In Vitro Membrane Assembly of a Polytopic, Transmembrane Protein Results in an Enzymatically Active Conformation

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Abstract. In vitro integration of the polytopic, transmembrane lactose permease into membrane vesicles from *Escherichia coli* is demonstrated. To this end the enzyme was synthesized in a homologous, cell-free transcription-translation system. In this system, synthesis occurred in an essentially membrane-free environment leading to the formation of lactose permease aggregates, which were resistant to protease digestion and detergent solubilization. However, if inverted membrane vesicles from *E. coli* were included in the synthesis reaction, most de novo-synthesized lactose permease could be recovered from a membrane-containing subfraction (enriched in leader [signal] peptidase activity). This membrane association of lactose permease was Na_2CO_3 resistant, detergent sensitive, and yielded a distinct pattern of proteolytic cleavage peptides. Moreover, membrane vesicles when present cotranslationally during synthesis of lactose permease, acquired the capability to accumulate lactose, strongly suggesting a correct in vitro assembly of the enzyme.

Because of the extensive aggregation of lactose permease synthesized in the absence of membranes, only low amounts originating from the soluble enzyme pool integrated posttranslationally into the membrane vesicles. Unlike the translocation of the outer membrane protein LamB into membrane vesicles, integration of lactose permease was found to be independent of the H^+-motive force.

OUR present knowledge of how and when newly synthesized integral membrane proteins are integrated into the lipid bilayer stems mostly from studies with mammalian endoplasmic reticulum. For numerous proteins a signal recognition particle-dependent integration into microsomal vesicles has been demonstrated (2, 3, 6, 21, 25, 26, 33, 34, 44, 45, 48, 53). In all but one case (2), these proteins are synthesized without a cleavable signal sequence, suggesting that the signal recognition particle must recognize an internal segment of the amino acid sequence functionally equivalent to a signal sequence. The signal recognition particle dependence of the membrane integration is also consistent with the notion that the integration of membrane proteins into and translocation of secretory proteins across the endoplasmic reticulum membrane initially proceed along the same molecular pathway (5). Only the interruption of an ongoing translocation process by distinct stop-transfer sequences emerging on the translocated polypeptide chain would lead to anchorage within the membrane proteins (5, 14, 63). The final topography of a given membrane protein appears to depend on structural features determining the orientation of the most proximal transmembrane segment (NH_2 terminus inside or outside) and on the number of internal signal and stop-transfer sequences (4, 21).

Fewer details are available on how prokaryotic proteins integrate into bacterial membranes. Outer membrane proteins of gram-negative bacteria are in a sense secretory proteins because they have to traverse the plasma membrane before reaching the outer membrane. As such, they are synthesized with cleavable signal sequences and require two forms of energy and active SecA and SecY proteins for their export. On the other hand, most inner membrane proteins (1, 7, 17, 18, 22, 30, 32, 46, 52, 60) are not proteolytically processed when incorporated into the lipid bilayer of the plasma membrane. The only representative of these proteins studied in detail so far is the bacterial signal peptidase (leader peptidase, signal peptidase I). The NH_2-terminal third of the polypeptide chain has been reported to anchor the enzyme within the plasma membrane with the remainder extending into the periplasm. Integration requires a membrane potential as well as SecA and SecY (summarized in 59).

Lactose permease (lactose carrier) is a polytopic, inner membrane protein of *E. coli*, which catalyzes the symport of galactosides and H^+ (29, 62). The lactose permease-encoding gene (*lacY*) has been sequenced (8), and the protein purified (56, 61) and reconstituted into liposomes (12, 61). A model of the secondary structure has been proposed in which the 417-amino acid polypeptide is organized into 14 transmembrane, α-helical segments. The 10 most hydrophobic segments are believed to form, within the lipid bilayer, a cylinder-like structure built around the remaining transmembrane stretches which accommodate the binding sites for the hydrophilic substrates (39, 57). Both the NH_2 and the COOH terminus protrude into the cytoplasm.

Little is known about the assembly mechanism of lactose

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permease. A search for internal signal sequences has recently been started (40, 49, 51). Here, we report on the in vitro synthesis and assembly into inverted membrane vesicles (INV)[1] of lactose permease, based on a careful analysis of its association with the membranes. We demonstrate that a polytopic, plasma membrane protein integrates authentically in vitro into membrane vesicles, giving rise to an enzymatically active transmembrane protein.

Materials and Methods

Materials

Plasmid pACYC184 (9) contains the *cat* gene encoding chloramphenicol-acetyltransferase. It was prepared using spectinomycin (100 mg/liter) for amplification. Plasmid pGM21 was derived from pACYC184 by inserting a fragment of the *lac*-operon containing an intact *lac*-promotor-operator region and the lactose permease-encoding *lacY* gene into the *cat* gene sequence of pACYC184 (55). Plasmids pHI-5 and pLB7012 coding for alkaline phosphatase and the lambda receptor, respectively, have been described elsewhere (37).

In Vitro Transcription-Translation and Integration into INV

Published procedures were used for preparing plasmid DNA (35); salt-washed, gradient-purified INV (35); and an S-135 of a cell extract from *Escherichia coli* K12 strain MC4100 (38) grown at 30°C (35). DNA-dependent protein synthesis using the S-135 was performed in 25-μl reactions as described previously (37). When the *lacY* gene was to be expressed, cAMP was added from a 12.5-mM stock solution neutralized with KOH, yielding a final concentration of 0.5 mM. For posttranslational integration studies, 25 μl of INV-free reactions were subsequently incubated with INV for an additional 30 min at 37°C, to which ATP, creatine phosphate, and creatine kinase were added to give the previous concentrations within the total new volume of 27.5 μl. INV-free controls were treated identically, whereas cotranslational controls were kept on ice during this period.

Subfractionation of Translation Products

After chilling on ice, 25-μl samples were applied to a two-step sucrose gradient in an airfuge tube (Beckman Instruments, Inc., Fullerton, CA) consisting of 100 μl 20% (wt/vol) and 50 μl 50% sucrose in 40 mM triethanolamine acetate (TeaOAc), pH 7.5, 100 mM KOAc, respectively. After a 10-min centrifugation at 30 psi in an A100/18 rotor at 4°C, the following fractions were withdrawn sequentially from the top: 90 μl representing soluble material, 70 μl to give the membrane fraction, and 15 μl which was discarded. Pelleted material was directly dissolved in 30 μl of SDS-PAGE loading buffer (see below). Proteins contained in the supernatant fractions were precipitated by the addition of 1 vol of 10% TCA, unless stated otherwise.

