In Vitro Biotinylation Provides Quantitative Recovery of Highly Purified Active Lactose Permease in a Single Step

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ABSTRACT: Consler et al. [Consler, T. G., Persson, B. L., et al. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90,* 6934−6938] described a one-step purification of lactose permease, a hydrophobic membrane transport protein, from *Escherichia coli.* Permease constructs containing a biotin acceptor domain are biotinylated in vivo, followed by solubilization and avidin affinity purification. Although a high degree of purity is obtained, only about 15−20% of the permease is recovered due to incomplete biotinylation. In this communication, a simple modification is described that allows quantitative recovery of highly purified permease. Membranes containing permease with the biotin acceptor domain from the *Klebsiella pneumoniae* oxaloacetate decarboxylase are extracted with 5 M urea or treated with dicyclohexylcarbodiimide to inactivate F_1/F_o ATPase and biotinylated in vitro with biotin ligase, ATP and *d*-biotin. Subsequently, the membranes are harvested, washed to remove free biotin and solubilized with 2% *n*-dodecyl-β-D-maltopyranoside. Biotinylated permease is then purified in one step by affinity chromatography on monomeric avidin−Sepharose. The purified material is homogeneous and exhibits full activity with respect to ligand binding and counterflow.

One of the most extensively studied secondary membrane transport proteins is the lactose permease (lac permease)[1] of *Escherichia coli* (reviewed in refs *1*−*3*). This hydrophobic integral membrane protein consisting of 417 amino acid residues catalyzes the coupled stoichiometric translocation of β-galactosides and H^+ (i.e., H^+/substrate symport), thereby converting the free energy stored in an electrochemical H^+ gradient ($\Delta\bar{\mu}_{H^+}$) into work in the form of a concentration gradient. The *lacY* gene which encodes the permease has been cloned (*4*) and sequenced (*5*), and the permease has been purified, reconstituted into proteoliposomes (reviewed in ref *6*), and shown to be solely responsible for β-galactoside transport as a monomer (*7*). All available evidence is consistent with a general secondary-structure model derived from hydropathy profiling (*8*) in which the permease contains 12 α-helical domains that traverse the membrane in zigzag fashion connected by hydrophilic loops with the N and C termini on the cytoplasmic surface of the membrane (reviewed in ref *1*).

Site-directed and Cys-scanning mutagenesis have allowed delineation of amino acid residues in the permease that are important for active transport and/or substrate binding (*3*, *9*−*11*). However, structural and dynamic information at high resolution are required to understand the role of these residues in the transport mechanism. Since hydrophobic membrane proteins are difficult to crystallize, a high-resolution structure of the permease is not available, and development of alternative methods for obtaining structural information is essential. In this respect, a helix packing model of the permease has been formulated (*9*, *10*). Proximity relationships are based on a battery of site-directed approaches, including excimer fluorescence (*12*, *13*), chemical cleavage (*14*), spin−spin interactions (*13*, *15*) engineered divalent metal binding sites (*16*−*19*), metal−spin label interactions (*20*−*22*), thiol-specific cross-linking (*23*−*27*), and identification of discontinuous mAb epitopes (*28*).

Many of the approaches used for obtaining structure/function data on the permease rely on a rapid, convenient method for obtaining highly purified wild-type permease, as well as a variety of site-directed mutants. For this purpose, lac permease has been constructed with a biotin acceptor domain (BAD) from the oxaloacetate decarboxylase of *Klebsiella pneumoniae* (*29*) inserted at either the C terminus (CXB) or in the middle cytoplasmic loop (L6XB) (*30*) (Figure 1). By this means, the permease is biotinylated in vivo and purified to a high state of purity by avidin affinity chromatography in a single step after solubilization. However, only 15−20% of the permease is biotinylated in vivo, resulting in considerable loss, and a similar situation has been described for a plant sucrose transporter expressed in yeast (*31*). Although metal−chelate chromatography can also be used to purify lac permease with six contiguous His residues at the C terminus, the method does not yield homogeneous preparations in a single step, necessitating further manipulation with consequent decreases in yield (*32*).

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[1] Abbreviations: Ab, antibody; AP, alkaline phosphatase; ATP, adenosine triphosphate; BAD, biotin acceptor domain; BirA, biotin ligase; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DM, *n*-dodecyl-β-D-maltopyranoside; DTT, dithiothreitol; ECF, enhanced chemifluorescence; IPTG, isopropyl 1-thio-β-D-galactopyranoside; lac permease, lactose permease; MIANS, 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid; NaDodSO$_4$−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride) membranes; TDG, β-D-galactopyranosyl-1-thio-β-D-galactopyranoside.

