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Reconstitution of an Active Lactose Carrier In Vivo by Simultaneous Synthesis of Two Complementary Protein Fragments†

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Escherichia coli **lactose permease mediates the proton-driven translocation of galactosides across the cytoplasmic membrane. To define regions important for membrane insertion as well as for biological function, we constructed plasmids encoding different portions of the lactose carrier. Among several** ***lacY*** **deletions, two were obtained that encoded mutant proteins with complementary amino acid sequences. The truncated polypeptide Y71/1 (amino acid residues 1 to 71) comprises the first two α-helices predicted for the intact protein, and polypeptide ΔY4-69 carries an internal deletion of this region. Regulated coexpression of these** ***lacY*****-DNA segments governed by separate but identical** ***lacOP*** **control regions resulted in functional complementation with the following characteristics. (i) Simultaneous synthesis of both incomplete proteins restored transport activity in transport-negative cells, measured as accumulation of [^{14}C]lactose. (ii) Under complementing conditions, but not in the absence of the smaller N-terminal protein, specific radiolabeling of the larger polypeptide by** ***N*****-ethylmaleimide was prevented by substrate. (iii) The presence of the complementing N-terminal polypeptide was also required for the detection of the larger C-terminal protein by antibodies directed against the C terminus of lactose permease, indicating a stabilizing effect contributed by the smaller N-terminal fragment. Thus, coexpression of** ***lacY*** **mutant genes encoding two nonoverlapping portions of the lactose carrier resulted in reconstitution of a two-subunit protein in the cytoplasmic membrane exhibiting biological properties of intact lactose permease.**

The lactose carrier of *Escherichia coli* (also referred to as lactose permease) is a polytopic protein of the cytoplasmic membrane. This polypeptide catalyzes proton-galactoside symport across the lipid bilayer. In recent years, kinetics, substrate binding, and amino acid side chains essential for transport have been analyzed in great detail (11, 32). Based on biochemical and biophysical data, models for the structure of the lactose carrier have been developed. They predict that the highly hydrophobic protein is composed of at least 10 membrane-spanning α-helices and that most of the polypeptide is embedded within the lipid bilayer (1, 8, 31) (Fig. 1). Both the N terminus and the C terminus of lactose permease are exposed to the cytoplasmic face of the membrane (1, 24), and only few sites of the protein are accessible to proteolytic enzymes and to site-specific antibodies (1, 10, 22, 26).

In view of its important role as a model system, we studied the biosynthesis and membrane insertion of the lactose carrier. Lactose permease is synthesized without a cleavable N-terminal signal sequence (7). However, the N-terminal region of the protein mediates membrane attachment of the nascent polypeptide chain during its biosynthesis. Analyses of truncated proteins have demonstrated that the first 120 amino acid residues contain sufficient information for membrane targeting of the protein (27). Even shorter truncated polypeptides comprising 50 or 71 N-terminal amino acid residues of lactose permease were intimately associated with the lipid bilayer (27, 28). These data suggested that the N-terminal region exhibits a certain autonomy in interacting with the lipid bilayer. This interpretation has gained further support from the study of a series of LacY-OmpA hybrid proteins. Several N-terminal segments of lactose permease were found to mediate membrane attachment of C-terminal OmpA segments. Evidently, the N-terminal LacY segments were able to insert into the membrane and to attain the correct topological orientation in the absence of the remainder of the protein (15).

In this study, we analyzed mutant forms of the lactose carrier in more detail. Besides investigating membrane association, we addressed the question of biological function exhibited by the different polypeptides. We could demonstrate that simultaneous expression of two incomplete *lacY* DNA segments led to reconstitution of an active carrier.

(Preliminary accounts of these results have been presented: W. Wrubel, U. Stochaj, U. Sonnewald, C. Theres, and R. Ehring, Biol. Chem. Hoppe-Seyler **370:**636, 976, 1989.)

MATERIALS AND METHODS

Abbreviations. IPTG, Isopropyl-β-D-thiogalactopyranoside; NEM, *N*-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TDG, D-galactopyranosyl-β-D-thiogalactopyranoside.

Enzymes. Restriction endonucleases and enzymes used in the construction and analysis of plasmids described below were purchased from Boehringer GmbH (Mannheim, Federal Republic of Germany), New England BioLabs (Schwalbach, Federal Republic of Germany), and Bethesda Research Laboratories "GIBCO-BRL" (Freiburg, Federal Republic of Germany). They were used as recommended by the suppliers.

Radioactive materials. [^{14}C]lactose and [^{35}S]methionine

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† This paper is dedicated by R.E. to Prof. Dr. Wilhelm Menke on the occasion of his 80th birthday.