Radioalkylation with [^{3}H]N-Ethylmaleimide (NEM)

Cholate-extracted membranes of the *E. coli* strain T206 containing plasmid pGM21 (55) were a generous gift of Dr. Peter Overath, Max-Planck-Institute of Biology, Tübingen, FRG. 1 μl of membranes (9.2 μg protein) was diluted with 8 μl 50 mM TeaOAc, pH 7.5, and incubated with 1 μCi/μl H_2O of [^{3}H]NEM (40 Ci/mmol; Amersham Corp., Arlington Heights, IL) for 30 min at 25°C. Samples were then either TCA precipitated after adding 3 μl of carrier membranes or prepared for reaction with antibodies.

Immunoprecipitation

Samples (25 μl of a translation reaction and 10 μl of an alkylation reaction) were diluted threefold with 3.3% (wt/vol) SDS containing 2 mM PMSF. After incubation for 30 min at 37°C, 10 vol of Triton buffer (25 mM Tris-HCl, pH 6.8, 5 mM EDTA, 154 mM NaCl, 1% Triton X-100) were added, and the solution, now cleared of aggregates, was incubated with 25 μl antilactose permease antiserum and 10 μl 0.1 M PMSF/DMSO at 4°C overnight. Immunoglobulins were bound to 5–10 mg Protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ) by rotating the samples end-over-end at room temperature for 60 min. The beads were collected and washed successively three times with 1 ml of Triton buffer and twice with 1 ml of 25 mM Tris-HCl, pH 6.8, each. After the final wash, remaining liquid was carefully removed with a drawn-out pasteur pipette, and bound proteins were released in SDS-PAGE loading buffer (see below).

Quantitation of Radioactivity Associated with Individual Bands on SDS Gels

Bands were located on the dried gels by alignment with an exposed X-ray film, excised, rehydrated, and extracted with tissue solubilizer TSI (Zinsser Analytic, Frankfurt, FRG) as described (58).

Lactose Uptake by INV

The method described previously (31) was used with minor modifications. For assaying in vivo-assembled lactose permease, INV were prepared from strain T206 containing plasmid pGM21 as described (35). Cells were grown in the presence of 20 μg/ml tetracycline, to an A_{600} = 0.2, at which point 30 μg/ml isopropyl-1 thio-β-D-galactoside was added to induce the synthesis of lactose permease. Cells were harvested at an A_{600} = 1.3, and salt-washed, gradient-purified INV were prepared (35). For each individual assay INV (0.04–0.07 A_{280} U) were diluted into 30 μl 50 mM TeaAOc, pH 7.5, 7.5 mM $MgCl_2$. The reaction was started by adding 3 μl containing 0.6 μCi [^{14}C]lactose (57 mCi/mmol; Amersham Corp.). Control samples, always run in parallel, received 1 μl of 0.1 M $HgCl_2$ before the radioactive substrate. Inhibitors (1 μl of 0.1 M NEM and 1 μl of 0.5 M β-galactosyl 1-thio-β-D-galactoside, respectively) were added 5–10 min before starting the reaction. Reactions were allowed to proceed for 5 min at 25°C, and stopped by adding 1 μl of 0.1 M $HgCl_2$ – if appropriate – and by the addition of 1 ml of ice-cold wash buffer (10 mM TeaOAc, pH 7.5, 3 mM $MgCl_2$). Samples were applied to nitrocellulose filters (0.45-μm pore size, 24-mm diam; Millipore Continental Water Systems, Bedford, MA) which had been prewetted in H_2O, placed on a filter holder, and prerinsed with 1 ml of wash buffer each. After sample application, each filter was rinsed three times with 1 ml of wash buffer. Filters were air dried and radioactivity was determined by liquid scintillation counting in 10 ml Rotiszint 11 (Roth, Karlsruhe, FRG). The lowest possible amount of membranes which still gave reproducible figures for INV-associated radioactivity sufficiently high over background, as well as the optimal time of reaction, had first been determined in a series of preliminary experiments.

For assaying in vitro-assembled lactose permease, in vitro translation reactions were prepared as described above, except that 1 μl of 1 mM nonradioactive methionine per 25 μl was used instead of the ^{35}S-labeled isotope. INV were collected from 250-μl reactions by centrifugation in the airfuge (Beckman Instruments; 10 min, 30 psi at 4°C), washed once with 50 mM TeaOAc, pH 7.5, 1 mM DTT containing 250 mM sucrose, and finally resuspended in 60 μl 50 mM TeaOAc, pH 7.5, 7.5 mM $MgCl_2$ to give a pair of cognate samples, one serving as the Hg^{2+}-treated control (see above).

SDS-PAGE

TCA-precipitated and Protein A-Sepharose-bound proteins were dissolved and denatured in 30 μl each of loading buffer containing 0.133 M Tris base, 3.3% SDS, 12% glycerol, 0.01 M EDTA, 0.01% bromophenol blue, 0.1 M DTT, and 0.33 mg/ml chymostatin. The latter proved to be indispensable in keeping the proteinase K, which was present in protease-treated samples, inactive during the subsequent incubation for 30 min at 37°C. SDS-PAGE and fluorography were performed as described (36). SDS-PAGE in the presence of urea was conducted with minor modifications of the method described (28). The separation gel contained 22.3% (wt/vol) acrylamide, 0.085% *N,N'*-methylenebisacrylamide, 0.41 M Tris-HCl, pH 8.7, 6.3 M urea, and 0.1% SDS. The stacking gel contained 5% acrylamide, 0.13% *N,N'*-methylenebisacrylamide, 0.06 M Tris-HCl, pH 6.8, 6.3 M urea, 0.1% SDS, and 6.25% sucrose.

Results

Lactose permease was synthesized in vitro by plasmid pGM21-directed transcription-translation in a high speed supernatant (S-135) obtained from an *E. coli* lysate (Fig. 1).

1. *Abbreviations used in this paper*: DCCD, *N,N'*-dicyclohexyl carbodiimide; INV, inverted plasma membrane vesicles from *E. coli*; NEM, *N*-ethylmaleimide; TeaOAc, triethanolamine acetate.