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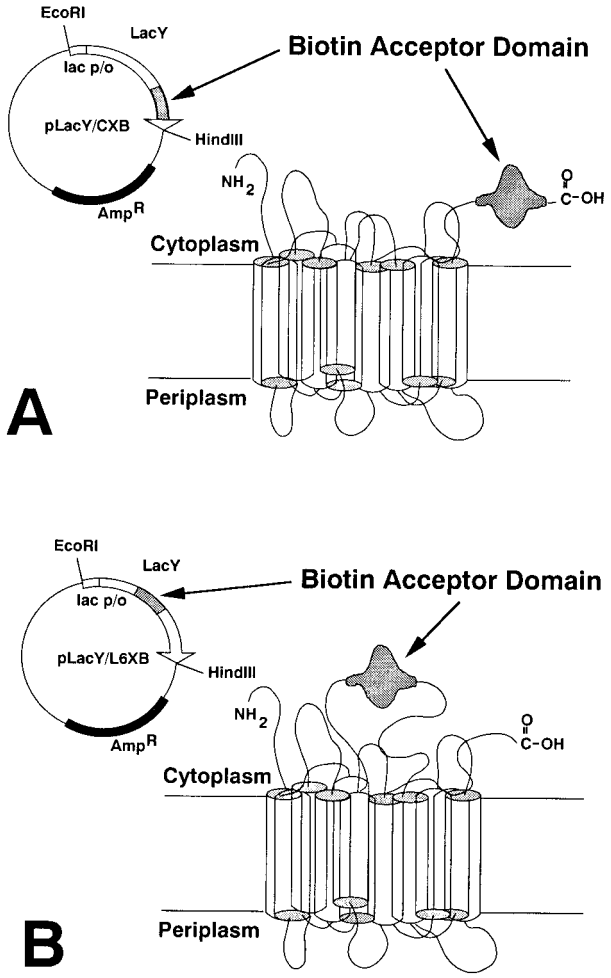

FIGURE 1: Schematic representation of plasmids encoding wild-type lac permease with the BAD from the oxaloacetate decarboxylase of *Klebsiella pneumoniae* inserted at the C-terminus (pLacY/CXB) (A) or in the middle cytoplasmic loop (pLacY/L6XB) (B) and schematic representations of the chimeric proteins. A factor Xa protease cut site is located upstream of the BAD (*30*), but is not shown.

Biotin ligase is a soluble enzyme that utilizes ATP to activate biotin (biotinyl-AMP) which is then transferred to the ϵ-amino group of a unique Lys residue (*33*) in the BAD of an acceptor protein (Figure 2) (reviewed in refs *34* and *35*). By using fusion constructs of soluble proteins with an engineered BAD, Cronan and co-workers (*29*, *36*) have shown that protein biotinylated in vivo can be purified in one-step by avidin affinity chromatography. In this paper, we describe a method for biotinylating lac permease in vitro that leads to quantitative recovery of highly purified material with full ligand binding and counterflow activity. An important aspect of the method involves inactivation of the membrane-bound F_1/F_o ATPase in order to allow biotinylation in situ.

EXPERIMENTAL PROCEDURES

Materials. Isopropyl 1-thio-β-D-galactopyranoside (IPTG) was purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Pefablock SC (AEBSF) was purchased from Boehringer GmbH (Mannheim, Germany). Avidin-conjugated alkaline phosphatase (avidin−AP), *N*,*N*′-dicyclohexylcarbodiimide (DCC), ATP, inorganic pyrophosphatase,

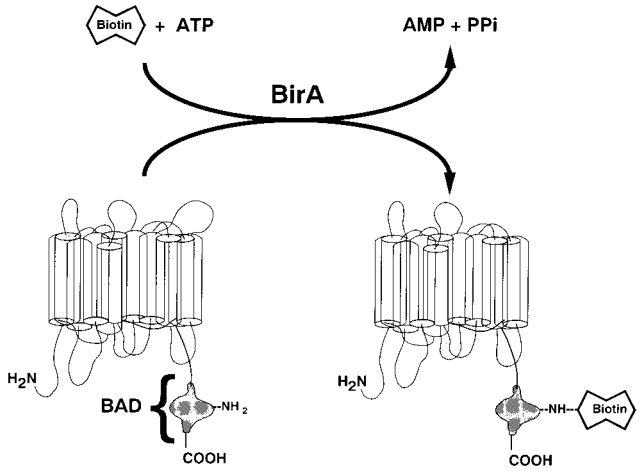

FIGURE 2: Schematic representation of the biotinylation reaction catalyzed by biotin ligase. BirA activates biotin with ATP forming biotinyl-5′-AMP (not shown). Biotin is then transferred to ϵ-amino group of a specific Lys residue located in the biotin acceptor domain at the C terminus of lac permease.

and *d*-biotin were from Sigma Chemicals (St. Louis, MO). Immobilized monomeric avidin was obtained from Pierce (Rockford, IL). Prestained molecular weight markers were from Bio-Rad (Richmond, CA). Poly(vinylidene difluoride) membranes (Immobilon-PVDF) were from Millipore (Bedford, MA). Site-directed rabbit polyclonal antiserum against a dodecapeptide corresponding to the C terminus of the permease (*37*) was prepared by BabCo (Richmond, CA). Anti-rabbit−alkaline phosphatase linked antibody and an Enhanced Chemifluorescence (ECF) detection kit were obtained from Amersham (Sunnyvale, CA). Valinomycin was obtained from Calbiochem (La Jolla, CA). Phosphatidylethanolamine (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine) and phosphatidylglycerol (1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]) were purchased from Avanti (Alabaster, AL). Sypro dyes were purchased from Molecular Probes (Eugene, OR). All other materials were reagent grade and obtained from commercial sources.

