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Biochemistry

Intramolecular dislocation of the COOH terminus of the *lac* carrier protein in reconstituted proteoliposomes

(*Escherichia coli*/site-directed polyclonal antibody/monoclonal antibody/active transport)

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ABSTRACT **A dodecapeptide corresponding to the carboxyl terminus of the *lac* carrier of *Escherichia coli* was synthesized, coupled to thyroglobulin, and the conjugate was used to generate site-directed polyclonal antibodies. The antibodies react with the carboxyl-terminal peptide and with the *lac* carrier protein, while monoclonal antibody 4B1 reacts with intact *lac* carrier protein, but not with the carboxyl-terminal peptide. Antibody 4B1 binds preferentially to right-side-out membrane vesicles relative to inside-out vesicles, confirming the presence of the 4B1 epitope on the periplasmic surface of the membrane. Alternatively, anti-carboxyl-terminal antibody binds preferentially to inside-out vesicles, demonstrating that the carboxyl terminus of the *lac* carrier protein is on the cytoplasmic surface. Surprisingly, both antibodies bind to proteoliposomes reconstituted with purified *lac* carrier protein, and quantitative binding assays indicate that the epitopes are equally accessible. When proteoliposomes containing purified *lac* carrier protein are digested with carboxypeptidases A and B, binding of anti-carboxyl-terminal antibodies decreases by >80%, while binding of antibody 4B1 and various transport activities remain essentially unchanged. It is suggested that during reconstitution, the *lac* carrier protein undergoes intramolecular dislocation of the carboxyl terminus with no significant effect on its catalytic activity.**

The *lac* carrier protein (i.e., *lac* permease) in *Escherichia coli* is an intrinsic membrane protein, the product of the *lacY* gene, that catalyzes the coupled translocation of β-galactosides with hydrogen ion in a symport reaction (cf. ref. 1 for a recent review). As such, this protein is representative of a large class of substrate-specific polypeptides that couple downhill movement of a cation to uphill transport of solute in response to a transmembrane electrochemical ion gradient.

lac permease has been purified to homogeneity in a functional state, and proteoliposomes reconstituted with a single polypeptide species catalyze all of the transport activities observed in intact cells and right-side-out (RSO) membrane vesicles with full efficiency (1–7). In addition, other similarities between native and purified *lac* carrier protein have been documented (6, 7), and it is evident from the findings as a whole that β-galactoside transport in *E. coli* requires a single gene product, that of the *lacY* gene.

The permease is a 46.5-kDa polypeptide containing 417 amino acid residues of known sequence (8). Based on circular dichroic measurements indicating that the protein has an exceptionally high helical content and based on analysis of the sequential hydropathic character of the protein, a secondary structure model has been proposed (9). The model suggests that the protein consists of 12 or 13 hydrophobic α-helical segments that traverse the membrane in a zigzag fashion connected by more hydrophilic loops. Accordingly, the model makes explicit predictions regarding those portions of the molecule that should be accessible to the solvent at the surfaces of the membrane. To test these predictions, monoclonal antibodies against purified *lac* carrier protein have been prepared and characterized (10–14), and more recently, peptides corresponding in sequence to various portions of the molecule have been synthesized and used to generate site-directed polyclonal antibodies (15, 16).

The combined results of these studies demonstrate that the *lac* carrier protein is inserted asymmetrically into the membrane. Thus, using RSO (17–21) and inside-out (ISO) (22–24) membrane vesicles, it has been shown that at least two monoclonal antibodies bind to independent epitopes in the *lac* carrier situated on the periplasmic surface of the membrane. In contrast, studies with site-directed polyclonal antibodies against the COOH terminus of the permease demonstrate that the ultimate COOH terminus is accessible from the cytoplasmic surface (15, 16). Finally, quantitative binding studies with a monoclonal antibody (4B1) that inhibits lactose/proton symport support the contention that purified *lac* carrier in reconstituted proteoliposomes probably has a conformation similar to that of the protein in the native membrane (12–14).

The findings presented here show unexpectedly that the COOH terminus of the *lac* permease in proteoliposomes is dislocated to the outer surface of the membrane with no effect on the disposition of the 4B1 epitope and no apparent effect on the catalytic activity of the protein.