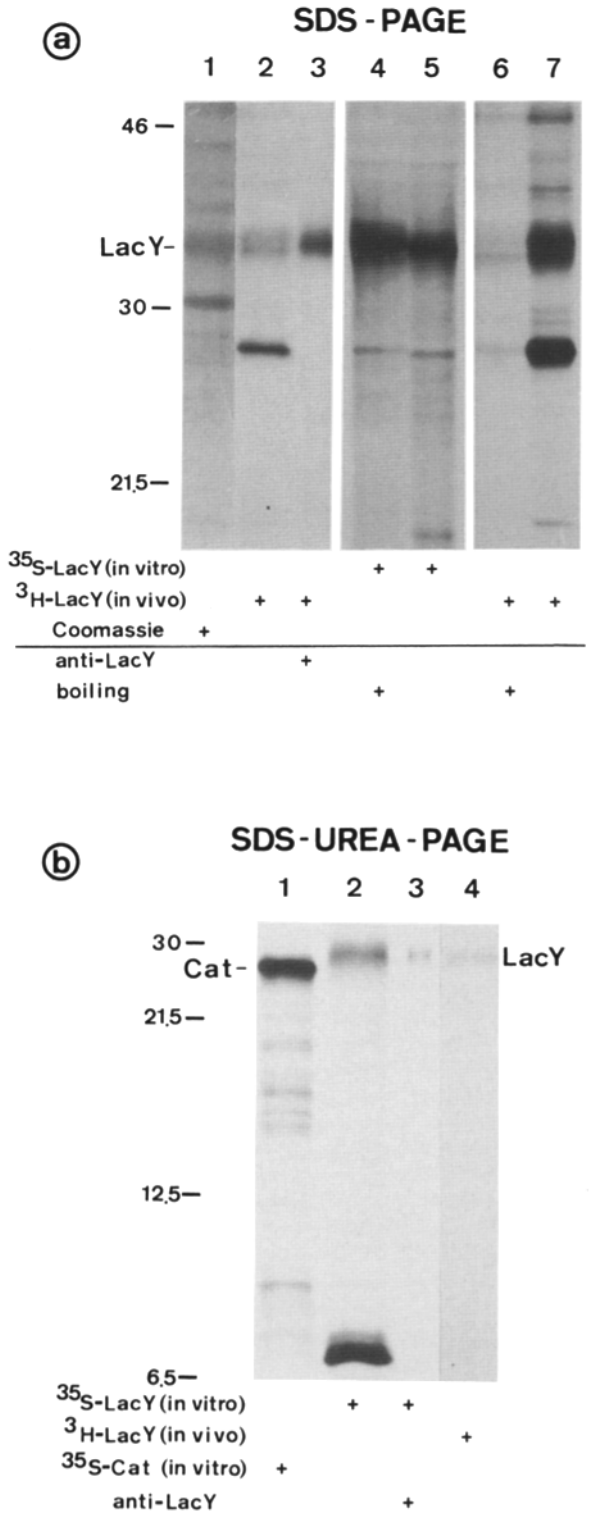

Figure 1. Plasmid pGM21-dependent synthesis of full-length lactose permease in a membrane-deprived extract from *E. coli*. (*a*) Proteins were separated by SDS-PAGE. The gel was first stained with Coomassie blue (lane *1*) and subsequently prepared for fluorography (lanes *2–7*). ^{3}H-labeled proteins (lanes *2*, *3*, *6*, and *7*) required a longer exposure of the film than ^{35}S-labeled ones (lanes *4* and *5*). (Lane *1*) Proteins present in 5 μl cholate-extracted membranes from the lactose permease–overproducer T206 (see Materials and Methods). (Lane 2) T206 membrane proteins (1 μl) radioalkylated with [^{3}H]NEM. (Lane *3*) Same as lane *2*, after reaction with anti–lactose permease antibodies. (Lanes *4* and *5*) Lactose permease (*LacY*) synthesized in a pGM21-programmed, membrane-free S-135 derived from an *E. coli* extract (25-μl reaction volume). (Lanes *6* and *7*) same as lane *2*. (*b*) Proteins were separated by SDS-PAGE in the presence of urea and visualized by fluorography. In vitro synthesis was directed by plasmid pACYC184 encoding the *cat* gene product (*Cat* lane *1*) and pGM21 (lanes *2* and *3*). Proteins were TCA-precipitated (lanes *1* and *2*) or immunoprecipitated (lane *3*). (Lane *4*) same as lane *2* of *a*. The marker proteins used were [^{14}C]methylated ovalbumin (46 kD), carbonic anhydrase (30 kD), trypsin inhibitor (21.5 kD), cytochrome C (12.5 kD), and aprotinin (6.5 kD).

The major ^{35}S-labeled translation product, visualized by fluorography after SDS-PAGE, had an apparent molecular mass of 33 kD (Fig. 1 *a*, lane *5*; *LacY*). This protein was identified as lactose permease by its comigration on SDS-PAGE with the most abundant membrane protein of the lactose permease–overproducing strain T206 (Fig. 1 *a*, lane *1*; *Coomassie blue staining*), which could be labeled fairly specifically (one of two proteins; reference 20) with [^{3}H]-NEM (Fig. 1 *a*, lane *2*), and as such was recognized by antibodies raised against lactose permease (Fig. 1 *a*, lane *3*). The mobility on SDS-PAGE of the in vitro–synthesized lactose permease (33 kD) is in good agreement with values reported previously for the native enzyme isolated from membranes (18, 61), which reflects the fact that the enzyme is synthesized without a cleavable signal sequence (8, 18). The actual molecular mass of lactose permease, calculated from the known amino acid sequence, is 46,500 D (8). The much lower value obtained on SDS gels is due to an incomplete unfolding by SDS and is typical for highly hydrophobic, integral membrane proteins (27).

Synthesis of lactose permease in the S-135 occurred in a largely membrane-free environment. This is indicated by the finding that the S-135—in contrast to an S-30 used by others (18)—does not contain detectable amounts of signal peptidase activity (35). Furthermore, an absence of membranes from the S-135 is suggested by the different temperature sensitivities observed for native and in vitro–synthesized lactose permease. In accordance with what had previously been described for this (54) and other membrane proteins (27), lactose permease derived from [^{3}H]NEM-labeled membranes could only be detected if boiling before electrophoresis was avoided (Fig. 1 *a*, compare lanes *6* and *7*). On the other hand, the protein synthesized in our in vitro system proved to be completely insensitive to heat (Fig. 1 *a*, lanes *4* and *5*). This finding is consistent with different conformations of lactose permease depending on whether it is embedded in the lipid bilayer of the membrane or synthesized in vitro in a lipid-free milieu. Clearly, the availability of a membrane-free in vitro system was a prerequisite to demonstrate integration of lactose permease into exogenously added membrane vesicles.