Bacterial Strains and Plasmids. E. coli T184 [*lacI*$^+$-*O*$^+$*Z*$^-$*Y*$^-$*(A), rpsL, met, thr, recA, hsdM, hsdR/F*′, lacI^q*O*$^+$*Z*^{D118}-*(Y*$^+$*A*$^+$*)*] (*38*) harboring plasmid pLacY/L6XB or pLacY/CXB (*30*) was used for expression and purification of lac permease (Figure 1). *E. coli* X90 [*araD(lac-pro), nalA argEam rif thi-1/F*′ lacI^q *lac*$^+$ *pro*$^+$] (*39*) harboring plasmid pJMR1 (*40*) was used for expression and purification of biotin ligase (BirA).

Growth of Cells and Preparation of Membranes. L6XB lac permease and CXB lac permease were expressed in *E. coli* T184 harboring plasmid pLacY/L6XB or pLacY/CXB, respectively. One liter of an overnight culture grown in Luria-Bertani (LB) broth with streptomycin (10 μg/mL) and ampicillin (100 μg/mL) was diluted into 11 L of LB broth and grown for 2 h at 37 °C. The culture (OD_{600} of 0.8−1.0) was then induced with 0.3 mM IPTG and grown for an additional 3 h at 37 °C. Cells were harvested by centrifugation (30 g wet weight) and crude membranes were prepared as described (*41*).

Where indicated, *E. coli* T184 harboring pLacY/C-H6 which encodes wild-type permease with six contiguous histidine residues at the C terminus was grown and treated as described (*32*).

BirA Extraction. The ammonium sulfate precipitate from cells expressing BirA was prepared as described (*40*) with the following modification: *E. coli* X90 harboring plasmid pJMR1 were disrupted by using a French pressure cell at 20 000 psi (*41*). The ammonium sulfate precipitate was pelleted as described (*40*), resuspended in 100 mM sodium phosphate (pH 6.5)/5% glycerol/1.0 mM DTT, dialyzed three times against the same buffer and once against 50 mM Tris·HCl (pH 7.5)/5% glycerol/0.1 mM DTT/200 mM KCl (storage buffer), concentrated, and stored at −70 °C in the presence of 0.5 mM Pefablock (protease inhibitor). Judging from Sypro-stained sodium dodecyl sulfate/12% polyacrylamide gel electrophoresis ($NaDodSO_4$−PAGE), with gels analyzed with a STORM 860 phosphoimager (Molecular Dynamics), the concentration of BirA in the preparation used was estimated to be approximately 1.2 mg/mL (32 μM).

In Vitro Biotinylation. Crude membranes from 30 g wet weight of cell pellet were resuspended in 15 mL of 100 mM KP_i (pH 7.5)/20 mM $MgSO_4$/20 mM β-mercaptoethanol/1 mM Pefablock. Fifteen milliters of 10 M urea was added to inactivate F_1/F_o ATPase (*42*), and the suspension was sonified on ice for 1 s on and 2 s off for 3 min with a probe sonicator. The suspension was left on ice for 30 min with continuous stirring and centrifuged at 160000g_{max} for 16 h. Alternatively, F_1/F_o ATPase was inactivated by incubating the membrane suspension with dicyclohexylcabodiimide (DCC) at a final concentration of 70 μM for 30 min at room temperature (*42*) prior to centrifugation. The membrane pellet was resuspended in 50 mM KP_i (pH 7.5)/2.5 mM $MgCl_2$/0.1 mM dithiothreitol (DTT)/0.5 mM Pefablock (biotinylation buffer). Inorganic pyrophosphatase (120 mg of protein/mL of stock solution), biotin (10 mM stock solution at pH 7.5), and ATP (200 mM stock solution, pH 7.5) were added to the membrane suspension to final concentrations of 0.9 mg/mL, 0.2 mM, and 3 mM, respectively. The suspension was then divided into two identical samples. Partially purified BirA was added to one sample at a final concentration of 2 μM, and both samples were then sonified on ice for 1 s on and 2 s off for 3 min with a tip sonicator and then incubated at 20 °C for 90 min, with continuous stirring. Aliquots (4 μL) were removed at given times, mixed with $NaDodSO_4$−PAGE sample buffer and frozen immediately in liquid N_2. The suspension was centrifuged at 200000g_{max} for 4 h, and the membrane pellet was resuspended in 50 mM KP_i (pH 7.5)/20 mM lactose/10 mM $MgSO_4$/0.5 mM Pefablock/10 mM β-mercaptoethanol.

Purification of Lac Permease. Membranes were solubilized with 2% (w/v) *n*-dodecyl-β,D-maltopyranoside (DM) by incubation at 4 °C for 30 min with continuous stirring. The sample was then centrifuged at 150000g_{max} for 1 h, the supernatant carefully decanted, and the pellet discarded. Purification of lac permease by immobilized monomeric avidin affinity chromatography was performed as described (*43*). Briefly, monovalent avidin−Sepharose was washed sequentially with 100 mM KP_i (pH 7.5)/150 mM NaCl/0.02% DM (column buffer). The DM-soluble fraction was mixed with preequilibrated avidin−Sepharose (0.1 mL of packed resin/mg of membrane protein) for 30 min at 4 °C with continuous rotation. The slurry was packed into a small column, and unbound material was removed by extensive washing with column buffer. Bound permease was eluted with 5.0 mM *d*-biotin in column buffer, dialyzed overnight against column buffer, and kept at 4 °C for immediate use or frozen in liquid N_2 and stored at −80 °C.