MATERIALS AND METHODS

Growth of Cells and Preparation of Membrane Vesicles. *E. coli* ML 308-225 ($i^-z^-y^+a^+$) (17) and *E. coli* T206 (25), which carries the *lacY* gene on a recombinant DNA plasmid, were grown as described. RSO vesicles were prepared by osmotic lysis (17, 18), and ISO vesicles were prepared by passing cells through a French pressure cell at 5000 psi (24).

Purification and Reconstitution of *lac* Permease. *lac* permease was purified from T206 membranes and reconstituted into proteoliposomes with *E. coli* phospholipids by octyl-β-D-glucopyranoside (octylglucoside) dilution, followed by freeze–thaw/sonication (1–4).

Transport Assays. Carrier-mediated lactose efflux down a concentration gradient, equilibrium exchange, and membrane potential-driven lactose accumulation in proteoliposomes were determined as described (2–6).

Antibody 4B1. Antibody 4B1 and 4B1 Fab fragments were prepared and iodinated as described (10, 13, 14).

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Abbreviations: RSO, right-side-out; ISO, inside-out; SP-RIA, solid-phase radioimmunoassay; NPG, *p*-nitrophenyl-α-D-galactopyranoside.

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Synthesis of the COOH-Terminal Peptide of the *lac* Carrier Protein. A dodecapeptide corresponding to the COOH terminus of the *lac* carrier protein was synthesized in accordance with the amino acid sequence predicted from the nucleotide sequence of the *lacY* gene (H_2N-Leu-Ser-Leu-Leu-Arg-Arg-Gln-Val-Asn-Glu-Val-Ala-COOH) (8). Solid-phase synthesis was carried out (26), and the peptide was purified by high-performance liquid chromatography (27). The purity of the peptide was ascertained by hydrolysis in 6 M HCl at 110°C for 72 hr *in vacuo*, followed by amino acid analysis.

Conjugation of the COOH-Terminal Peptide to Thyroglobulin. One micromole of COOH-terminal peptide was dissolved in 500 μl of 0.1 M sodium phosphate (pH 7.2) containing 20 nmol of bovine thyroglobulin (Sigma); 22.5 μmol of glutaraldehyde (Sigma; grade I, 25%) was added and the mixture was incubated overnight at 4°C. Unreacted material was separated by gel filtration on Sephadex G-75. The final product had a molar ratio of COOH-terminal peptide to thyroglobulin of 50:1.

Antibody Production. Antibody was obtained by immunizing female New Zealand White rabbits with 0.65 mg of COOH-terminal peptide/thyroglobulin conjugate emulsified in 1 ml of complete Freund's adjuvant. The mixture was injected intradermally at ≈20 different sites, and the animals were given booster injections after 4–5 weeks by intradermal injection of the same amount of conjugate in incomplete Freund's adjuvant. Blood was drawn ≈10 days thereafter.

Purification of IgG and Preparation of Fab Fragments. IgG was purified from serum by affinity chromatography on protein A/Sepharose (28), followed by affinity chromatography on a column containing COOH-terminal peptide conjugated to Sepharose (29). To conjugate the COOH-terminal peptide, 3.4 μmol of peptide was dissolved in 300 μl of phosphate-buffered saline (pH 12.5), mixed with 240 μl of epoxy-activated Sepharose (Pharmacia), and the mixture was incubated for 20 hr at 37°C. Antiserum was diluted 1:5 with 0.1 M sodium phosphate (pH 8.1) and applied to the column, which was washed with the same buffer until no protein was detected in the eluant. Bound IgG was then eluted with 0.1 M sodium citrate (pH 3.5), adjusted to pH 7.4 with 1 M Tris·HCl (pH 9.5), concentrated by evaporation under vacuum, and dialyzed overnight against 1000 vol of 0.1 M potassium phosphate (pH 7.5) with 3 changes. Monovalent Fab fragments were prepared from purified IgG by papain digestion (30), and the Fc portion was removed by chromatography on protein A Sepharose (28). Protein was determined as described (31) with bovine serum albumin as standard, and the purity of the preparations was assessed by $NaDodSO_4$/polyacrylamide gel electrophoresis (32). Aliquots of purified IgG and Fab fragments were frozen and stored in liquid nitrogen.