Fig. 1 *b*, like all subsequent figures, shows in vitro synthesized lactose permease after resolution by SDS-PAGE in the presence of urea. Under these conditions the *lacY* translation product migrated with increased mobility as a 29-kD protein (Fig. 1 *b*, lane *2*). This protein, however, was also pGM21 specific: the parental plasmid pACYC184 lacking the intervening *lacY* gene, gave rise to the 25-kD chloramphenicol acetyltransferase protein (Fig. 1 *b*, lane *1*). The 29-kD pro-

tein directed by pGM21 comigrated with the [^{3}H]NEM-labeled lactose permease integrated in vivo into membranes (Fig. 1 *b*, compare lanes *2* and *4*) and was recognized by anti-lactose permease antibodies (Fig. 1 *b*, lane *3*). Since these antibodies recognize only the COOH-terminal peptide of lactose permease (47), their interaction with the in vitro–synthesized lactose permease is the strongest indication for the 29-kD protein being the full-length transcription–translation product of the *lacY* gene. The weak antigenicity of in vitro–synthesized lactose permease (Fig. 1 *b*, compare lanes *2* and *3*) might be the result of an aggregation of the protein (see below) which buries most of the COOH termini. This view is supported by the much higher antigenicity of lactose permease integrated in vivo into membranes (Fig. 1 *a*, lanes *2* and *3*) properly exposing the COOH-terminal epitope. The nature of the nonantigenic, pGM21-dependent, 7-kD protein (Fig. 1 *b*, lane *2*) which was consistently produced is not clear; it could be an NH_2-terminal fragment of lactose permease due to premature termination.

To find out whether the 29-kD translation product could integrate into plasma membranes from *E. coli*, inverted plasma membrane vesicles (INV) were added during or after the in vitro synthesis of lactose permease. It was apparent that because of the lack of a cleavable signal sequence (8), integration into membranes would be difficult to ascertain experimentally, since it would not simply be indicated by proteolytic maturation nor was the integrated protein expected to be completely protease resistant, like proteins sequestered within the lumen of the vesicles. In addition, cosedimentation, as a means to demonstrate membrane integration, also proved to be of no use. When in vitro reactions were resolved by centrifugation into soluble and pelletable subfractions, as shown in Fig. 2, the bulk of lactose permease (*LacY*) was recovered from the sedimentable fractions, irrespective of whether INV were omitted or added co- or posttranslationally (Fig. 2, compare lanes *1–3* with lanes *4–6*). However, it was possible to discriminate between lactose permease sedimenting with INV, added either co- or posttranslationally, if the sediments were digested with proteinase K (Fig. 2, lanes *7* and *8*) or thermolysin (Fig. 2, lanes *9* and *10*). When INV were present during synthesis, both proteases digested the 29-kD lactose permease almost completely, giving rise to a number of proteolytic fragments, the most prominent of them being a 13-kD peptide (Fig. 2, lanes *8* and *10*). Very little of this 13-kD cleavage product was found in the digests of reactions, in which INV were added posttranslationally: the 29-kD lactose permease was in these cases largely resistant to proteolysis (Fig. 2, lanes *7* and *9*). The same result was obtained with lactose permease kept as a control in the complete absence of INV (Fig. 2, lanes *11* and *12*). The 13-kD degradation product is likely to be the NH_2-terminal, ~140-amino acid, stable peptide obtained under a variety of proteolytic conditions (50).

The extensive protease-resistance of lactose permease synthesized in the absence of INV (Fig. 2, lanes *11* and *12*) probably is due to an aggregation of the hydrophobic protein which masks proteolytic cleavage sites. Aggregation is in fact indicated by the insolubility of the protein in the absence of INV. Conversely, protease-susceptibility of lactose permease synthesized in the presence of membranes suggests a more open conformation of the enzyme with the exposure of protease cleavage sites, which are known (50) to exist on the surface of the native, membrane-embedded protein. Protease sensitivity, therefore, can be taken as an indication for the integration of lactose permease into the added membranes.

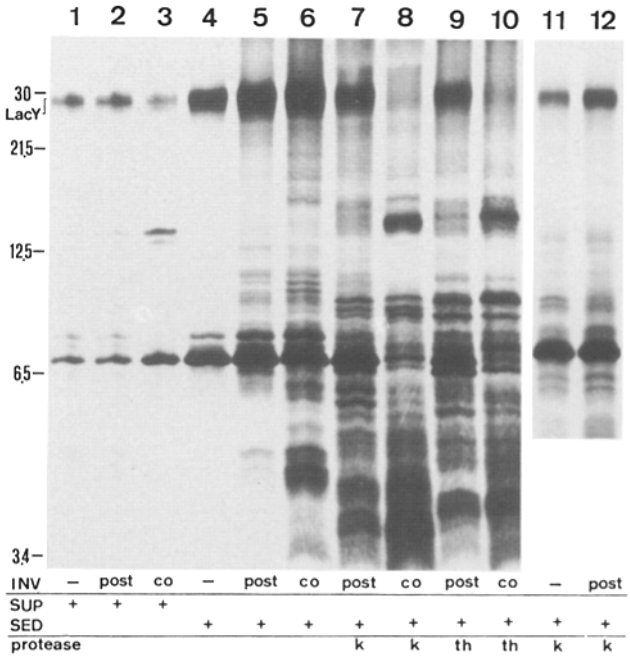

Figure 2. Lactose permease synthesized in an S-135 from *E. coli* forms protease-resistant aggregates unless INV are cotranslationally added. INV were present during the in vitro synthesis of lactose permease (*LacY*; *co*), thereafter (*post*), or completely omitted (see Materials and Methods). Reactions were stopped at 4°C. INV-free samples received carrier-INV, and the volumes of all samples were adjusted for any posttranslational supplement. Pelletable material was separated from soluble proteins by centrifugation through 50 μl of 0.5 M sucrose prepared in gradient buffer (40 mM TeaOAc, pH 7.5, 110 mM KOAc, 20 mM NH_4OAc, 11 mM $Mg[OAc]_2$) in the airfuge. Supernatant fractions (load + 5 μl cushion) were recovered and TCA precipitated (*SUP*). The remaining cushions were discarded, the pellets were washed once in 0.25 M sucrose prepared in gradient buffer, adjusted to pH 11.5, and collected again. They were resuspended in 50 mM TeaOAc, pH 7.5, 250 mM sucrose, 1 mM DTT, and sub-aliquots (*SED*) were TCA precipitated or treated with proteases. Proteinase K (*k*) and thermolysin (*th*) were added from 8.3 mg/ml stock solutions to give final concentrations of 0.5 mg protease/ml. Incubations were for 30 min on ice (*k*) and at 25°C (*th*). Reactions were stopped by addition of 1 vol of 10% TCA. Samples equivalent to 42 μl (lanes *3*, *6*, *8*, and *10*) or 25 μl (all other lanes) of original translation mix were applied to the SDS–urea gel. This was done to compensate for the INV-induced inhibition of lacY synthesis usually observed when INV were added cotranslationally. The 3.4-kD marker was insulin B chain.