For purification of lac permease by metal chelate chromatography, the DM extract was mixed with 3.0 mL of Ni^{2+}-NTA-agarose (washed previously with 5 volumes of water) for 30 min at 4 °C with continuous rotation. The mixture was poured into a column, and unbound material was removed by washing with 100 mL of 50 mM KP_i (pH 7.5)/1% glycerol/0.008% DM followed by 100 mL of the same buffer containing 5 mM histidine. Bound permease was then eluted with 100 mM histidine in the same buffer and kept at 4 °C for immediate use or frozen in liquid N_2 and stored at −80 °C (*32*).

Electrophoresis. The purity of each preparation was assessed by $NaDodSO_4$−PAGE (*44*), followed by staining with either silver or Coomassie blue.

Western Blots. Membrane proteins separated by $NaDodSO_4$−PAGE (*44*) were electroblotted to poly(vinylidene difluoride) (PVDF) membranes and probed with a site-directed polyclonal antibody against the C-terminus of lac permease (*45*). The PVDF membrane was subsequently incubated with alkaline phosphatase-conjugated anti-rabbit antibody and finally developed with enhanced chemifluorescence (ECF) substrate. To quantitate the amount of biotinylated permease, PVDF membranes with electroblotted proteins were incubated with alkaline phosphatase-conjugated avidin (avidin−AP) and developed with ECF substrate. Quantitation was accomplished by using a STORM 860 Phosphoimager (Molecular Dynamics).

Fluorescence Measurements. Fluorescence was measured at 30 °C with an SLM 8000C spectrofluorometer (SLM-Aminco Instruments Inc., Urbana, IL), using 8- and 4-nm slits for excitation and emission, respectively. Purified Cys148 permease was preincubated for 5 min on ice with either 20 mM lactose, 20 mM sucrose, or 10 mM TDG, and the rate of labeling with 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid (MIANS) was determined by mixing 400 μL of protein (40 μg/mL) with 2 μL of 1 mM MIANS (*43*). Fluorescence emission was monitored continuously for 150 s at 415 nm, with excitation at 330 nm.

Reconstitution. Purified permease was reconstituted into proteoliposomes as previously (*46*). Briefly, purified permease in DM was added to 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (3:1, w/w) in 1% DM (w/w) to yield a phospholipid-to-protein ratio of 12:1. The sample was then incubated on ice for 15 min and rapidly diluted 180-fold into 50 mM KP_i (pH 7.5). The sample was stirred for 15 min at room temperature and centrifuged at 158000g_{max} for 4 h, the pellet was washed once in 50 mM KP_i (pH 7.5), and resuspended in a small volume of the same buffer.

Counterflow. Counterflow (*6*) was employed to measure transport activity. Proteoliposomes containing 330 μg of permease (lipid-to-protein ratio of 12:1 (w/w)) were prepared as described and resuspended in 200 μL of 50 mM KP_i (pH 7.5)/20 mM lactose/1 mM DTT/20 μM valinomycin and incubated 30 min on ice. After 5-s sonification, the suspension was diluted 1:10 into 50 mM KP_i (pH 7.5)/2 mM $MgSO_4$/1 mM DTT containing 0.6 mM [1-^{14}C]lactose (10 mCi/mmol) at room temperature. At given times, 100 μL of the above suspension was filtered through the center of a nitrocellulose filter (pore size, 0.45 μm; Sartorius) and

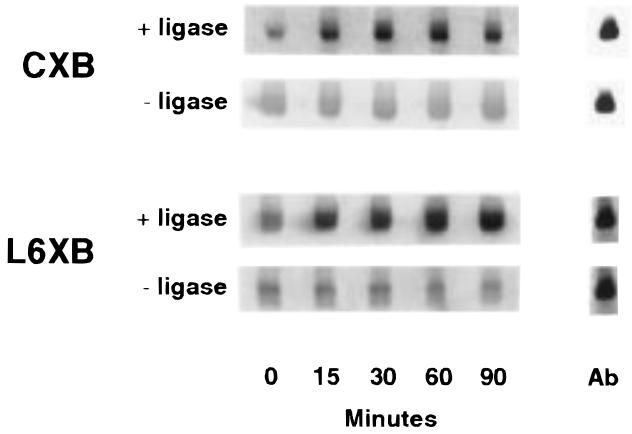

FIGURE 3: Time course of in vitro biotinylation of membrane embedded CXB or L6XB lac permease. Reactions were performed in the presence (upper line) or absence (lower line) of biotin ligase. Aliquots of 2 μL were taken at time points indicated (90 min for column designated Ab), immediately mixed with sample buffer and subjected to $NaDodSO_4$−PAGE and Western blotting. The blots were probed with avidin−AP or polyclonal antibody directed against the C terminus of lactose permease (Ab), and the complexes were visualized using ECF as the detection system. Immunoreactive material corresponding to lac permease with the BAD migrates as a broad band at an M_r of about 45 kDa.