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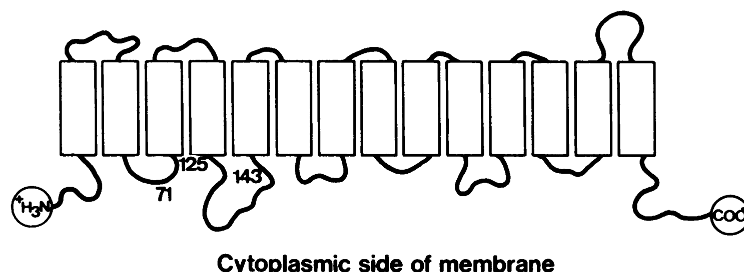

FIG. 1. Schematic representation of the structure of lactose permease. Simplified form of a model for the structure of lactose permease proposed by Vogel et al. (31). According to these authors, the more hydrophilic α-helices 7, 8, 9, and 12 are not predicted to span the membrane with certainty (31). The figure is taken from reference 28, with minor modifications (used by permission). Numbers indicate the approximate locations of amino acid residues 71, 125, and 143 of lactose permease.

were obtained from Amersham-Buchler (Braunschweig, Federal Republic of Germany).

Media. M9 medium contained 7 g of $Na_2HPO_4 \cdot 2H_2O$, 3 g of KH_2PO_4, and 1 g of NH_4Cl per liter. In addition, the following ingredients were supplied (indicated as final concentrations): $CaCl_2$ (0.1 mM), $MgCl_2$ (1 mM), $FeCl_3$ (0.5 μM), thiamine hydrochloride (1 μg/ml), L-tryptophan (20 μg/ml), glycerol (0.85%, vol/vol), and Casamino Acids (Difco Laboratories, Detroit, Mich.) (6 g/liter). NB medium contained 8 g of nutrient broth (Difco) and 5 g of NaCl per liter. Indicator plates were prepared from MacConkey agar base as recommended by the manufacturer (Difco) (1% lactose).

Bacteria and plasmids. *E. coli* T184 and plasmid pGM21 (29) were kindly provided by P. Overath, and strain HB101 (2) was provided by P. Starlinger. The RecA phenotype of both strains was verified by tests of their UV sensitivity. Strain T184 (29) carries a deletion of the lactose operon. It was used as the bacterial host for plasmid constructions, lactose transport assays of plasmid-containing cells, and DNA and membrane preparations described below. The presence of the *lacI*^{q} allele on an episome ensures overproduction of Lac repressor. The *lacZ* strain HB101 (2), which contains a wild-type *lacZ* allele, was used as the recipient in transformation tests, when a large number of clones transformed by *lacY*-related mutant plasmids were scored for their Lac phenotype by plating on MacConkey lactose indicator plates. *E. coli* DS410 harboring a *lacI*^{q} allele on an F′ episome (20, 26) was used to prepare *E. coli* minicells.

Plasmid pVI-1 (carrying the *lacY* wild-type allele), plasmids pY71/1 and pY143 encoding truncated forms of lactose permease, and the shortened vector pV142 have been described previously. They were derived by standard techniques from plasmid pGM21 (28), which confers tetracycline resistance to host cells and contains a *lacY* wild-type allele under the control of the *lacOP* region (16, 29).

Construction of plasmids pΔY4-69, pAY, and pBY. Essentially the same methods described previously (28) were used in similar constructions. To obtain plasmid pΔY4-69, a unique *Sma*I recognition site following codon 3 was provided by random linker insertion (28) into the *lacY* gene of plasmid pVI-1 and used in conjunction with a unique *Ava*I site at codon 70 in the *lacY* sequence (4) to delete codons 4 to 69. In the resulting plasmid pΔY4-69, two codons specifying phenylalanine and proline separate codons 3 and 70 of the *lacY* sequence (Fig. 2b).

Plasmids pAY and pBY were constructed as follows. Corresponding fragments bordered by unique *Bgl*II and *Hin*dIII sites from either pY71/1 or pY143 were used to replace in both cases the short fragment of pΔY4-69 bordered by unique *Bam*HI and *Hin*dIII sites as illustrated in Fig. 2a. Both newly created plasmids encode the *lacY* deletion polypeptide ΔY4-69 and, in addition, one of the truncated proteins Y71/1 or Y143 (Fig. 2). Each of the *lacY*-related DNA segments carries the authentic *lacOP* region as present on plasmid pGM21. Note that repression was maintained during all stages of plasmid construction and that no selection for a LacY-positive phenotype was exerted during transformations and DNA amplifications in strain T184. Several clones of the *recA* strain T184 independently transformed with each of the plasmids pAY and pBY and selected for tetracycline resistance were analyzed for transport activity with similar results.

Complementation assays on indicator plates. HB101 (2) carries a *lacY* mutation and produces an active β-galactosidase. This host was transformed with the different *lacY* mutant plasmids to test their capacity to provide a lactose transport function. pAY-containing clones were found to exhibit a lactose-positive phenotype on MacConkey indicator plates. This was observed with several clones transformed with pAY plasmids reisolated from T184 host cells, after their capacity to accumulate [^{14}C]lactose had been established (see below).