Integration of biosynthetically produced lactose permease into the lipid bilayer of cotranslationally added INV is further suggested by the data depicted in Fig. 3. Pelleting of lactose permease synthesized in the absence of INV could not be avoided by treatment with dodecyl-O-β-D-maltoside (Fig. 3, lanes *1* and *2*), a detergent that can successfully be used to solubilize lactose permease from membranes (61). Dodecyl-O-β-D-maltoside insolubility was also characteristic for lactose permease incubated posttranslationally with INV (Fig. 3, lanes *3* and *4*). In contrast, considerable amounts of lactose permease were converted to a soluble form by the de-

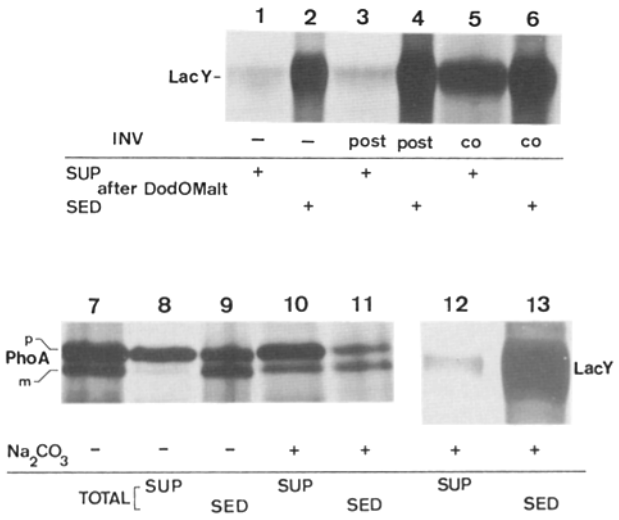

Figure 3. Lactose permease cotranslationally associates with INV in a detergent-sensitive and carbonate-resistant manner. (Lanes *1–6*) Lactose permease (*LacY*) was synthesized in vitro. To each 25-μl sample (an INV-free, a posttranslational, and a cotranslational reaction, prepared as described in Materials and Methods and the legend to Fig. 2) 1 vol of a 15-mg/ml solution of dodecyl-O-β-D-maltoside (*DodOMalt*) in H_2O was added followed by a 15-min incubation on ice. 45 μl was subsequently centrifuged in the airfuge through 50 μl of 0.5 M sucrose in 50 mM TeaOAc, pH 7.5, 1 mM DTT containing 7.5 mg/ml dodecyl-O-β-D-maltoside. Supernatant fractions were recovered and TCA precipitated (*SUP*). Pellets (*SED*) were directly dissolved in SDS-PAGE loading buffer. (Lanes *7–11*) Alkaline phosphatase (*PhoA*) was expressed in the S-135 from plasmid pHI-5, with INV cotranslationally added. Indicated are precursor (*p*) and mature form (*m*) of the enzyme. Total alkaline phosphatase translation products (lane *7*) were separated into supernatant (lane *8*) and pellet (lane *9*). Recovery of pre–alkaline phosphatase from the pellet is partially due to translocation into INV observed in general for precursors of bacterial export proteins (37). Alternatively the finding could reflect incomplete separation into SUP and SED or aggregation of some precursor. In the experiment shown in lanes *10* and *11*, centrifugation was preceded by diluting 24 μl of translation mix with 150 μl 250 mM Na_2CO_3 and incubating for 15 min on ice. The pH after addition of Na_2CO_3 was found to be 12. The supernatant fraction was neutralized with HOAc before TCA precipitation. (Lanes *12* and *13*) Lactose permease (*LacY*) synthesized in vitro and cotranslationally integrated into INV was treated as described for alkaline phosphatase, except that carbonate treatment was done subsequent to the removal of soluble proteins by resuspending the pellet in 175 μl 0.1 M Na_2CO_3. Shown are Na_2CO_3-extractable (lane *12*) and Na_2CO_3-resistant lacY (lane *13*).

tergent when synthesis had been allowed to proceed in the presence of INV (Fig. 3, lanes *5* and *6*). Evidently integration into the lipid bilayer of INV occurred only concurrently with synthesis.

According to previous results demonstrated with membranes from different sources (23), proteins incorporated into the lipid bilayer of cellular membranes should be resistant to extraction with 0.1 M Na_2CO_3 at pH 11–12. This does not apply to peripherally associated membrane proteins and proteins entrapped in closed membrane vesicles which usually are opened up during carbonate treatment. The applicability of this method to INV from *E. coli* was verified using in vitro–synthesized alkaline phosphatase, the precursor of which is proteolytically processed upon translocation into INV (Fig. 3, lane *7*; *p* and *m*). When translation products were separated into soluble and pelletable proteins, all of the mature enzyme sedimented with INV (Fig. 3, lane *9*), while most of the precursor remained in the supernatant (Fig. 3, lane *8*) as expected for a translocated protein (35). (Reasons for the appearance of precursor in the pellet fraction [Fig. 3, lane *9*] are indicated in the legend to Fig. 3). If, however, centrifugation was preceded by treatment with Na_2CO_3 at an alkaline pH, roughly 50% of the mature, INV-enclosed alkaline phosphatase was released into the supernatant (Fig. 3, compare lanes *10* and *11* with lanes *8* and *9*). It is not clear why part of the mature alkaline phosphatase remained in the pellet, but this could be due merely to an incomplete separation of the two phases. Under conditions that released at least half of the alkaline phosphatase (Fig. 3, lanes *10* and *11*), lactose permease synthesized in the presence of INV was almost completely Na_2CO_3 resistant as shown in Fig. 3, lanes *12* and *13*. This Na_2CO_3 resistance was not mimicked by a conceivable denaturation, and hence aggregation, of lactose permease after being liberated from a loose membrane association by Na_2CO_3: the protease sensitivity, characteristic of cotranslationally integrated lactose permease (Fig. 2, lanes *8* and *10*), was maintained during Na_2CO_3 treatment.