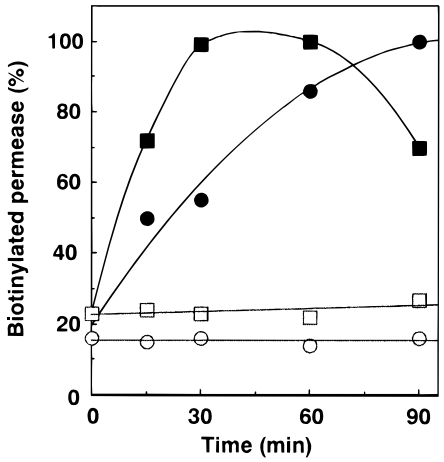

FIGURE 4: Time course of in vitro biotinylation of L6XB and CXB. The data shown in Figure 3 were quantitated by using a STORM 860 Phosphoimager (Molecular Dynamics) as described under Experimental Procedures: ■, membranes containing CXB mutant was incubated in the presence of biotin ligase; ●, membranes containing L6XB mutant was incubated in the presence of biotin ligase; □, membranes containing CXB mutant was incubated in the absence of biotin ligase; ○, membranes containing L6XB mutant was incubated in the absence of biotin ligase. The percentage of biotinylated permease in each band was calculated assuming that 100% of the CXB was biotinylated at 30 min and 100% of L6XB was biotinylated at 90 min. The assumptions are based on the observation that no lac permease is detected in the flow-through from the monovalent avidin affinity columns (see Figure 5). The decrease in the amount of biotinylated CXB at 90 min is very likely artifactual and was not seen in other experiments.

washed immediately with 5 mL of 100 mM KP_i (pH 5.5)/100 mM LiCl/10 mM $MgSO_4$. Controls were obtained by diluting the loaded proteoliposomes into the same solution containing 10 mM β-D-galactopyranosyl-1-thio-β-D-galactopyranoside (TDG).

Protein Determinations. Protein was assayed as described (*47*) using bovine serum albumin as standard. Standards included DM column buffer and 5 mM biotin when relevant.

RESULTS

In Vitro Biotinylation. The effect of incubating membrane suspensions containing L6XB permease or CXB permease but devoid of F_1/F_o ATPase activity with biotin ligase (BirA), ATP, and *d*-biotin was determined by probing Western blots with avidin−AP (Figure 3). ATP and biotin were added, the reaction mixture was divided into two samples, and BirA was added to one. Clearly there is a dramatic increase in the amount of biotinylated permease observed in the presence of BirA, relative to incubation in the absence of BirA (Figures 3), and the time course of the reaction appears to be somewhat faster with the CXB construct (Figure 4). In contrast, when the blots are probed with anti-C-terminal antibody (Figure 3; Ab), it is apparent that equal amounts of permease are present. Therefore, the increased intensity observed with avidin−AP can be attributed only to increased biotinylation. Importantly, unless the membrane-bound F_1/F_o ATPase is inactivated by urea extraction or treatment with DCC, only about a 2-fold increase in biotinylation is observed (data not shown).

Affinity Chromatography. Membrane suspensions containing the permease were incubated in the absence or presence of BirA and washed to remove excess free biotin. The pellets were resuspended and solubilized in DM, followed by affinity chromatography on immobilized monomeric avidin. Samples from the DM extract (Figure 5, lanes 1 and 4), the flowthrough from the avidin column (Figure 5, lanes 2 and 5) and the material eluted with *d*-biotin (Figure 5, lanes 3 and 6) were subjected to $NaDodSO_4$−PAGE and probed with anti-C-terminal antibody to estimate the amount of permease in each fraction. Comparable amounts of permease are evident in the starting material. In striking contrast, about a 7.5-fold increase (ca. 0.7 mg versus ca. 5.3 mg of protein; Table 1) is recovered in the fraction eluted with *d*-biotin. Moreover, a substantial amount of unbiotinylated permease is observed in the flowthrough from the sample incubated with BirA (lane 2) which is completely absent in the flowthrough from the sample incubated with BirA (lane 5). Thus, after biotinylation in vitro, essentially

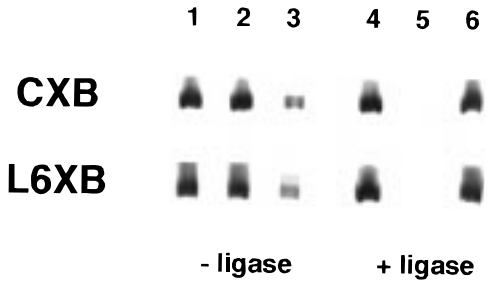

FIGURE 5: Effect of in vitro biotinylation on affinity purification of lac permease. Solubilized membrane protein containing CXB or L6XB permease was incubated in the absence (lanes 1, 2, and 3) or presence (lanes 4, 5, and 6) of 2 μM BirA as indicated under Experimental Procedures. The samples were then subjected to monomeric avidin affinity chromatography. Aliquots (1%) of the DM extract (lanes 1 and 4), the flowthrough (lanes 2 and 5), and the protein eluted with *d*-biotin (lanes 3 and 6) were subjected to SDS−PAGE and immunoblotting. The blots were probed with a polyclonal antibody directed against the C terminus of lactose permease, and the complexes were visualized using the ECF detection system as described under Experimental Procedures.

Table 1: Lactose Permease Purification by Monomeric Avidin Affinity Chromatography versus Metal Chelate Chromatography

	avidin (−BirA)	avidin (+BirA)	Ni^{2+}
initial membrane protein (mg)	55	55	55
total permease in 5 mL of eluant (mg)	0.7	5.3	3.1
purified permease per gram of cell pellet (μg)	40	300	200
% of total permease expressed	14	100[a]	66

[a] Based on absence of lactose permease inflowthrough fractions of treated samples (see Figure 5).

