Properties and purification of an active biotinylated lactose permease from *Escherichia coli* (topology/affinity chromatography/lactose–H⁺ symport)

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ABSTRACT A simplified approach for purification of functional lactose permease from *Escherichia coli* is described that is based on the construction of chimeras between the permease and a 100-amino acid residue polypeptide containing the biotin acceptor domain from the oxaloacetate decarboxylase of *Klebsiella pneumoniae* [Cronan, J. E., Jr. (1990) *J. Biol. Chem.* 265, 10327–10333]. Chimeras were constructed with a factor Xa protease site and the biotin acceptor domain in the middle cytoplasmic loop (loop 6) or at the C terminus of the permease. Each construct catalyzes active lactose transport in cells and right-side-out membrane vesicles. Moreover, the constructs are biotinylated in vivo, and in both chimeras, the factor Xa protease site is accessible from the cytoplasmic surface of the membrane. Both biotinylated permeases bind selectively to immobilized monomeric avidin and are eluted with free biotin in a high state of purity, and the loop 6 chimera catalyzes active transport after reconstitution into proteoliposomes. The methodology described should be applicable to other membrane proteins.

The lactose permease of *Escherichia coli* is a polytopic hydrophobic membrane protein that catalyzes the coupled translocation of a single β-galactoside molecule with a single H⁺ (i.e., symport or cotransport). As such, it is a paradigm for membrane proteins that transduce the energy stored in an electrochemical ion gradient into work in the form of a concentration gradient (see refs. 1 and 2 for reviews). The permease is encoded by the lacY gene, which has been cloned (3) and sequenced (4), and the protein has been solubilized from the membrane, purified, and reconstituted into proteoliposomes in a fully functional state (5). Based on circular dichroism of the purified protein and the sequential hydrolysis of the deduced amino acid sequence (6), a secondary structure was proposed in which the polypeptide is organized into 12 α-helical domains that traverse the membrane in a zigzag fashion connected by more hydrophilic domains (loops). Evidence supporting the general aspects of the model and demonstrating that both the N and C termini are on the cytoplasmic face of the membrane has been obtained through a variety of experimental approaches (1, 2), and analysis of lactose permease–alkaline phosphatase (lacY-phoA) constructs has provided unequivocal support for the topological predictions of the 12-helix model (7).

Although lactose permease can be solubilized from the membrane, purified, and reconstituted in a functional state, a simpler, more rapid purification is needed for biochemical and spectroscopic studies involving cysteine (8–12) or tryptophan (13) replacement mutants, as well as attempts at crystallization. Cronan (14) has described a method for the construction of soluble chimeric proteins from either *E. coli* or *Saccharomyces cerevisiae* with biotin acceptor domains from one of four different carboxylases fused to the C termini and demonstrated that the proteins are biotinylated in vivo. The biotinylated fusion proteins can be readily purified in native form by binding to columns of monomeric avidin followed by elution with buffers containing biotin. This report describes applications of the approach to lactose permease. Preliminary reports on some of this work have been presented (15, 16).

MATERIALS AND METHODS

Materials. Mutagenic oligodeoxynucleotide primers were synthesized on an Applied Biosystems DNA synthesizer and used without further purification. *Eco*RI, *Bam*HI, and *Hind*III restriction endonucleases, Klenow DNA polymerase, *T4* polynucleotide kinase, and *T4* DNA ligase were purchased from New England Biolabs. Sequenase was from United States Biochemical. Protein A-conjugated horseradish peroxidase (PA-HRP), enhanced chemiluminescence (ECL) detection kits, and [1-¹⁴C]lactose were from Amersham. Adenosine 5′-[α-³²P]thiotriphosphate and [³²P]methylene are from NEN. Immobilized monomeric avidin and avidin-conjugated horseradish peroxidase (avidin-HRP) were from Pierce. Factor Xa protease, *Sal I* restriction endonuclease, *Tag* DNA polymerase, and calf intestinal alkaline phosphatase were from Boehringer Mannheim. GeneClean glassmilk DNA purification kits were obtained from Bio 101 (La Jolla, CA), and Magic Mini Prep kits from Promega. Purified *E. coli* phospholipids were purchased from Avanti Polar Lipids and were washed with acetone/ether (5) prior to use. All other materials were reagent grade and obtained from commercial sources.