Iodination. IgG and Fab fragments specific for the COOH-terminal dodecapeptide were iodinated while bound to COOH terminus/Sepharose affinity resin using chloramine-T. IgGs or Fab fragments (100 μg of protein) were incubated with 25 μl of resin in a Pasteur pipette and iodinated with 2 mCi of $Na^{125}I$ (300–600 mCi/ml; 1 Ci = 37 GBq; Amersham) (29, 33). Unbound material was washed through the resin with phosphate-buffered saline, and bound material was then eluted with 0.2 M glycine·HCl (pH 2.5) and adjusted immediately to pH 7.0 by addition of 0.2 M Tris·HCl (pH 8.6). The specific activities of the ^{125}I-labeled proteins ranged from 0.5 to 1.5 μCi per μg of protein, and >90% of the radioactivity was precipitated by trichloroacetic acid.

Miscellaneous Procedures. Binding of ^{125}I-labeled IgG and ^{125}I-labeled Fab fragments was assayed by filtration using 0.22-μm nitrocellulose filters (GSTF; Millipore) (14).

Solid-phase radioimmunoassays (SP-RIA) were carried out as described (10), except that 10 pmol of either purified *lac* carrier protein, COOH-terminal peptide, or proteoliposomes reconstituted with purified *lac* carrier protein were dried in the wells of polyvinylchloride plates.

Immunoblotting of purified *lac* carrier protein or membrane vesicles from $NaDodSO_4$/polyacrylamide gels to nitrocellulose (BA85; Schleicher & Schuell) was done electrophoretically for 12 hr at constant current (50 mA) (10), except that the buffer did not contain detergent.

Binding of *p*-nitro[2-^{3}H]phenyl-α-D-galactopyranoside (NPG) was measured under nonenergized conditions by flow dialysis (34).

Materials. [^{3}H]NPG was synthesized by Yu-Ying Liu (Isotope Synthesis Group, Hoffmann–La Roche) under the direction of Arnold Liebman. Carboxypeptidases A and B were purchased from Sigma, and camphorquinone was from Aldrich. All other materials were reagent grade obtained from commercial sources.

RESULTS

Properties of Site-Directed Polyclonal IgG Against the COOH Terminus of the *lac* Carrier. A dodecapeptide corresponding in sequence to amino acids 406 to 417 at the COOH terminus of the *lac* carrier protein (8) was synthesized, coupled to thyroglobulin, and used to produce site-directed polyclonal antibodies in a manner similar to that described by Lerner *et al.* (35). Crude antisera were tested for reactivity against the COOH-terminal peptide and against purified *lac* carrier protein by SP-RIA using ^{125}I-labeled protein A to detect bound IgG, and positive results were obtained in both instances (not shown). IgG was then purified by affinity chromatography on protein A Sepharose, and the anti-COOH-terminal-specific IgG was further purified by affinity chromatography on COOH terminus/Sepharose. The purified material was tested again for reactivity against the COOH-terminal peptide and against purified *lac* carrier protein by SP-RIA with ^{125}I-labeled protein A (Fig. 1). As shown, anti-COOH-terminal IgG reacts identically with the COOH-terminal peptide and intact *lac* permease. In contrast, monoclonal antibody 4B1 reacts with intact permease but not with the COOH-terminal peptide.

In the experiments presented in Fig. 2, membrane vesicles from *E. coli* T206, which contain amplified levels of *lac* permease, were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis, and the proteins were electroblotted onto nitrocellulose. The nitrocellulose was then incubated with affinity-purified anti-COOH-terminal IgG, followed by ^{125}I-labeled protein A (lane A), ^{125}I-labeled anti-COOH-terminal