A large number of HB101 clones harboring independent isolates of pAY were obtained by direct use of the ligation reaction mixture to transform this complementing strain. Among more than 1,000 of these primary transformed pAY-containing clones, obtained by plating onto MacConkey lactose plates containing tetracycline, about 2% exhibited a lactose-negative phenotype on these indicator plates. This small fraction can be accounted for by cells receiving reconstituted parental plasmids and by cloning artifacts. The lactose-positive clones obtained with plasmid pAY exhibited a uniform phenotype which was characterized by a slightly delayed development of the red color on the indicator plates when compared with host cells transformed with the *lacY* wild-type plasmid. This uniform phenotype did not involve sectored colonies or papillae. The same host cells transformed by the vector or by individual parental plasmids were negative in parallel assays.

Transport assay. Uptake of [^{14}C]lactose was measured essentially as described previously (3). For these transport assays, the transport-negative strain T184 was used, which carries the *lacI*^{q} allele on an F′ episome. Derivatives harboring one of the plasmids indicated in Fig. 3 were grown to an optical density at 600 nm of 0.6 to 1.0 in M9 medium supplemented as described above. Unless otherwise indicated, inducer (0.5 mM IPTG) was present for at least two generations before harvest. Cells were washed in 0.1 M potassium phosphate buffer (pH 7.0) and resuspended in the