Bacterial Strains. The following strains of *E. coli* K-12 were used: T206 [lacI*O*Z′Y− (A), rpsL₅, met, thr, recA₅, hsdM, hsa-R] F− lacI*O*Z Δ(lac8 Y′ A⁺) harboring plasmid pGM21 [lac(lacO*F*P*Δ(Z)Y*Δ(A)tetR)] (17); T184 [T206 cured of pGM21]; and HB101 [hsdS20 (r-B, m-B), recA₁₃, ara-14, proA₂, lacY₁, galK₂, rpsL20(Sm²), xyl-5, mtl₁, supE₄₄, l−]/F⁺ (18).

Abbreviations: PA-HRP, protein A-conjugated horseradish peroxidase; avidin-HRP, avidin-conjugated horseradish peroxidase; RSO, right-side-out; OG, octyl-β-D-glucopyranoside; ΔH⁺, H⁺ electrochemical gradient; Dns-Gal, dansylaminohexyl β-D-thiogalactopyranoside; CCCP, carbonylcyanide m-chlorophenylhydrazone; mAb, monoclonal antibody.

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Construction of Chimeras. (i) C-terminal biotin acceptor domain (CB perosme). Plasmid pLacY/CB was constructed by using lacY from pGM21 (17) and pKR35 (19) as the source of DNA encoding the biotin acceptor domain. pGM21 has a 2.3-kb EcoRI fragment containing the lacY gene preceded by the lac promoter/operator and a Shine–Dalgarno sequence. By bacteriophage M13 site-directed mutagenesis (20), an EcoRI site was introduced immediately upstream of the lac promoter/operator and a Sal I site was created at the 3' end of the lacY coding sequence. The Sal I site converts the lac for the last residue of the perosme from alanine to aspartate. The new restriction sites uniquely flank a 1.5-kb DNA fragment containing the lac promoter/operator and the lacY gene and allow mobilization from M13 replicative-form (RF) DNA. pKR35 contains a 0.3-kb DNA fragment encoding the biotin acceptor domain from the Klebsiella pneumoniae oxaloacetate decarboxylase gene. The new EcoRI site was introduced immediately upstream of the lac promoter/operator and a Sal I site was created at the 3' end of the lacY coding sequence. The Sal I site converts the lac for the last residue of the perosme from alanine to aspartate. The new restriction sites uniquely flank a 1.5-kb DNA fragment containing the lac promoter/operator and the lacY gene and allow mobilization from M13 replicative-form (RF) DNA. pKR35 contains a 0.3-kb DNA fragment encoding the biotin acceptor domain from the Klebsiella pneumoniae oxaloacetate decarboxylase gene.

(ii) Insertion of factor Xa protease site (CBX perosme). The polymerase chain reaction (PCR) was used to introduce a factor Xa "restriction" protease site (Ile-Glu-Gly-Arg) between the C terminus of the perosme and the biotin acceptor domain. PCR primers were designed to introduce a factor Xa site at the N terminus of the biotin acceptor domain and to create a new Sal I site downstream of the termination codon. The ends of the PCR product were trimmed with Sal I. Screening of the ligation products for correct insertion orientation identified plasmid pLacX/CBX (Fig. 1 Left). One hundred nanograms of pLacX/CBX DNA was used as template with 100 pmol of each primer for 50 PCR cycles utilizing 1 unit of Taq DNA polymerase in buffer with 0.2 mM dNTPs. The thermal cycle was as follows: denature at 94°C for 30 sec, ramp anneal to 37°C over 2 min, extension to 72°C, and extension at 72°C for 30 sec. An identical PCR protocol was used for the construct described below.

(iii) Perosme with a factor Xa site and the biotin acceptor domain in loop 6 (CBX perosme). A general strategy was devised to insert the biotin acceptor domain into any position in lactose perosme—in this case, the middle cytoplasmic loop. First, a unique Sal I site was introduced changing codons 194 and 195 of lacY in pGM21 by two-stage PCR mutagenesis. The 2.3-kb EcoRI fragment encoding mutated lacY (S194V/A195D) was subcloned into the unique EcoRI site of pKR0 (derived from pKR35 by removal of the BamHI-HindIII DNA fragment encoding the biotin acceptor domain). Second, pLacX/CBX was used as a template with synthetic PCR primers appropriate for amplifying the DNA encoding the factor Xa protease site and the biotin acceptor domain. The primers were also designed to remove the termination codon and introduce a second in-frame Sal I site at the 3' end of the fragment. After digestion of the PCR product with Sal I, a 0.3-kb DNA fragment encoding the factor Xa protease site and the biotin acceptor domain flanked by Sal I sites was obtained. The fragment was then ligated into the newly created, unique Sal I site within lacY. Ligation products were screened by restriction fragment analysis to verify the orientation of fragment insertion, yielding pLacY/L6XB (Fig. 1 Right).