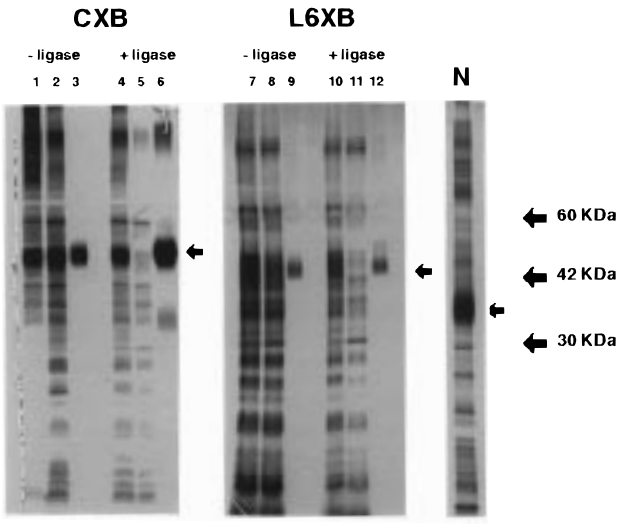

FIGURE 6: Effect of in vitro biotinylation on purification of L6XB and CXB lac permease. $NaDodSO_4$−polyacrylamide gel electrophoresis was performed as described under Experimental Procedures. The gels were stained with silver and the most significant bands visualized migrate at an approximate M_r of 45 KDa corresponding to the complexes detected in Figures 2 and 5. DM extracts containing 400 μg of total membrane protein were loaded on lanes 1, 4, 7, and 10. Aliquots from flowthroughs (350 μg protein) were loaded on lanes 2, 5, 8, and 11. Aliquots containing 200 μg of eluted protein were loaded on lanes 3, 6, 9, and 12. No contamination is observed in these lanes, except minor one in lane 6. Migration positions of prestained molecular weight markers are indicated. Lac permease with 6 contiguous His residues at the C terminus (CH6 lac permease) purified by metal chelate chromatography is shown for comparison (lane N). Due to the smaller size of the His-tag, CH6 lac permease migrates at an approximate M_r of 34 KDa. Small arrows designate the positions of lactose permease bands.

all of the permease is bound to the avidin affinity column and eluted quantitatively with *d*-biotin.

As shown by $NaDodSO_4$−PAGE and silver-staining, permease biotinylated in vivo (Figure 6, lanes 3 and 9) or in vitro (lanes 6 and 12) and eluted with *d*-biotin migrates as a single band with an M_r of about 45 kDa, a mobility identical to that observed on Western blots probed with avidin−AP or anti-C-terminal antibody. Moreover, permease subjected to purification by avidin affinity chromatography is significantly purer than permease purified by metal−chelate chromatography (compare lanes 6 and 12 with lane N).

Ligand Binding. As shown (*43*), site-directed fluorescence labeling with 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid (MIANS) is useful for measuring ligand binding to permease containing a single native-Cys residue at position 148. Therefore, single Cys148/L6XB permease was biotinylated in vitro, purified and reactivity with MIANS was

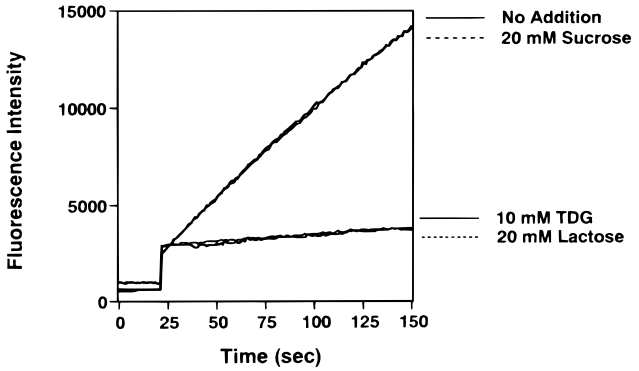

FIGURE 7: Effect of in vitro biotinylation MIANS reactivity of single Cys148 permease and protection by substrate. Single Cys148 permease was purified by avidin affinity chromatography after biotinylation in vitro and preincubated on ice with given sugars as described in Experimental Procedures. MIANS labeling was carried out with 0.4 mL of purified permease (40 μg of protein/mL, final concentration), and reactions were initiated by adding MIANS to a final concentration of 5 μM. Fluorescence was recorded continuously at 415 nm (excitation 330 nm) as described under Experimental Procedures. Where indicated the permease was incubated with 20 mM sucrose, 20 mM lactose, or 10 mM TDG.