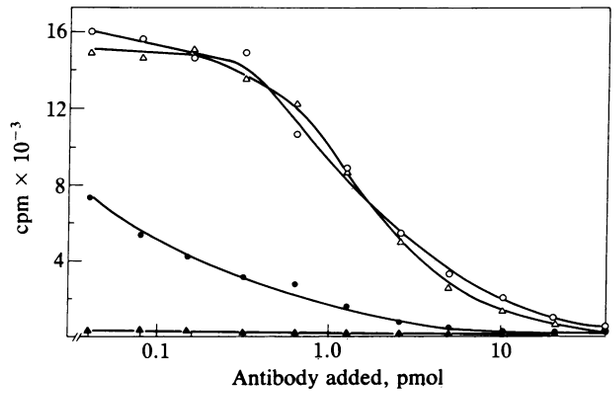

FIG. 1. Specificities of anti-COOH-terminal IgG and monoclonal antibody 4B1 for intact *lac* carrier protein and the COOH-terminal peptide. Proteoliposomes containing purified *lac* carrier protein (○, ●) or COOH-terminal peptide (△, ▲) were applied to the wells of a polyvinylchloride plate (10 pmol of protein or peptide per well). Immunoreactivity against purified anti-COOH-terminal IgG (○, △) and monoclonal antibody 4B1 (●, ▲) was assayed by SP-RIA using ^{125}I-labeled protein A as described (10).

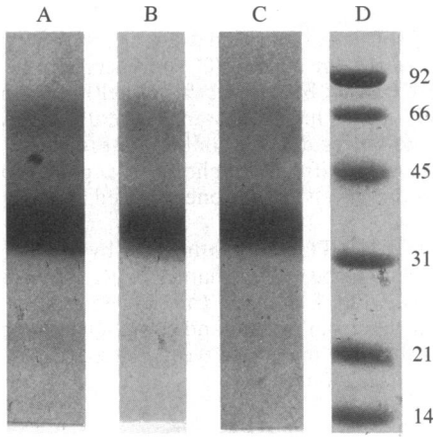

FIG. 2. Specificity of anti-COOH-terminal IgG and anti-COOH-terminal Fab fragments for the *lac* carrier protein. ISO membrane vesicles from *E. coli* T206 were extracted with 5 M urea, dissolved in $NaDodSO_4$, and electrophoresed in $NaDodSO_4$/12% polyacrylamide gels (25 μg of protein per lane). Proteins were then transferred to nitrocellulose by electroblotting. Individual strips were incubated with the following: lane A, 5 pmol of affinity-purified anti-COOH-terminal IgG, followed by ^{125}I-labeled protein A (*ca.* 2×10^5 cpm); lane B, ^{125}I-labeled anti-COOH-terminal IgG (*ca.* 10^5 cpm); lane C, ^{125}I-labeled anti-COOH-terminal Fab fragments (*ca.* 10^5 cpm). Incubation was carried out for 1 hr at room temperature as described (10), and autoradiography was carried out at −70°C overnight. Lane D, molecular size standards after $NaDodSO_4$/polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue (phosphorylase, 92.5 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa).

IgG (lane B), or ^{125}I-labeled anti-COOH-terminal Fab fragments (lane C). In each instance, positive reactions occur exclusively with bands that migrate at about 33 kDa and 65 kDa, although numerous bands are observed by Coomassie blue or silver staining (not shown). Since the 33-kDa band is the immediate product of the *lacY* gene and the 65-kDa band is an aggregate of *lac* permease (2, 10, 36), it is evident that anti-COOH-terminal IgG and its Fab fragments are highly specific.

Topology of the *lac* Permease in RSO and ISO Membrane Vesicles. Although the *lac* carrier protein catalyzes lactose/proton symport in both directions across the membrane (i.e., it is functionally symmetrical), the protein is inserted into the membrane asymmetrically, as evidenced by binding studies with monoclonal (10, 13, 14) and site-directed polyclonal IgGs (15, 16) in RSO and ISO membrane vesicles. Monoclonal antibodies 4B1 and 5F7 bind preferentially to nonoverlapping epitopes on the surface of spheroplasts and RSO membrane vesicles (i.e., the periplasmic surface) relative to ISO vesicles (i.e., the cytoplasmic surface). Alternatively, anti-COOH-terminal IgG binds preferentially to the surface of ISO vesicles relative to RSO vesicles. These conclusions are confirmed and extended by the results shown in Fig. 3. Clearly, ^{125}I-labeled 4B1 binds preferentially to RSO vesicles relative to ISO vesicles (Fig. 3*A*). Furthermore, binding saturates at about 10 pmol of IgG. Since intact 4B1 binds bivalently to 2 mol of *lac* carrier protein (13, 14), the membrane samples used in the titration contain ≈20 pmol of *lac* carrier, a quantity similar to that determined by [^{3}H]NPG binding (ref. 14; data not shown). On the other hand, ^{125}I-labeled anti-COOH-terminal IgG binds preferentially to ISO vesicles, but saturation also occurs at about 10 pmol (Fig. 3*B*). Thus, the COOH terminus of the *lac* carrier is on the opposite side of the membrane from the epitope for 4B1 (i.e., on the cytoplasmic surface, as opposed to the periplasmic surface). With monovalent Fab fragments from 4B1 and anti-COOH terminus, binding to RSO and ISO vesicles, respectively, saturates at ≈20 pmol, a value consistent with that observed for binding of the bivalent IgGs (Fig. 3*C*).