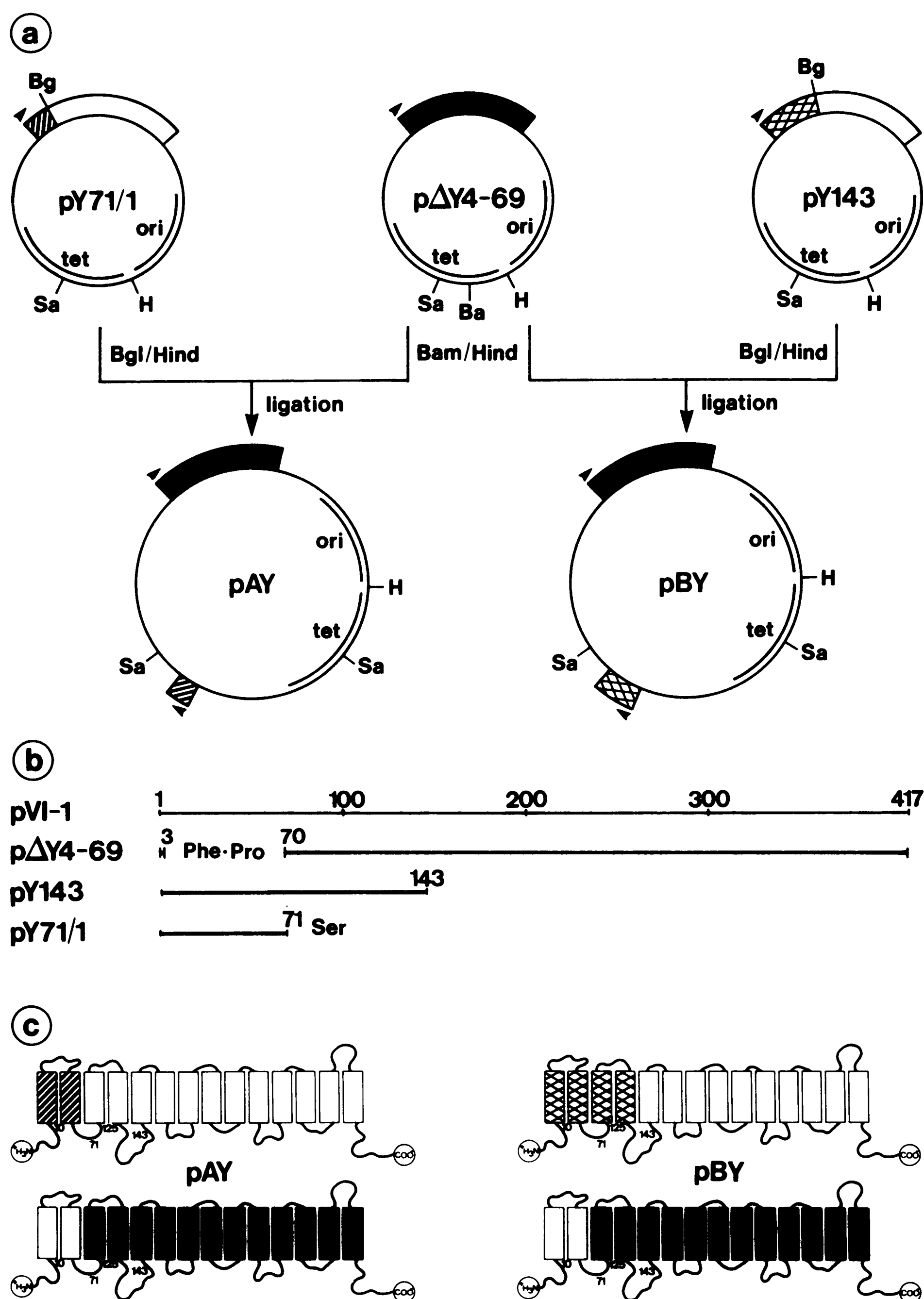

FIG. 2. Diagrammatic representation of construction and *lacY*-related polypeptides encoded by plasmids pAY and pBY. (a) Positions of origin of replication (ori) and of the tetracycline resistance gene (tet) are marked as well as recognition sites for restriction enzymes *Bam*HI (Ba), *Bgl*II (Bg), *Hin*dIII (H), and *Sal*I (Sa). Coding sequences of the *lacY* DNA segments are indicated by corresponding hatched, cross-hatched, and filled-in segments on parental plasmids, and pAY and pBY and the direction of their translation are indicated. Nontranslated segments on pY71/1 and pY143 located downstream of the terminator codons are shown as open segments. They have been deleted in the construction of pAY and pBY. (b) Relative lengths of the deletion polypeptide ΔY4-69 and of the truncated proteins Y71/1 and Y143 encoded by the parental plasmids are compared with that of the wild-type carrier encoded by plasmid pVI-1. Numbers indicate the position of the C-terminal amino acid of the authentic *lacY* sequence (4). Additional amino acid codons which are present in PY71/1 (Ser) and pΔY4-69 (Phe-Pro) as a result of the DNA manipulations are also indicated. (Panel b is modified from reference 28.) (c) Segments of the lactose carrier encoded by the *lacY* sequences of plasmids pAY and pBY are marked in the structure model for the intact protein (cf. Fig. 1). Symbols correspond to panel a. Segments of the native lactose permease which are not encoded by pAY and pBY are left as open rectangles.