Additional evidence for the membrane integration of in vitro–synthesized lactose permease is provided by the experiment shown in Fig. 4. This experiment was designed to allow separation of membrane-associated from aggregated permease. To prepare an INV-enriched subfraction, total translation products (Fig. 4, *tot*) were resolved into soluble (*sol*), membrane-associated (*mbs*), and pelletable (*pel*) material by means of centrifugation through a two-step sucrose gradient in the airfuge, as outlined in Materials and Methods. The expected resolution into the three subfractions was first confirmed for the outer membrane protein LamB (Fig. 4 *a*), in which the presence of INV was verified by signal peptidase activity: almost all the mature LamB, generated by cleavage of the signal peptide upon transport into INV, was recovered from the membrane fraction. Subfractionation of lactose permease, synthesized in the absence of INV, revealed that (similar to the results depicted in Fig. 2): 82% pelleted (Fig. 4 *b*), 14% remained soluble, and negligible amounts (4%) sedimented with the carrier INV added. When INV were added cotranslationally to the in vitro translation reaction (Fig. 4 *c*), 51% of the lactose permease was now found in the membrane fraction at the expense of pelletable enzyme (37%), while the soluble fraction (12%) remained constant. (A comparison of the results illustrated in *b* and *c* also demonstrates how few endogenous INV are present in the S-135). After posttranslational incubation with INV (Fig. 4 *d*), 82% of lactose permease was recovered from the pellet, whereas part of the soluble lactose permease was now found associated with INV (13% in Fig. 4 *d* compared with 4% in Fig. 4 *b*).

To corroborate that small amounts of nonaggregated lactose permease could posttranslationally associate with INV, soluble lactose permease was obtained by high-speed centrifugation of translation products synthesized in the absence of membranes. (Soluble lactose permease thus prepared appears [Fig. 4 *e*, lane *1*] as a much more intense band than previously [Fig. 4 *b*; *sol*] because of a comparably long exposure of the X-ray film.) When this material was incubated for an additional 30 min in the absence of INV and subse-

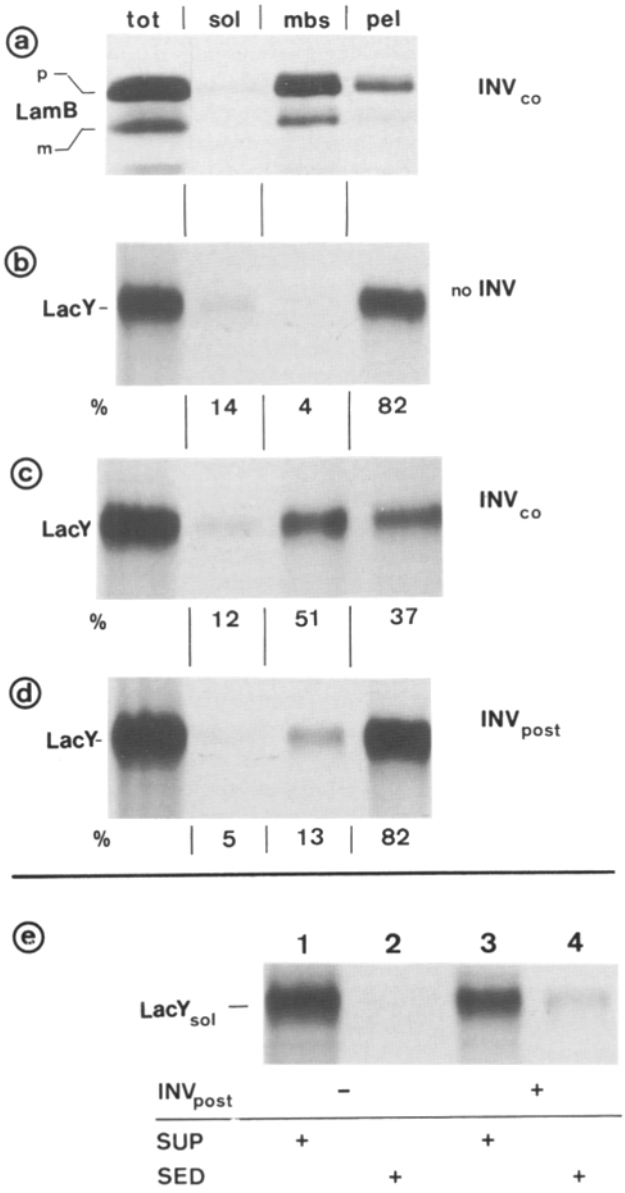

Figure 4. Lactose permease integrates posttranslationally into INV at low efficiency from a soluble precursor pool. The lambda receptor (*LamB*; precursor [*p*] and mature form [*m*], respectively) and lactose permease (*LacY*) were synthesized in vitro with INV added as indicated. Total translation products (*tot*) were subfractionated into soluble (*sol*), membrane-bound (*mbs*), and pelletable proteins (*pel*), and the respective partitioning was quantitated as described in Materials and Methods. The INV-free sample received an equal amount of carrier INV before centrifugation. The total recovery (i.e., sum of the three subfractions with respect to total) was usually between 70 and 80%. In the experiment depicted in *e*, posttranslational incubation with INV was done only after removing pelletable material. Subsequent to the incubation, supernatant (*SUP*) and pellet fractions (*SED*) were again prepared. Pellets were washed in 0.1 M Na_2CO_3 before SDS-urea-PAGE.

quently subjected to differential centrifugation, yielding only a supernatant and a pellet fraction, it remained entirely in the supernatant (Fig. 4 *e*, lanes *1* and *2*). A small amount of soluble enzyme, however, became pelletable upon incubation with INV (Fig. 4 *e*, lanes *3* and *4*), which must reflect INV association since no aggregation occurred under these conditions (Fig. 4, lane *2*). These results unequivocally demonstrate that a posttranslational membrane association of lactose permease can occur only from a soluble protein pool, which because of the centrifugation conditions of the experiment depicted in Fig. 4 *e* is likely to be ribosome free. This posttranslational association of lactose permease with INV was also Na_2CO_3 resistant (see legend to Fig. 4 *e*), suggesting true incorporation into the lipid bilayer. A substantial posttranslational integration of lactose permease into INV, however, was obviously prevented by the extensive denaturation of the hydrophobic protein in the aqueous environment (Fig. 4 *d*), a phenomenon which is likely to hold true for many other membrane proteins.

Table I. Assay of ^{14}C-Lactose Uptake by INV: In Vivo-assembled Lactose Permease

	T206 INV (lacY$^+$)				MRE600* INV (lacY$^-$)
Treatment:	–	NEM	TDG‡	no Hg^{2+}	–
	821§	51	373	0	56

* Wild-type strain not induced for the synthesis of lactose permease.
‡ TDG, β-galactosyl 1-thio-β-D-galactoside.
§ INV-associated cpm corrected for the values of Hg^{2+} controls.