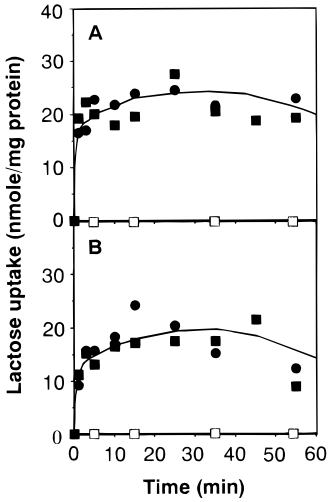

FIGURE 8: Counterflow activity of reconstituted CXB (A) or L6XB lac permease (B). Time courses of lactose counterflow in reconstituted proteoliposomes with a protein-to-lipid ratio of 1:12 (w/w) or 1:756 (mol/mol) were measured as described in Experimental Procedures: ■, proteoliposomes were prepared with in vitro biotinylated protein; ●, proteoliposomes were prepared with protein biotinylated in vivo only; □, counterflow activity in the presence of 10 mM TDG. The data were corrected for control samples that were boiled for 5 min prior to use.

measured in the absence or presence of different sugars (Figure 7). In the absence of sugar, Cys148 permease reacts rapidly with MIANS, as evidenced by the linear increase in fluorescence after addition of the probe. Strikingly, preincubation with β-D-galactopyranosyl-1-thio-β-D-galactopyranoside (TDG) or lactose completely blocks MIANS reactivity, while sucrose which is not a substrate for the permease has no effect whatsoever.

Counterflow. The data presented in Figure 8 show counterflow activity of lactose permease mutants CXB (panel A) and L6XB (panel B) biotinylated in vitro in comparison with a control sample biotinylated in vivo only. Both samples contain the same amount of purified permease and were reconstituted at the same protein-to-lipid ratio. Counterflow activity comparable to that observed previously (*46*)

is observed, and no significant differences are apparent between the two samples. Moreover, counterflow is completely blocked by the high affinity ligand TDG.

DISCUSSION

Cronan and co-workers (*29*) pioneered the use of soluble fusion proteins containing a BAD that can be biotinylated in vivo and in vitro for purification and to study conformational changes as a result of biotinylation (*36*). Subsequently, Consler et al. (*30*) demonstrated that lac permease fusions with the BAD in either the middle cytoplasmic loop (L6XB) or at the C terminus (CXB) catalyze active transport and become biotinylated in vivo. By utilizing this approach, biochemical quantities of functional lac permease have been purified by detergent extraction of membranes and affinity chromatography on immobilized monomeric avidin. This development is central to the application of a battery of biochemical and biophysical techniques to purified single and double-Cys mutants that have allowed the development of a helix packing model for the molecule, as well as a variety of structure/function studies which demonstrate that lac permease undergoes widespread conformational changes upon ligand binding (reviewed in refs *9* and *11*). Although in vivo biotinylation leads to material of high purity, the yield of purified permease is estimated to be only about 20% (*30*; G. G. Privé and G. E. Verner, unpublished information).

This report is the result of a systematic study following preliminary observations (*30*), indicating that L6XB permease in sonified membrane preparations incorporates a significant amount of [^{14}C]biotin when incubated with purified BirA. Western blots developed with avidin−AP demonstrate that 1 h incubation of sonified membranes with saturating concentrations of biotin, ATP and biotin ligase is sufficient to biotinylate the remaining 80% of the lactose permease that is unbiotinylated in vivo. Alternatively, in vitro biotinylation can be carried out with membranes solubilized with DM, as the detergent has little or no effect on BirA activity at concentrations that solubilize lac permease (data is not shown). However, under these conditions, free biotin must be removed by dialysis which is time-consuming relative to washing membranes free of biotin by centrifugation.

Since ATP is required for BirA-catalyzed biotinylation, significant ATPase activity decreases the yield of biotinylated permease. When the target protein is soluble, F_1/F_o ATPase is removed with the membranes by centrifugation after cell disruption (*36*). However, when the acceptor protein is a membrane protein, biotinylation in situ is relatively ineffective unless the membrane-bound F_1/F_o ATPase is inactivated by either urea extraction or treatment with DCC (*42*). With membranes containing lac permease, urea extraction is preferred, since a significant negative purification of the permease is accomplished in addition by this means (*44*).

Complete biotinylation of the permease results in a 7.5-fold increase in yield, from 40 μg to 300 μg per gram of cell pellet (Table 1), and the preparation exhibits a high state of purity, as evidenced by silver-stained protein gels. Importantly, the increase in yield is not associated with any loss in activity, as evidenced by substrate protection against MIANS labeling of purified single-cys148 permease and counterflow activity. In addition to providing a means to obtain higher yields of wild-type permease and mutants that are well expressed, it is apparent that in vitro biotinylation will be useful both for obtaining mutant permeases that are not expressed well and for identifying and purifying membrane proteins from pro- and eukaryotic cells that may contain low levels of ligase.