DNA Sequencing. Double-stranded DNA sequencing (21) was carried out by the dideoxynucleotide termination method (22, 23).

Membrane Morphology. E. coli HB101 (lacZ− Y−) was transformed with plasmids encoding given constructs, and the cells were grown on MacConkey indicator plates containing 25 mM lactose.

Lactose Transport in Cells and Right-Side-Out (RSO) Vesicles. Lactose transport was measured in E. coli T184 harboring given plasmids as described (24). Transport assays with RSO membrane vesicles were carried out with ascorbate/phosphate buffer under oxygen (25, 26). In both cases, [1-14C]lactose (10 μCi/mmol; 1 Ci = 37 GBq) was used at a final concentration of 0.4 mM, and radioactivity was assayed by liquid scintillation spectrometry.

SDS/PAGE and Blotting. Freshly grown cells were washed in 50 mM potassium phosphate (KP, pH 7.5) and resuspended to an OD600 of 1.0-1.25% (wt/vol) OG/100 mM KCl/1.25% (vol/vol) OG/100 mM KCl/1.25% (wt/vol) OG/100 mM KCl/phosphate buffer (pH 7.5) with 0.2 mM dNTPs and 1.25% octyl β-D-glucopyranoside (OG), without pretreatment with urea and cholate, and the biotinylated perosme was purified by affinity chromatography on a 5-ml column of immobilized monovalent avidin in the following manner. The column was washed sequentially with 10 bed volumes of 100 mM NaPi, pH 7.2, 150 mM NaCl (PBS), followed by 10 volumes each of 2 mM (+)-biotin in PBS, 100 mM glycine/HCl (pH 2.8), and PBS. Prior to sample application, the column was equilibrated with 20 bed volumes of column buffer [50 mM KP, pH 7.5/150 mM KCl/1.25% (wt/vol) OG/100 mM KCl/1.25% (wt/vol) OG/100 mM KCl/phosphate buffer (pH 7.5)]. The sample (−20–20 ml of OG extract containing ≈26 mg of protein) was then applied to the column, and unbound protein was removed by washing with column buffer until A280 returned to baseline. Bound material was then eluted with 2 mM (+)-biotin in column buffer. The flow rate was 1 ml/min, and 2-ml fractions were collected. Protein-containing fractions eluted with (+)-biotin were pooled and reconstituted into proteoliposomes with acetone/ether-washed E. coli phospholipids by detergent dilution, followed by two cycles of freeze–thaw/sonication (5).

Transport in Proteoliposomes. A H+ electrochemical gradient (ΔμH+; interior negative and alkaline) was generated by diluting proteoliposomes prepared in 50 mM KP, pH 7.5, and treated with 20 μM valinomycin 200-fold into 30 mM NaPi, pH 5.5 containing 0.4 mM [1-14C]lactose (20 μCi/mmol) (5).
Transport assays were terminated at a given time by quenching
of the reactions with 3 ml of ice-cold 50 mM NaP$_2$(pH 7.5)
and immediate filtration. The filters were washed once with
the same cold buffer, and radioactivity retained was mea-
sured by liquid scintillation spectrometry.

Fluorescence of dansylaminohexyl β-d-thiogalactopyran-
ose (Dns-Gal) was also measured (5). Proteoliposomes
were concentrated 5-fold in 50 mM KP$_2$(pH 7.5) and diluted
200-fold into a cuvette containing 50 mM NaP$_2$(pH 7.5)
and 5 μM Dns-Gal. A membrane potential (interior negative) was
then generated by addition of 20 μM valinomycin. Where
indicated, 20 μM carbonylcyanide m-chlorophenylhydra-
zone (CCCP) was added. In control experiments, proteoli-
posomes were diluted into 50 mM KP$_2$(pH 7.5). Fluorescence
was recorded with an Aminco SLM 8000C spectrofluorimeter
using 1 × 1-cm cuvettes. Excitation was at 340 nm, and
emission was measured at 500 nm. The sample chamber
was maintained at 25°C with a circulating water bath.