Clearly, the best way to test whether or not a membrane protein has correctly integrated is to examine its biological activity. To this end, an established assay for the lactose permease-dependent uptake of [^{14}C]lactose into INV (31) was adapted (Tables I and II). This assay makes use of the enzyme-catalyzed, facilitated diffusion of substrate down a concentration gradient, leading to an accumulation of substrate within INV, until the exterior and interior concentrations are balanced. Subsequent poisoning of lactose permease by Hg^{2+} prevents later efflux of substrate during recovery of the INV, so that quantitation of the INV-associated radioactivity reflects uptake of [^{14}C]lactose into the lumen of the INV, provided that the obtained values are corrected for unspecific binding (controls in which Hg^{2+} was given before the substrate). As shown in Table I, INV prepared from the lactose permease-overproducing strain T206 accumulated [^{14}C]lactose, which was abolished by inhibiting lactose permease with NEM and substantially reduced by the substrate analogue β-galactosyl 1-thio-β-D-galactoside. No radioactive substrate remained within INV when treatment with Hg^{2+} was omitted. Most importantly, INV pre-

Table II. Assay of ^{14}C-Lactose Uptake by INV: In Vitro-assembled Lactose Permease

MRE600-INV*	+	+	+	–
LacY-DNA	–	+	+	+
NEM	–	–	+	–
Exp. 1	<0	1,784‡		
Exp. 2	<0	798	270	110
Exp. 3		415		
Exp. 4	<0	1,277	348	
Exp. 5		227		85

* Cotranslationally added to in vitro synthesis of lactose permease.
‡ INV-associated cpm as detailed in Table I (SEM, 900 ± 637).

pared from strain MRE 600, which had not been induced for the synthesis of lactose permease, did not accumulate significant [^{14}C]lactose, clearly indicating that the uptake of radioactivity into T206-INV was dependent upon the presence of lactose permease.

To find out whether in vitro–assembled lactose permease was active, MRE 600-INV that had been present during synthesis of lactose permease were collected from fivefold scaled-up reactions and subjected to the [^{14}C]lactose uptake

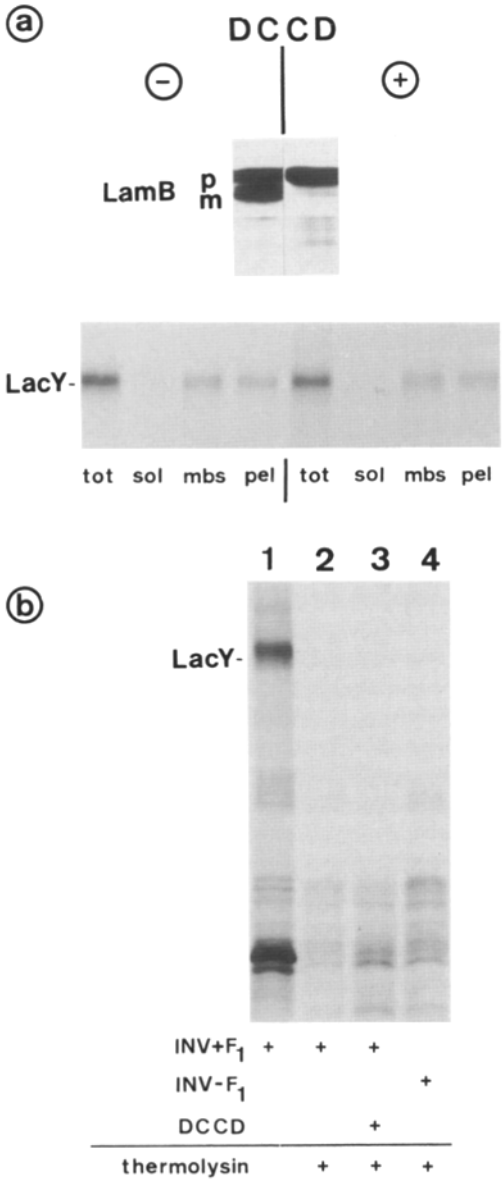

Figure 5. Cotranslational integration of lactose permease into INV does not require the H^+-motive force. (*a*) Lambda receptor (*LamB*) and lactose permease (*LacY*) were synthesized in vitro in the presence of INV. Where indicated, DCCD was included at 0.5 mM final concentration as described (37). LacY was subfractionated as outlined in the legend to Fig. 4. (*b*) LacY was synthesized in the presence of INV (lane *1*). The pellet fraction was digested with thermolysin (lane *2*) as detailed in the legend to Fig. 2. (Lane *3*) Same as lane *2*, except that 0.5 mM DCCD had been included into the translation mix. (Lane *4*) Same as lane *2*, except that INV deprived of F_1-ATPase by low-salt stripping (37) were used.

described above. In five independent experiments (Table II), MRE 600-INV accumulated radioactivity if biosynthetically reconstituted with lactose permease. No accumulation occurred either if lactose permease was not synthesized because of an absence of the lacY DNA, or if INV were omitted. As expected for the enzyme-mediated reaction, uptake of radioactivity was subject to inhibition by NEM. Therefore, the INV-associated radioactivity is the result of an enzyme-catalyzed transport of [^{14}C]lactose into INV, demonstrating that in vitro–synthesized lactose permease can assemble into isolated membrane vesicles in a native (i.e., functional) configuration.

To obtain initial information on the mechanism of membrane integration occurring in our in vitro system we studied the dependence of this process on the H^+-motive force. Many authors have recently demonstrated that protein export in *E. coli* requires two sources of energy: an H^+-motive force and ATP (references summarized in 37). The dependence of protein transport into INV on a H^+ gradient can be shown by the use of *N,N'*-dicyclohexylcarbodiimide (DCCD), which preferentially inhibits the H^+-translocating F_1F_O-ATPase (37). As illustrated in Fig. 5 *a*, processing of preLamB by INV was completely prevented by 0.5 mM DCCD. Under identical conditions, however, lactose permease integration into membranes was unaffected by the presence of DCCD, as shown by the recovery of ~50% of the protein from the membrane fraction. Furthermore, INV-associated lactose permease displayed identical patterns of proteolytic cleavage peptides regardless of whether native INV (Fig. 5 *b*, lane *2*), DCCD-poisoned INV (lane *3*), or F_1-depleted INV (lane *4*) were used. In all cases 29-kD lactose permease (*LacY*; lane *1*) was completely digested, suggesting a native tertiary structure and consequently an H^+-motive force–independent integration into INV.