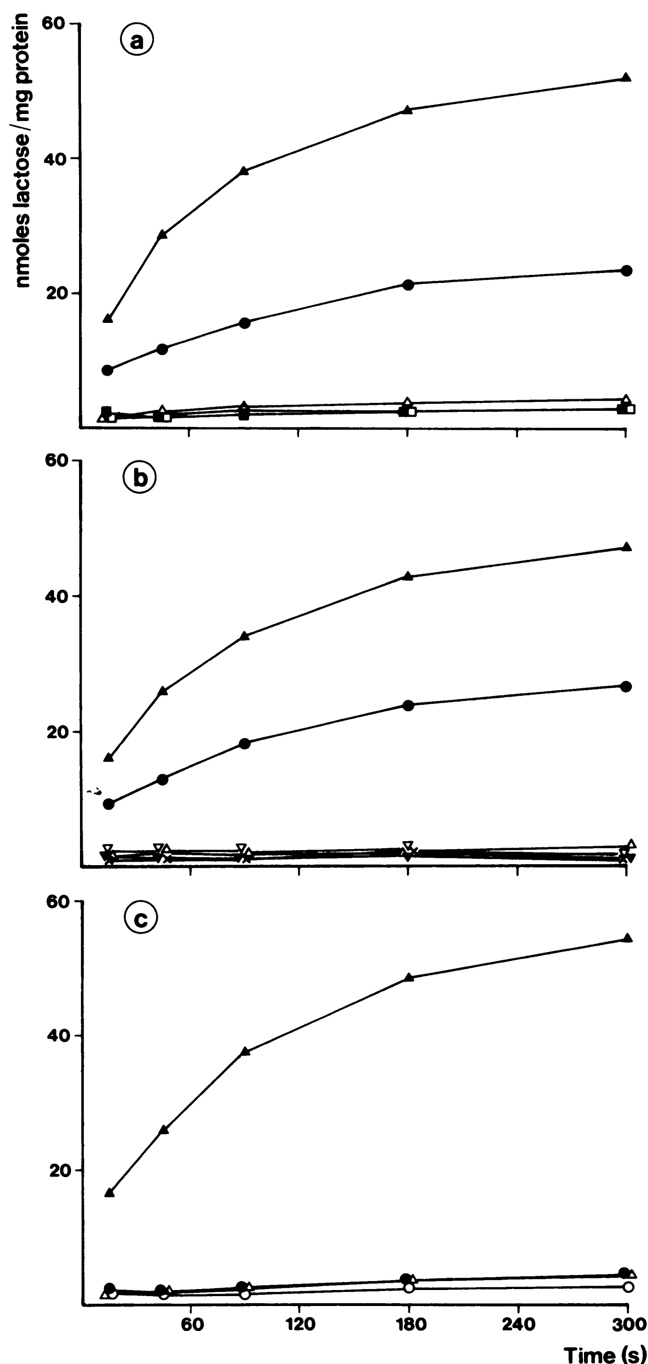

FIG. 3. Accumulation of [^{14}C]lactose by *E. coli* T184 cells harboring plasmids encoding different *lacY*-related polypeptides. Accumulation of [^{14}C]lactose was measured as described in Materials and Methods. (a) Symbols: △, cells harboring the vector pV142; ▲, cells harboring the *lacY* wild-type plasmid pVI-1; ●, cells harboring plasmid pAY (cf. Fig. 2); □, cells harboring parental plasmid pΔY4-69; ■, cells harboring parental plasmid p71/1. (b) Values obtained with cells carrying the vector pV142 or plasmid pVI-1 (wild type) or plasmid pAY are indicated by the same symbols as used in panel a. In addition, cells carrying plasmid pAY were tested in the presence of NEM (▽) or TDG (▼). Either compound was added simultaneously with [^{14}C]lactose at the start of the reaction to final concentrations of 5 mM NEM and 40 mM TDG. ×, Values obtained from cells carrying plasmid pAY but grown in the absence of the inducer IPTG. (c) Values obtained with cells carrying either plasmid pVI-1 (wild type) or the vector pV142 indicated as above are compared with values from cells harboring either plasmid pBY (○) or pY143 (●). Cells carrying plasmid pΔY4-69, the second precursor plasmid of pBY (cf. Fig. 2), have been included in the experiment shown in panel a.

same buffer to an optical density of about 0.7 (600 nm, 1-cm light path). After 5 min of equilibration at 25°C, [^{14}C]lactose was added to a final concentration of 0.1 mM (specific activity, 5 mCi [0.185 GBq]/mmol). At the times indicated in Fig. 3, equal amounts were diluted into buffer containing 5 mM NEM. Cells were collected by suction onto membrane filters (0.65-μm diameter; no. 11305; Sartorius GmbH, Göttingen, Federal Republic of Germany) and washed with buffer at room temperature. Radioactivity retained on the filters was determined by liquid scintillation counting and normalized to the total protein present in the assay (14). As a control, the same assay was performed with cells harboring the vector pV142.

Membrane preparation. T184 transformed with the different plasmids as indicated was grown in NB medium in the presence of inducer (0.25 mM IPTG). The cells were converted to spheroplasts (29) and broken by passage through a French press (10,000 to 12,000 lb/in^2). After the cell debris was removed by centrifugation (4,000 × *g*, 10 min, 4°C), the crude membranes were pelleted by ultracentrifugation (120,000 × *g*, 1 h, 4°C). Fractions enriched for cytoplasmic membranes and obtained after sucrose gradient centrifugation essentially as described previously (29) were combined. The yield of membrane protein was determined (14).

Protection by substrate against modification by NEM. Binding of the substrate TDG was determined essentially by the method of Fox et al. (9). Preparations enriched for cytoplasmic membranes derived from T184 cells harboring different plasmids as indicated were incubated with nonradioactive NEM in the presence of the high-affinity substrate TDG. After removal of both compounds, equal amounts of each preparation were incubated with [^{14}C]NEM either in the presence or in the absence of TDG. After termination of the reaction, the proteins were separated by SDS-PAGE.

Immunological detection of proteins harboring the C terminus of lactose permease. Samples of the membrane preparations used for the NEM protection assay were separated by SDS-PAGE (11 to 20% acrylamide), and the proteins were electrotransferred to nitrocellulose membranes with a Biometra fast blot apparatus (12, 30). Nonspecific binding sites were blocked by immersing the filters in a 5% (wt/vol) solution of lipid-free milk powder, and filters were treated with antibodies directed against the C terminus of lactose permease (24). Bound antibodies were subsequently visualized by incubation with a secondary antibody conjugated to alkaline phosphatase.

Analysis of plasmid-encoded proteins. *E. coli* minicells harboring an F′ *lacI*^{q} episome and the different plasmids to be analyzed were prepared, stored, and used essentially as described previously (26). Incorporation of L-[^{35}S]methionine into proteins was terminated by the addition of trichloroacetic acid (5% final concentration). Total proteins of the unfractionated minicells were subjected to SDS-PAGE (11 to 20% gradient of acrylamide) and autoradiography. *E. coli* lactose permease is known to exhibit an anomalous electrophoretic mobility on SDS gels (32) which was also observed for deletion mutant proteins (27, 28). Their sizes are underestimated by comparison with standard marker proteins.

RESULTS

Construction of plasmids encoding mutant *lacY* proteins. To define regions important for membrane insertion and for

biological function of the lactose carrier, we specifically constructed and analyzed plasmids expressing *lacY*-related mutant polypeptides (27, 28; this study). We were particularly interested in truncated proteins and in deletion mutant proteins that comprise well-defined structural elements predicted for the intact protein (11, 31).

Plasmids pAY and pBY were designed to test the potential of incomplete polypeptides for mutual complementation. Plasmid pAY was constructed (Fig. 2) from parental plasmids that encoded two complementary portions of the lactose carrier. Both *lacY* DNA segments contained the authentic *lacOP* sequence controlling their simultaneous regulated expression. In pAY, one segment specified the truncated protein Y71/1 (amino acid residues 1 to 71 of lactose permease), and the other segment specified protein ΔY4-69, which is lacking residues 4 to 69 of the wild-type lactose carrier. *lacY* segments of plasmid pBY encoded the longer truncated protein Y143 (amino acid residues 1 to 143 of lactose permease) in addition to the deletion polypeptide ΔY4-69 (Fig. 2 and Materials and Methods).