**Protein Determination.** Protein was assayed by a modified
Lowry procedure (28).

**RESULTS**

**Verification of Constructs by DNA Sequencing.** Genetic
constructs were verified by sequencing through all of the
ligation junctions in double-stranded DNA.

**Colony Morphology.** The ability of the constructs to trans-
locate lactose “downhill” was estimated by transforming E.
coli HB101 (lacZ’ Y”) with pLacY/L6XB or pLacY/CXB
and growing the transformants on MacConkey indicator
plates containing 25 mM lactose. Cells expressing functional
lactose permease hydrolyze the imported lactose, and me-
tabolism of the monosaccharides released causes acidifica-
tion which makes the colonies appear red. Cells impermeable
to lactose appear as white colonies, while mutants with low
activity grow as red colonies with a white halo. HB101 cells
expressing either L6XB or CXB permease grow as dark-red
colonies indistinguishable from cells expressing wild-type
permease.

**Active Lactose Transport.** Time courses of [1-14C]lactose
transport by E. coli T184 (lacZ’ Y”) expressing wild-type,
L6XB, or CXB permease are shown in Fig. 2. Clearly, cells
with either chimera transport lactose at initial rates and to
steady-state levels of accumulation that are identical to those
of wild-type permease within experimental error, while cells
transformed with plasmid devoid of lacY transport the disac-
charide to a negligible extent.

Similarly, RSO membrane vesicles containing the loop 6
chimera or the C-terminal fusion protein catalyze active
lactose transport (data not shown). In the absence of electron

![Fig. 2. Lactose transport in E. coli T184 cells harboring pACYC
(plasmid without lacY) ( ), pLacY/CXB ( ), pLacY/L6XB ( ), or
pGMT21 (wild-type lacY) ( ).](image)

**Fig. 2.** Lactose transport in E. coli T184 cells harboring pACYC
(plasmid without lacY) ( ), pLacY/CXB ( ), pLacY/L6XB ( ), or
pGMT21 (wild-type lacY) ( ).

**Membrane Insertion, Biotinylation, and Topology.** Mem-
brane insertion, biotinylation, and the topology of the in-
serted domains in each construct were evaluated by Western
blot analyses using monoclonal antibody (mAb) 4A1OR (27)
and PA-HRP or avidin-HRP (Fig. 3). Membranes containing
the loop 6 chimera or the C-terminal fusion protein react with
avidin-HRP, demonstrating that the constructs are biotinyl-
ated in vivo. No other biotinylated proteins are observed in
the membranes (data not shown). The fusion proteins exhibit
molecular masses of about 48 and 45 kDa, respectively,
whereas wild-type permease apparent or relative exhibits a
molecular mass of 33 kDa. In addition, while both proteins
react with mAb 4A1OR, L6XB permease reacts with anti-C
terminus polyclonal antibody (27), whereas CXB permease
does not (data not shown). Thus, the C terminus of the
permease is apparently inaccessible in the C-terminal fusion
protein.

The factor Xa site in each chimera is exclusively accessible
from the cytoplasmic surface of the membrane. When
spheroplasts containing either construct are prepared under
conditions where the external surface of the cytoplasmic
membrane is accessible to antibody (29) and exposed to
factor Xa, minimal cleavage is observed. In marked contrast,
when the spheroplasts are disrupted by sonication, the factor
Xa sites in both chimeric proteins become completely sus-
ceptible to cleavage by the protease. Similar results are
obtained when binding of avidin is measured directly

[35S]Methionine pulse-chase experiments (30) demon-
strate that L6XB and CXB permeases are stable after inser-
tion into the membrane (data not shown). After labeling in
vivo with [35S]methionine, followed by addition of excess
unlabeled methionine, both constructs, like wild-type
permease, are completely stable for at least 12-14 hr.