Topology of the *lac* Permease in Proteoliposomes. Proteoliposomes reconstituted with purified *lac* carrier catalyze all of the transport activities typical of the β-galactoside transport system in a manner comparable to RSO membrane vesicles (1–7). Furthermore, 4B1 IgG and 4B1 Fab fragments bind to the reconstituted system with stoichiometries essentially identical to those observed with RSO vesicles (12, 14). Based on these observations, it was concluded that the orientation of the permease in proteoliposomes is similar to that in the native membrane. Since the COOH terminus of the permease is clearly present on the cytoplasmic surface of the bacterial membrane, the side opposite from the epitope for 4B1, it was expected that proteoliposomes reconstituted with purified *lac* carrier would not bind anti-COOH-terminal

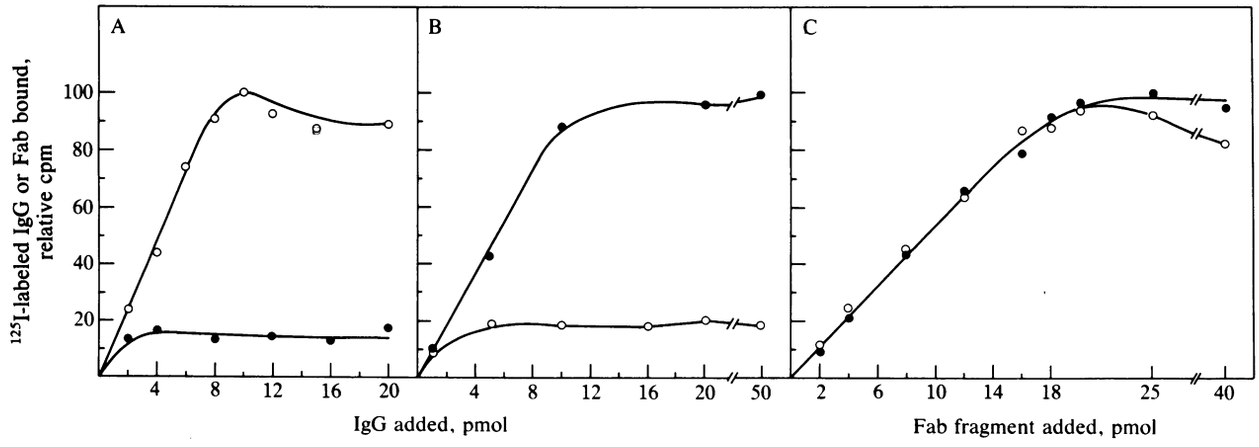

FIG. 3. Binding of 4B1 and anti-COOH-terminal IgGs and Fab fragments to RSO and ISO membrane vesicles. All manipulations were done at room temperature. RSO (○) and ISO (●) membrane vesicles prepared from *E. coli* T206 (18.6 μg of membrane protein per sample containing ≈20 pmol of *lac* carrier protein) were incubated in 50 mM potassium phosphate (pH 7.5) containing 5% bovine serum albumin for 1 hr. Given amounts of ^{125}I-labeled IgG or ^{125}I-labeled Fab fragments were added and the incubations were continued for 2 hr. The samples were then flocculated with poly-L-lysine, filtered, and assayed for bound radioactivity as described (14). Data were corrected for nonspecific binding, which was determined by incubating the vesicles with a 10-fold molar excess of the appropriate unlabeled IgG or Fab fragment relative to *lac* carrier. (*A*) ^{125}I-labeled 4B1; (*B*) ^{125}I-labeled anti-COOH-terminal IgG; (*C*) ^{125}I-labeled anti-COOH-terminal Fab fragments (●) or ^{125}I-labeled 4B1 Fab fragments (○) incubated with ISO and RSO vesicles, respectively.