Two noncovalently bound segments of lactose permease can reconstitute an active carrier protein. Cells of the transport-negative strain T184 were transformed with either plasmid pAY or pBY. Those harboring pAY were able to transport D-lactose. The sugar was accumulated to 30 to 50% of the level observed with cells carrying the corresponding *lacY* wild-type plasmid pVI-1 (Fig. 3a). The uptake of [^{14}C]lactose did not merely represent equilibration with the outside substrate. Energy dependence of the lactose uptake was demonstrated by the use of carbonyl cyanide *m*-chlorophenylhydrazone. Preincubation of cells harboring plasmid pAY with this compound at 20 μM final concentration resulted in the loss of the transport activity (data not shown).

In contrast to pAY, plasmid pBY did not restore the capacity to accumulate [^{14}C]lactose. Cells carrying this plasmid retained only unspecific background levels of radioactivity as seen with cells carrying the vector plasmid pV142 (Fig. 3c). Cells harboring one of the parental plasmids encoding only one of the incomplete polypeptides Y71/1, Y143, or ΔY4-69 were analyzed in parallel. Incorporation of [^{14}C]lactose in no case exceeded unspecific background levels (Fig. 3a and c). Note that no blank values were subtracted from the values shown in Fig. 3.

T184 cells (transport negative, *recA*) regained transport activity without selection for the LacY-positive phenotype. Several independent clones transformed by pAY yielded transport activities similar to those shown in Fig. 3a (data not shown). The activity was plasmid linked as demonstrated by transformation of T184 cells with pAY DNA reisolated from several primary transformed clones.

HB101, another *recA* strain, was used to screen a large number of clones independently transformed by pAY (as described in Materials and Methods). This *lacZ*-complementing host yielded clones of a uniform lactose-positive phenotype on indicator plates. In contrast, HB101 clones transformed either by plasmid pBY or by one of the parental plasmids and tested in parallel were indistinguishable from HB101 transformed by the vector. Simultaneous synthesis of the incomplete *lacY*-related proteins Y71/1 and ΔY4-69 encoded separately by two coexisting plasmids was also found to restore transport activity in T184 as well as in HB101 cells (data not shown).

Taken together, these results obtained with *recA* strains rule out the assumption that regain of transport activity might result from a rare recombinational event rather than reflect the intrinsic properties of cells transformed by plasmid pAY.

In T184 host cells, appearance of transport activity was completely dependent on prior induction of the *lacY*-related genes (Fig. 3b). Cells of this strain overproduce Lac repressor due to the presence of the *lacI*^{q} episome. Other characteristics of transport activity resembled those described previously (32) for wild-type lactose permease (Fig. 3b). Accumulation of [^{14}C]lactose was prevented by the addition of nonradioactive TDG, a high-affinity substrate of the carrier, and it was abolished by treatment with NEM (see below).

Lactose carrier reconstituted from noncovalently associated fragments was protected by substrate against chemical modification by NEM. To study the active lactose carrier formed by interaction between proteins Y71/1 and ΔY4-69 in more detail, we analyzed binding of the high-affinity substrate TDG. TDG is known to protect residue Cys-148 of the wild-type protein against chemical modification by NEM (9, 32). Membrane vesicles from cells harboring different plasmids were treated in the presence or absence of this substrate (see Materials and Methods). Membranes from cells containing either the *lacY* wild-type plasmid pVI-1 or plasmid pAY showed a band specifically protected by substrate (Fig. 4a, lanes 1 to 4, bands marked by arrow and arrowhead). As expected, the band preferentially radiolabeled in the absence of substrate exhibited a higher electrophoretic mobility in the sample from cells harboring plasmid pAY (lane 4) compared with the corresponding band from *lacY* wild-type membranes (lane 2). Association of proteins Y71/1 and ΔY4-69 thus gave rise to a complex that specifically recognized the substrate TDG. This binding of TDG protected protein ΔY4-69 against modification by [^{14}C]NEM. Preferential radiolabeling of protein ΔY4-69 was only detected in the complementing situation (lanes 3 and 4) but not in membranes from cells harboring plasmid pBY or pΔY4-69 (Fig. 4a, lanes 5 to 8). Thus, the complementing truncated protein Y71/1 was required for both transport activity as well as binding of substrate.

To test for the presence of the larger deletion polypeptide by an independent method, samples of the same membrane preparations used for the substrate protection experiments described above were subjected to Western blotting (immunoblotting) and probed with antibodies directed against the C terminus of lactose permease (24). Only in membranes of cells carrying plasmid pAY could a protein of the electrophoretic mobility expected for protein ΔY4-69 be detected by this procedure (Fig. 4b, lane 2). Under identical conditions, no signal was obtained for cells harboring plasmid pBY or pΔY4-69. In summary, the intact protein ΔY4-69 was detected by anti-C-terminus-specific antibodies only in membranes from cells also producing the complementing protein Y71/1. The amount of protein ΔY4-69 found by this method appears to be lower than expected from the relative transport activity. The reactivity of lactose permease with these antibodies is lost upon mild proteolytic treatment which does not destroy substrate binding and transport activity (23). Other experiments have also shown that an intact C terminus is not required for transport activity of lactose permease (21, 25; unpublished experiments from our laboratory). We assume that in cells containing plasmid pAY stabilization of the C-terminal region may be incomplete. A fraction of protein complexes may contribute to the transport activity but not be detected by the anti-C-terminus-specific antibodies.