**Purification and Reconstitution.** L6XB permease was bio-
tinylated in vivo and purified by affinity chromatography on
immobilized monomeric avidin. Membranes were prepared

![Fig. 3. Surface topology of factor Xa-biotin acceptor domains.
Spheroplasts were prepared as described (28) from E. coli T184
transformed with pLacY/L6XB (lanes 1-4) or pLacY/CXB (lanes
5-8). Aliquots of each spheroplast preparation were used directly
(lanes 1, 2, 5, and 6) or subjected to sonication in a bath-type
sonicator (lanes 3, 4, 7, and 8). The preparations were either used
untreated (lanes 1, 3, 5, and 7) or digested with factor Xa protease
(2 μg of protease per mg of protein) for 30 min at 25°C (lanes 2, 4,
and 8). Samples were then subjected to SDS/PAGE and electroblot-
ing onto Immobilon P membranes (Millipore), followed by treatment
with either avidin-HRP (lanes 1-6) or mAb 4A1OR and PA-HRP
(lanes 7 and 8) and ECL reagents (Amersham). The L6XB and CXB
cleavage fragments migrate at about 28 and 33 kDa, respectively.
Although not shown, L6XB permease yields identical results with
mAb 4A1OR, and CXB permease yields identical results with avidin-
HRP. Moreover, results identical to those obtained with avidin-HRP
were obtained when mAb 4A1OR and PA-HRP were used to detect
cleavage of L6XB permease by factor Xa.](image)
and extracted directly with OG. The extract was then applied to a 5-ml avidin-Sepharose column, and unbound protein was eluted by extensive washing with column buffer (Fig. 4A). When \( A_{280} \) of the eluate returned to baseline, bound material was eluted with 2 mM (+)-biotin, and shortly thereafter, a sharp peak was observed. As shown by silver staining (Fig. 4B), the 280-nm-absorbing material eluted with biotin exhibits a major band at \( \approx 45 \) kDa with higher-order aggregates at 90 kDa and 135 kDa. Importantly, banding patterns identical to those observed with silver staining are obtained with avidin-HRP or mAb 4A10R (data not shown), thereby demonstrating that the bands observed at higher molecular mass are indeed aggregates of L6XB permease rather than contaminants. Further, with dodecyl maltoside in place of OG, almost all of the material migrates at 45 kDa (Fig. 4B), and similar results are obtained when membranes are subjected to SDS/PAGE directly (i.e., without OG extraction; see Fig. 3). Thus, the material obtained in the biotin eluate represents highly purified protein. Starting from \( \approx 20 \) g of cells (wet weight), \( \approx 2 \) mg of purified permease was obtained.

The fractions eluted with biotin were pooled, reconstituted with E. coli phospholipids, and subjected to freeze–thaw/sonication to prepare proteoliposomes (5). When proteoliposomes prepared in KPi (pH 7.5) are diluted into NaPi (pH 5.5) in the presence of valinomycin so that a \( \Delta \mu_{\text{iH}} \) (interior negative and alkaline) is created, lactose is taken up rapidly, and a maximum level of accumulation is observed in 1–2 min (Fig. 5). The level then decreases gradually due to dissipation of \( \Delta \mu_{\text{iH}} \). Lactose accumulation is not observed when the proteoliposomes are diluted into equimolar KPi (pH 7.5) or after treatment with p-chloromercuribenzenesulfonate.

Dns-Gal fluorescence studies were performed (5) to obtain independent confirmation for the functionality of purified L6XB (Fig. 6). A marked increase in Dns-Gal fluorescence is observed when valinomycin is added to proteoliposomes prepared in KPi and diluted into NaPi, but not when the proteoliposomes are diluted into equimolar KPi. Further, the increase in fluorescence observed in the presence of a K+ diffusion potential is completely reversed upon addition of the protonophore CCCP.

**DISCUSSION**

Although procedures have been described for purifying functional lactose permease (5), a simpler, more rapid method that yields purified material in more concentrated form would be useful for various applications. By utilizing the biotin

![Fig. 4.](image)  
**Fig. 4.** Monovalent avidin-affinity chromatography of L6XB permease. (A) Elution profile from the monovalent avidin-Sepharose column. An aliquot (2 ml) of OG extract from membranes containing L6XB permease (\( \approx 0.6 \) mg/ml) was applied to a 1.0 \( \times \) 6.4-cm monovalent avidin-Sepharose column. The column was developed as described in Materials and Methods, and 2-ml fractions were collected. After \( A_{280} \) returned to baseline, bound material was eluted with 2 mM (+)-biotin as indicated. (B) SDS/PAGE of OG extract and various fractions obtained during purification. Lane 1, OG extract (5 \( \mu \)l); lane 2, peak of unbound fraction (5 \( \mu \)l); lanes 3–10, fractions eluted with biotin (40 \( \mu \)l); lane 11 (DM), pooled fractions from an independent chromatography using dodecyl maltoside in place of OG. The gels were stained with silver, and identical results were obtained in blotting experiments with avidin-HRP or mAb 4A10R and PA-HRP.

