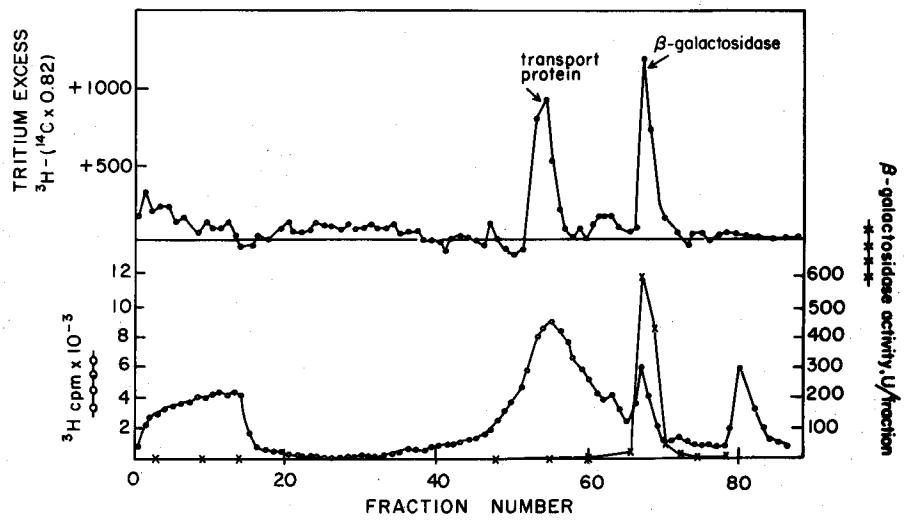

Figure 2. DEAE-cellulose chromatography of solubilized membrane proteins. 51 mg of membrane proteins in 15 mM Tris, 5 mM EDTA, 0.05% Triton X-100, 5 mM β-mercaptoethanol pH 7.0 were applied to a 1.5 x 45 cm column of DEAE-cellulose equilibrated and washed with the same buffer. Protein was eluted with a linear 0.0 - 0.3 M NaCl gradient in column buffer. A high salt wash, of Column Buffer plus 1 M NaCl, eluted the peak of β-galactosidase activity. A final wash of Column Buffer adjusted to pH 9.0, eluted more protein at fraction 80.

DEAE-cellulose had separated into two components during SDS gel filtration; one corresponding to a MW = 33,000 and the other to a MW = 15,000. The larger protein was subjected to preparative polyacrylamide gel electrophoresis in SDS and migrated as a broad diffuse band of MW 30,000-33,000. The most highly purified material, eluted from the gel, had a $^3H/^{14}C = 5.1$, compared to a $^3H/^{14}C = 0.82$ in the original envelope fraction. As shown in Table II, this represents a protein purity of 85%. The 15,000 dalton protein could not be further purified by PAGE due to contaminating glycoproteins of similar molecular weight.

DISCUSSION

The prediction from the DNA sequence that the lac Y gene product is a protein of MW = 46,500 has raised unresolved questions on the nature of the transport protein in the E. coli membrane. The Y gene product had been identified as a protein of MW = 30,000 by dissolving membrane proteins in SDS at room temperature and analyzing the mixture by SDS-PAGE or gel filtration in SDS.

TABLE II

Purification Table

Fraction	Total ^{3}H cpm x 10^{-6}*	Permease cpm x 10^{-6}	Purification	Yield
Whole cell	330	1.54	1.0	100
Envelope	113	1.54	2.9	100
Triton X-100 extract	96	1.25	2.8	81
DEAE-cellulose	4.8	.37	16.5	24
Bio-Gel A 1.5 M	3.24	.28	18.5	18
Sephadex G-150	.69	.15	47.8	10
Preparative PAGE	.11	.09	184	6

*The specific activity of this preparation was 1.9×10^{6} ^{3}H cpm/mg protein.

The MW was assigned by comparison with standard soluble proteins boiled in SDS. This MW assignment appears unjustified for two reasons. First, the stoichiometry of SDS binding to membrane proteins has been shown to deviate from the 1.4 g SDS/g protein observed for soluble proteins (12,13). Second, there is no evidence that the transport protein is fully denatured when dissolved at room temperature in the detergent. Bovine serum albumin and β-galactosidase retain their multimeric structures when dissolved in SDS at room temperature. These proteins are fully denatured only when boiled in SDS. Boiling has been avoided in studies of the lac transport protein because of its tendency to aggregate when heated (3). We found that aggregation was minimized in the presence of high concentrations of reducing agents or alkylating agents during the heating process. These treatments, followed by analysis by gel filtration, allowed complete denaturation and separation of two lac induced membrane polypeptides in Y^{+} strains. The sum of the MW's of these two peptides is approximately equal to the MW of the protein predicted from the Y gene sequence. Mutations in the Y gene affect these membrane polypeptides, while mutations in the Z gene and a related operon do not.

During partial purification in a non-denaturing detergent the two lac membrane proteins fractionate as a single component. The extent of purification in Triton X-100 is limited; probably due to formation of heterogeneous mixed micelles of protein and detergent. Gel filtration in SDS leads to separation of the 33,000 and 15,000 dalton components.

A reasonable hypothesis linking the genetic and protein composition data is that the Y gene product, a protein of MW = 46,500, is proteolytically processed or degraded into two fragments. Evidence that the 15,000 dalton protein is not a degradation product of the 33,000 dalton protein has been obtained from preliminary immunological experiments not presented in this paper in which antibody prepared to the larger protein did not cross-react with the 15,000 dalton protein. Conclusive identification of the membrane proteins requires further purification and comparison of peptide maps with the amino acid sequence predicted from the gene DNA sequence.

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