The mutant forms of lactose permease encoded either by

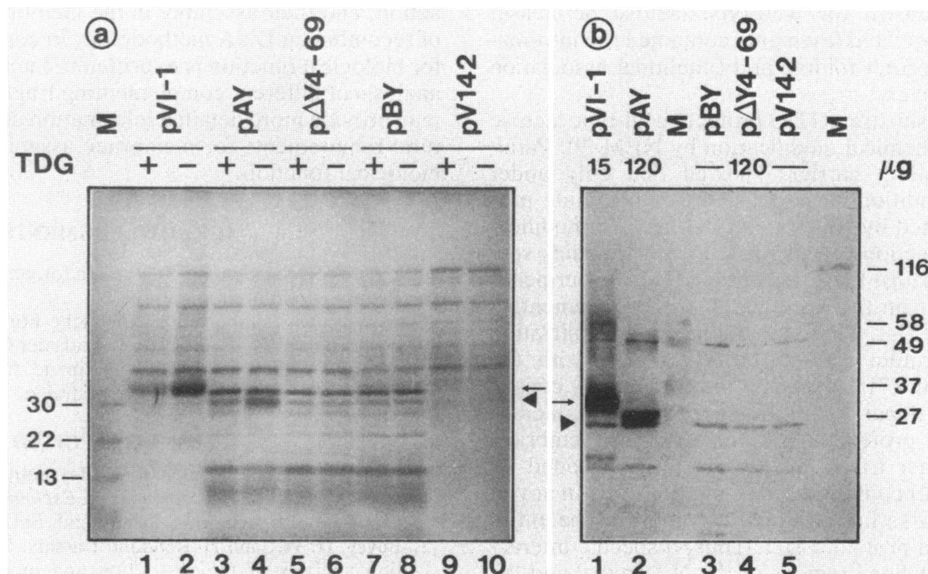

FIG. 4. Substrate binding and immunoreactivity of protein ΔY4-69 under complementing conditions. (a) Preparations enriched for cytoplasmic membranes derived from T184 cells harboring the different plasmids as indicated on the top of the figure were treated as described in Materials and Methods. TDG was present (+) or absent (−) during the reaction with [^{14}C]NEM (9) as indicated in the figure. Equal amounts of protein were applied to the gel and separated by SDS-PAGE. (b) Different amounts of the same membrane preparations used for panel a were applied to the gel as indicated on the top of the figure. After separation by SDS-PAGE, the proteins were transferred to nitrocellulose filters and detected by their reaction with immunoglobulin G directed against the C terminus of lactose permease as described in Materials and Methods. In both parts of the figure, an arrow and an arrowhead on the margin mark the positions of intact lactose permease and of protein ΔY4-69, respectively. M_r values of marker proteins (lanes M) are indicated (×10^3).

the individual plasmid pΔY4-69 or by plasmid pBY were not detected by any of the methods described so far. This cannot be attributed to the structure of the plasmids. Both plasmids direct the de novo synthesis of the expected *lacY*-related gene products upon derepression, as visualized by analysis of plasmid-bearing *E. coli* minicells (Fig. 5). In the presence of inducer, these proteins were preferentially radiolabeled and could be detected by SDS-PAGE followed by autoradiography. This method had been previously found to be well suited for the detection of incomplete proteins (27, 28).

So far, protein ΔY4-69 was observed in membranes from growing cells only under complementing conditions. Neither alone nor in the presence of the noncomplementing truncated protein Y143 was the deletion protein ΔY4-69 capable of folding and/or inserting into the membrane in a form sufficiently stable to be detected by substrate protection experiments or by reaction with the anti-C-terminus-specific antibodies.

DISCUSSION

E. coli lactose carrier most likely functions as a monomer in translocating galactosides across the cytoplasmic membrane (5, 6, 33). We created an artificial two-subunit structure to investigate the reconstitution of an active carrier molecule from its fragments. Simultaneous synthesis of the truncated protein Y71/1 and the deletion polypeptide ΔY4-69 led to their successful interaction, which was detected as their capacity to restore transport activity in transport-negative cells. Neither cells harboring the control plasmid pBY (see below) nor cells carrying the individual parental plasmids encoding only one of the two *lacY* segments regained this activity. Our data support the conclusion that two noncovalently bound portions of the lactose carrier can associate in the membrane and exhibit characteristics very