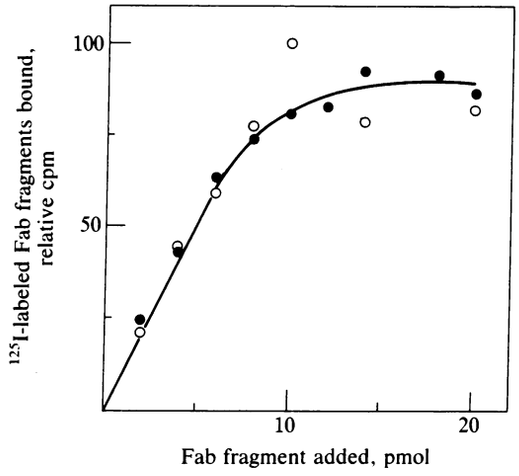

FIG. 4. Binding of anti-COOH-terminal Fab fragments and 4B1 Fab fragments to proteoliposomes reconstituted with purified *lac* carrier protein. Proteoliposomes containing 10 pmol of *lac* carrier protein per sample were assayed for binding of ^{125}I-labeled anti-COOH-terminal Fab fragments (●) or ^{125}I-labeled 4B1 Fab fragments (○) as described in Fig. 3 and in *Materials and Methods*.

IgG. Surprisingly, however, Fab fragments prepared from anti-COOH-terminal IgG bind to the reconstituted system (Fig. 4). Moreover, the proteoliposomes contain ≈10 pmol of permease per sample, and the binding of anti-COOH-terminal and 4B1 Fab fragments saturates at ≈10 pmol per sample. Thus, both epitopes appear to be quantitatively accessible from the external surface of the proteoliposomes.

Effect of Carboxypeptidases on IgG Binding and Transport Activity in Proteoliposomes Reconstituted with Purified *lac* Carrier. In view of the findings discussed above, it seems likely that the *lac* permease in the reconstituted system has undergone intramolecular dislocation of the COOH terminus with no significant effect on catalytic activity. To pursue this idea, proteoliposomes reconstituted with purified carrier were digested with carboxypeptidases A and B and subsequently assayed for binding of 4B1 and anti-COOH-terminal IgGs as well as transport activity (Fig. 5). Since binding of anti-COOH-terminal IgG decreases by >80% during the experiment [cf. Fig. 5 (*Inset*) in addition], it is evident that the COOH terminus of the *lac* carrier is accessible to the peptidases. In contrast, binding of 4B1 remains constant or increases slightly, and carrier-mediated lactose efflux is completely unaffected by digestion with the carboxypeptidases. Although not shown, it is also noteworthy that equilibrium exchange and lactose accumulation induced by an imposed potassium diffusion gradient ($K^+_{in} \rightarrow K^+_{out}$) in the presence of valinomycin are also completely unaffected by treatment of the proteoliposomes with carboxypeptidases A and B. Finally, none of the transport activities catalyzed by the permease is affected by anti-COOH-terminal IgG or Fab fragments.

DISCUSSION

Antibody 4B1 is the only one of >60 monoclonal antibodies tested that inhibits active transport of lactose (10–13), and the nature of the inhibition is unique. The antibody blocks only those reactions that involve net proton translocation with little or no effect on equilibrium exchange or on the ability of the carrier to bind substrate. In RSO vesicles, 4B1 binds with a stoichiometry of 1 mol of IgG per 2 mol of *lac* carrier protein, while 4B1 Fab fragments bind 1:1 (13, 14). Importantly, the intact antibody and its Fab fragments bind to proteoliposomes reconstituted with purified permease with stoichiometries virtually identical to those observed in