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REFERENCES

1. Kaback, H. R. (1992) in *International Review of Cytology* (Friedlander, M., and Mueckler, M., Eds.) pp 97−125, Academic Press, New York.
2. Kaback, H. R., Frillingos, S., Jung, H., Jung, K., Privé, G. G., Ujwal, M. L., Weitzman, C., Wu, J., and Zen, K. (1994) *J. Exp. Biol. 196*, 183−95.
3. Kaback, H. R. (1996) in *Handbook of Biological Physics: Transport Processes in Eukaryotic and Prokaryotic Organisms* (Konings, W. N., Kaback, H. R., and Lolkema, J. S., Eds.) pp 203−27, Elsevier, Amsterdam.
4. Teather, R. M., Müller-Hill, B., Abrutsch, U., Aichele, G., and Overath, P. (1978) *Mol. Gen. Genet. 159*, 239−48.
5. Buchel, D. E., Gronenborn, B., and Muller-Hill, B. (1980) *Nature 283*, 541−5.
6. Viitanen, P., Newman, M. J., Foster, D. L., Wilson, T. H., and Kaback, H. R. (1986) *Methods Enzymol. 125*, 429−52.
7. Sahin-Tóth, M., Lawrence, M. C., and Kaback, H. R. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 5421−5.
8. Foster, D. L., Boublik, M., and Kaback, H. R. (1983) *J. Biol. Chem. 258*, 31−4.
9. Kaback, H. R., Voss, J., and Wu, J. (1997) *Curr. Opin. Struct. Biol. 7*, 537−542.
10. Kaback, H. R., and Wu, J. (1997) *Q. Rev. Biophys. 30*, 333−364.
11. Frillingos, S., Sahin-Toth, M., Wu, J., and Kaback, H. R. (1998) *FASEB J.* (in press).
12. Jung, K., Jung, H., Wu, J., Privé, G. G., and Kaback, H. R. (1993) *Biochemistry 32*, 12273−8.
13. Wang, Q., Voss, J., Hubbell, W. L., and Kaback, H. R. (1998) *Biochemistry 37*, 4910−4915.
14. Wu, J., Perrin, D., Sigman, D., and Kaback, H. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 9186−9190.
15. Wu, J., Voss, J., Hubbell, W. L., and Kaback, H. R. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 10123−7.
16. Jung, K., Voss, J., He, M., Hubbell, W. L., and Kaback, H. R. (1995) *Biochemistry 34*, 6272−77.
17. He, M. M., Voss, J., Hubbell, W. L., and Kaback, H. R. (1995) *Biochemistry 34*, 15661−6.
18. He, M. M., Voss, J., Hubbell, W. L., and Kaback, H. R. (1995) *Biochemistry 34*, 15667−70.
19. He, M., Voss, J., Hubbell, W. L., and Kaback, H. R. (1997) *Biochemistry 36*, 13682−13687.
20. Voss, J., Hubbell, W. L., and Kaback, H. R. (1995) *Biophys. J. 68*, A423.
21. Voss, J., Salwinski, L., Kaback, H. R., and Hubbell, W. L. (1995) *Proc. Nat. Acad. Sci. U.S.A. 92*, 12295−12299.
22. Voss, J., Hubbell, W. L., and Kaback, H. R. (1997) *Biochemistry 37*, 211−216.
23. Wu, J., and Kaback, H. R. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 14498−502.
24. Wu, J., and Kaback, H. R. (1997) *J. Mol. Biol. 270*, 285−293.

25. Wu, J., Hardy, D., and Kaback, H. R. (1998) *J. Mol. Biol.* (in press).
26. Sun, J., and Kaback, H. R. (1997) *Biochemistry 36*, 11959−11965.
27. Sun, J., Kemp, C. R., and Kaback, H. R. (1998) *Biochemistry 37*, 8020−8026.
28. Sun, J., Li, J., Carrasco, N., and Kaback, H. R. (1997) *Biochemistry 36*, 274−80.
29. Cronan, J. E., Jr. (1990) *J. Biol. Chem. 265*, 10327−33.
30. Consler, T. G., Persson, B. L., Jung, H., Zen, K. H., Jung, K., Privé, G. G., Verner, G. E., and Kaback, H. R. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6934−8.
31. Stolz, J., Darnhofer-Demar, B., and Sauer, N. (1995) *FEBS Lett. 377*, 167−171.
32. Privé, G. G., and Kaback, H. R. (1996) *J. Bioenerg. Biomembr. 28*, 29−34.
33. Kaziro, Y., Leone, E., and Ochoa, S. (1960) *Proc. Natl. Acad. Sci. U.S.A. 46*, 1319−27.
34. Xu, Y., and Beckett, D. (1997) *Methods Enzymol. 279*, 405−21.
35. Beckett, D., and Matthews, B. W. (1997) *Methods Enzymol. 279*, 362−76.
36. Chapman-Smith, A., Turner, D. L., Cronan, J. E., Morris, T. W., and Wallace, J. C. (1994) *Biochem. J. 302*, 881−7.
37. Carrasco, N., Viitanen, P., Herzlinger, D., and Kaback, H. R. (1984) *Biochemistry 23*, 3681−7.
38. Teather, R. M., Bramhall, J., Riede, I., Wright, J. K., Furst, M., Aichele, G., Wilhelm, V., and Overath, P. (1980) *Eur. J. Biochem. 108*, 223−31.
39. Amman, E., Brosius, J., and Ptashne, M. (1983) *Gene 25*, 167−178.
40. Abbot, J., and Beckett, D. (1993) *Biochemistry 32*, 9649−9656.
41. Viitanen, P., Garcia, M. L., and Kaback, H. R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1629−33.
42. Patel, L., Schuldiner, S. and Kaback, H. R. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3387−91.
43. Wu, J., and Kaback, H. R. (1994) *Biochemistry 33*, 12166−71.
44. Newman, M. J., Foster, D. L., Wilson, T. H., and Kaback, H. R. (1981) *J. Biol. Chem. 256*, 11804−8.
45. Carrasco, N., Herzlinger, D., Mitchell, R., DeChiara, S., Danho, W., Gabriel, T. F., and Kaback, H. R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4672−6.
46. le Coutre, J., Narasimhan, L. R., Patel, C. K. and Kaback, H. R. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 10167−10171.
47. Peterson, G. L. (1977) *Anal. Biochem. 83*, 346−356.

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