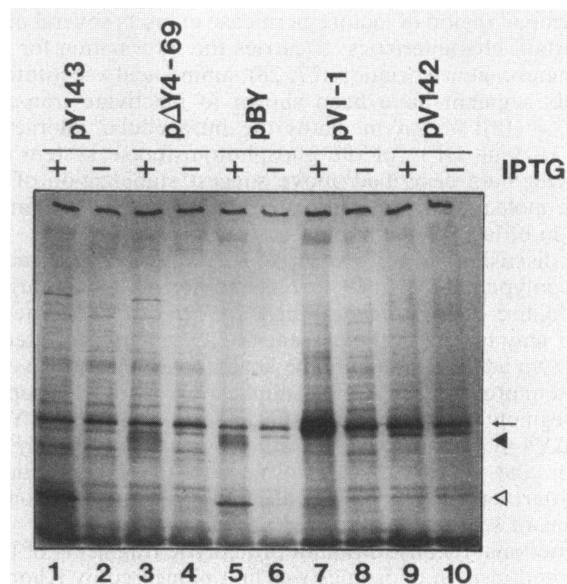

FIG. 5. Radiolabeling of pBY-encoded LacY-related proteins in *E. coli* minicells. Proteins from minicells harboring different plasmids were radiolabeled in the presence (+) or absence (−) of the inducer IPTG as indicated on the top of the figure. The autoradiograph shows total proteins separated by SDS-PAGE. Equal amounts of proteins were applied to the gel. An arrow on the margin indicates the position of the intact lactose permease, which comigrates under the conditions used here with the product of the tetracycline resistance gene (28); bands representing proteins Y143 and ΔY4-69 are marked by open and filled arrowheads, respectively. Only one-half the amount of radioactivity was added to the radiolabeling reaction mixture with minicells harboring the wild-type pVI-1.

similar to those known for wild-type lactose permease. Thus, the two *lacY*-related fragments contained the information required for correct folding and functional association within the lipid bilayer.

The high-affinity substrate TDG protects wild-type lactose permease against chemical modification by NEM (9). Parallel analyses of mutant carriers showed that only under complementing conditions was the larger polypeptide protein ΔY4-69 protected by substrate (TDG) against modification by NEM. Detection of this protein by antibodies specifically directed against the C terminus of lactose permease was also dependent on the presence of the complementing truncated protein Y71/1. These results indicate stabilization of the deletion protein ΔY4-69 by association with the N-terminal fragment Y71/1. Its effect may not merely consist in providing N-terminal hydrophobic portions of lactose permease to assist protein ΔY4-69 in initiating membrane insertion. The longer truncated protein Y143 encoded by plasmid pBY (Fig. 2) could not substitute for Y71/1 in any of the functions tested so far, although it comprises the entire sequence present in protein Y71/1. Thus, a specific interaction between well-defined regions of the N-terminal and the C-terminal parts of lactose permease was required to regain substrate binding and transport activity as well as for protein stabilization.

Roepe et al. (21) have recently shown that removal of certain amino acid residues from the C-terminal region of lactose permease resulted in a greatly increased sensitivity toward proteolytic degradation. Our data indicate that the N-terminal part may also contribute to the stabilization of the molecule. This interpretation is compatible with the suggestion of a ringlike structure formed by the hydrophobic membrane-spanning α-helices (6, 31, 32). Furthermore, the N-terminal region of lactose permease exhibits several other important characteristics: it carries the information for stable membrane association (27, 28); amino acid substitutions in this segment have been shown to inactivate transport activity (18); and it mediates the intracellular interaction with enzyme III^{Glc} of the phosphotransferase system (17, 18). Our data described above suggest stabilization of the entire molecule as an additional function for the N-terminal portion of lactose permease.

As discussed above, protein Y71/1 successfully interacted with polypeptide ΔY4-69 to reconstitute an active carrier. The failure of the truncated protein Y143 to substitute for Y71/1 may be due to the presence of a segment predicted to form two additional membrane-spanning α-helices also contained in protein ΔY4-69. The duplication of such hydrophobic segments might perturb the interaction between Y143 and ΔY4-69. Further studies will define more precisely the length and sequence specificity of N-terminal segments which are optimal for regain of lactose transport in combinations of specific *lacY*-related protein segments.

Functional reconstitution of proteolytic fragments of bacteriorhodopsin in liposomes was first pioneered by Khorana and co-workers and has been used more recently to study the refolding process and association of these membrane proteins (see reference 19 and references cited therein). Partially active split receptors have been synthesized and assembled in amphibian oocyte membranes from artificially constructed genes expressing different segments of the β-adrenergic receptor (13).

The construction of a two-subunit structure as exemplified here with *E. coli* lactose permease may be more generally applicable to similar polytopic proteins and represent an alternative technique to study essential regions, their interaction, and their assembly in the membrane. The versatility of recombinant DNA methodology in combination with tests for biological function is expected to facilitate the design and analysis of different complementing fragments. Such studies may provide more detailed information concerning the structural requirements for membrane association as well as for biological function.

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