Discussion

In vitro synthesis of lactose permease, previously achieved by others (18), was performed in the present study by programming a high speed supernatant (S-135) of an *E. coli* extract with plasmid pGM21. The major translation product was identified as full-length lactose permease by (*a*) its cross-reactivity with antibodies raised against the COOH terminus of the enzyme; (*b*) its comigration with lactose permease assembled in vivo into *E. coli* plasma membranes; and (*c*) its dependence on the *lacY* gene–containing plasmid pGM21. Unless supplemented with inverted membrane vesicles (INV), lactose permease synthesis in the S-135 occurred in a membrane-free environment, as inferred from a sedimentation velocity of the de novo–synthesized enzyme higher than membranes and a failure to demonstrate that the cell-free translation product exhibits the same temperature sensitivity as the membrane-embedded enzyme.

Lactose permease synthesized in the membrane-free S-135 was found to aggregate extensively. This was indicated by the masking of proteolytic cleavage sites, the stability towards detergents, and a sedimentation behavior suggesting particles denser than membrane vesicles. Cosedimentation of a protein with membranes, therefore, reflects functional association only if a technique is designed that allows discrimination between membranes and aggregated proteins (Fig. 4) (15, 24). Aggregation has also been found to occur with other

exported proteins synthesized in vitro (35), although to a considerably lesser extent than observed here for the extremely hydrophobic lactose permease (8).

The absence of membranes from the S-135, allowed the study of lactose permease integration into exogenously added INV. As proposed previously, on the basis of a different experimental approach (41), integration occurred predominantly in a cotranslational manner. Such in vitro–assembled lactose permease acquired a native conformation in the membranes of INV. This was verified by (*a*) a protease-cleavage pattern closely related to that of the in vivo enzyme which requires the exposure of a distinct domain with a cluster of proteolytic cleavage sites (50); (*b*) the solubilization by detergent; (*c*) the resistance towards Na_2CO_3 extraction, frequently used to probe membrane integration (3); (*d*) the recovery of lactose permease from a membrane-enriched subfraction of the in vitro reaction mix (the last two criteria were first examined for their suitability by the use of known exported proteins as controls); and (*e*) the enzymic activity of in vitro–integrated lactose permease. Certainly, the biological activity is the most convincing evidence for an authentic conformation of synthetically assembled lactose permease.

The activity assay used here is based on the enzyme's ability to catalyze facilitated diffusion of substrate. The fact that this assay measures the maximal amount of substrate accumulated after sufficient time, independent of enzyme concentration, proved to be particularly useful for the extremely small amounts of enzyme synthesized in vitro which would not allow determination of minute, initial uptake rates. This is also why the necessarily different amounts of permease assembled in vitro and in vivo exhibited similar enzymatic activities. The specificity of the reaction is undoubtedly reflected by the dependence on INV and lacY DNA, as well as by the inhibition using a substrate analogue or NEM.

The most plausible explanation for the extremely low rate of posttranslational integration into INV is that the synthesis of lactose permease in the absence of membranes resulted in massive aggregation. This notion is supported by the finding that properties characteristic of aggregated lactose permease, such as protease and detergent resistance, did not change upon posttranslational incubation with INV. Accordingly, the small amount of posttranslationally assembled lactose permease, detectable when membranes were isolated from the reaction mix (Fig. 4), was most probably derived from a soluble lactose permease pool. These results emphasize that, at least with hydrophobic proteins, the issue of whether incorporation into the lipid bilayer occurs concurrently with or subsequently to protein synthesis is merely academic. Rather, it seems likely that a mechanism operates which avoids the release of a nascent polypeptide chain from the ribosome until after the initiation of membrane integration, thus preventing denaturation in the aqueous milieu of the cytoplasm. This would also explain the recently described posttranslational integration of the human glucose transporter into mammalian endoplasmic reticulum that was demonstrated to occur efficiently only for a truncated form lacking the COOH-terminal 30% of the molecule (34).

An intriguing aspect is the mechanism which prevents denaturation; i.e., aggregation of the small fraction of in vitro–synthesized lactose permease which retains integration competence after termination of translation. It is conceivable that it is through an association with unfolding or renaturing proteins, such as those recently postulated (19, 42, 43) and described (10, 11, 13, 16, 65) both for prokaryotes and eukaryotes. Interestingly, soluble lactose permease was found to be largely protease resistant (Ahrem, B., and M. Müller, unpublished results) similar to what has been described for the association of M13 precoat with a 70-kD heat shock protein (65). The markedly different efficiencies of co- vs. posttranslational integration of lactose permease into INV suggest that renaturing activities must be low in the in vitro system described herein, whereas molecular components presumably involved in cotranslational integration of lactose permease into INV appear to be rather active. Whether this implies that different molecular pathways exist for the two temporal modes of protein integration also in vivo, cannot be answered by the in vitro studies presented here.

Integration of lactose permease into INV does not require the H^+-motive force. This finding is in remarkable contrast to all the other bacterial export proteins studied so far in vivo and in vitro (summarized in 37). Even inner membrane proteins such as the M13 major coat protein and the signal peptidase (59), appear to depend on a membrane potential for proper assembly. That integration of lactose permease into INV, observed under conditions which dissipate the H^+-motive force, might not lead to an authentically structured enzyme, are unlikely; protease-sensitive membrane association of lactose permease was found to occur both with native and deenergized INV, and this property was shown for native INV to correlate with the enzymatically active tertiary structure of the protein. Unfortunately, enzymatic activity could not be measured with DCCD-treated INV, because of an interference of DCCD with the uptake assay. A search for structural differences between lactose permease, on the one hand, and coat protein and leader peptidase, on the other, regarding their different requirements for a membrane potential, reveals that the major part of the leader peptidase molecule is not embedded in the membrane, but has to be translocated into the periplasm (59). It might be that this channeling of numerous polar residues through the membrane requires a H^+-motive force. Although the 50–amino acid coat protein does not exhibit large translocated domains, it is noteworthy that the presumably translocated part of the molecule contains several (mostly negatively) charged amino acids, one of which, when changed to an uncharged residue, renders integration of the coat protein independent of the membrane potential (64). Provided that our results obtained by use of an in vitro system truly reflect in vivo conditions, polytopic transmembrane proteins lacking extensive stretches of charged residues, such as the lactose permease, might in fact integrate into the bacterial plasma membrane without the aid of the H^+-motive force.

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