![Fig. 6.](image)  
**Fig. 6.** Membrane potential-induced Dns-Gal fluorescence. Proteoliposomes containing purified L6XB permease were diluted 200-fold into a cuvette containing 50 mM NaPi (pH 7.5) and 5 mM Dns-Gal (final protein concentration, 5 \( \mu \)g/ml) (upper trace). Fluorescence at 500 nm was recorded (excitation at 340 nm), and at a given time, 20 \( \mu \)M valinomycin was added, thereby generating a membrane potential (interior negative). At about 100 sec, 20 \( \mu \)M CCCP was added to dissipate the membrane potential. In a control experiment, the proteoliposomes were diluted into 50 mM KPi (pH 7.5) (lower trace). cps, Counts per second.
acceptor domain fusion approach described by Cronan (14), we have purified milligram quantities of functional lactose permease by detergent extraction of membranes, followed by a single column step using immobilized monovalent avidin. This report describes two chimeric forms of lactose permease, L6XB and CXB, with factor Xa protease sites at the N termini of the biotin acceptor domains. Both constructs are fully active in cells and RSO membrane vesicles, biotinylated in vivo, and stable after insertion into the membrane. Moreover, L6XB permease catalyzes active transport after purification and reconstitution into proteoliposomes. Although data are not presented, the CXB permease has also been purified with the same protocol, and in this case, the biotin acceptor domain can be removed by treatment with factor Xa. A preliminary report (12) describing the efficacy of the general approach for spectroscopic experiments with single- or double-cysteine replacement mutants has been presented.

Although we have not intensively studied the yield of permease obtained from the purification, it is unlikely that maximal conditions have been achieved. As indicated, ~2 mg of purified L6XB permease was obtained from 20 g (wet weight) of cells. If the permease comprises about 10% of the total membrane protein under the conditions utilized, a yield of about 20% can be calculated (i.e., 20 g wet weight = 1 g of protein = 0.1 g of membrane protein = 0.01 g of permease). To some extent, the relatively low yield may be due to incomplete biotinylation. Consistently, L6XB permease in sonicated membrane preparations incorporates a significant amount of [14C]biotin when incubated in the presence of purified biotin ligase. Therefore, by increasing the extent of biotinylation in vivo or in vitro, higher yields may be obtained. In any case, ease of preparation, the high state of purity of the material obtained, and the ability to elute the protein in relatively high concentrations at specified protein/phospholipid ratios in any chosen detergent make the procedure highly attractive.

The observations that L6XB and CXB permeases are biotinylated and that the constructs are digested by factor Xa from only the cytoplasmic surface of the membrane are consistent with other experiments (1, 2, 7, 27) demonstrating that both the middle hydrophilic domain of the permease and the C terminus are on the cytoplasmic face of the membrane. The results suggest that the construction of chimeras with the biotin acceptor domain inserted into various hydrophilic domains might be useful for studying the topology of polytopic membrane proteins. However, additional experiments (K.H.Z., T.G.C., and H.R.K., unpublished work) indicate that this is probably not the case. Although the N terminus of the permease is on the cytoplasmic face of the membrane (1, 2, 7, 31), when the biotin acceptor domain is placed at the N terminus, the chimera is not biotinylated but active transport is observed in cells expressing the construct. Furthermore, constructs with the factor Xa site and the biotin acceptor domain in loop 3 or 7 [loops that are on the periplasmic face of the membrane (1, 2, 7, 31)] are biotinylated. However, both constructs are inactive, and the factor Xa sites are accessible from the cytoplasmic face of the membrane but not from the periplasmic face. Thus, it appears that the presence of the biotinylation domain may block insertion of portions of the polypeptide, a finding that may be related to the run of Ala-Pro repeats at the N terminus of the domain (32). Therefore, although insertion of biotin acceptor domains may not be useful for studying the topology of polytopic membrane proteins per se, the approach may yield interesting information regarding the mechanism of insertion.

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