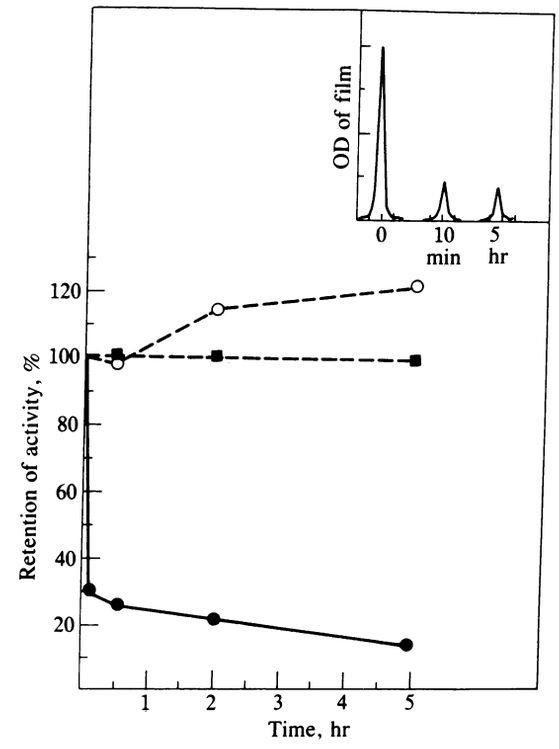

FIG. 5. Effect of carboxypeptidases A and B on binding of anti-COOH-terminal IgG, binding of 4B1 IgG, and carrier-mediated lactose efflux down a concentration gradient. Proteoliposomes (total vol, 320 μl) containing 80 μg of purified *lac* carrier protein per ml and 37.5 mg of *E. coli* phospholipid per ml in 50 mM potassium phosphate, pH 7.5/1 mM dithiothreitol were sonicated and loaded with 10 mM [1-^{14}C]lactose (11.8 mCi/mmol) by incubation at room temperature for 1 hr in the presence of 20 μM valinomycin (4). The sample was divided in half. To one aliquot, 10 and 5 units of carboxypeptidases A and B (in 10% LiCl), respectively, were added individually; to the other, an identical volume of 10% LiCl was added (8 μl). Incubation was continued at room temperature, and at the times indicated, samples were withdrawn and *o*-phenanthroline was added to 1 mM to inhibit the carboxypeptidases. Immunoreactivity against 4B1 IgG (○) and anti-COOH-terminal IgG (●) was then assayed by SP-RIA with ^{125}I-labeled protein A as described in Fig. 1 and in *Materials and Methods*, and rates of lactose efflux (■) were measured as described (4, 5). (*Inset*) In an independent experiment, proteoliposomes containing *lac* permease were treated with carboxypeptidases A and B for given times, as described above. Samples containing 5 pmol of permease were then immunoelectroblotted as described in Fig. 2. The autoradiogram was scanned using a Beckman DU-8 Spectrophotometer, and the tracings are shown.

RSO vesicles. With respect to the 4B1 epitope, therefore, it seems highly likely that the orientation of the *lac* carrier in the reconstituted system is similar to that in the bacterial cytoplasmic membrane.

Given the unique ability of antibody 4B1 to inhibit lactose/proton symport (10–13), its importance as a topological marker (12, 14), and the recent conjecture of Seckler *et al.* (15) that antibody 4B1 may be directed against the COOH terminus of the *lac* carrier because this portion of the molecule is immunodominant, it should be emphasized that the 4B1 epitope is completely unrelated to the COOH terminus. (*i*) Although both 4B1 and anti-COOH-terminal IgGs bind to proteoliposomes containing the *lac* carrier protein, only anti-COOH terminus binds to the COOH-terminal peptide itself, and no reactivity is observed with 4B1 (Fig. 1). (*ii*) Carboxypeptidase digestion of proteoliposomes reconstituted with purified *lac* carrier markedly decreases binding of anti-COOH-terminal IgG but has little effect on binding of 4B1 (Fig. 5). (*iii*) Since the COOH terminus contains two arginine

residues (8), both the COOH-terminal peptide and intact permease reacted with camphorquinone, a reagent that is relatively specific for arginine (37). As judged by SP-RIA, binding of anti-COOH-terminal IgG to the COOH-terminal peptide or intact *lac* carrier is diminished by ≈70% after reaction with camphorquinone, while binding of 4B1 is completely unaffected (data not shown).

As demonstrated previously (15, 16) and confirmed here, site-directed polyclonal antibodies against the ultimate COOH terminus of the *lac* carrier, in contradistinction to antibody 4B1, bind to ISO vesicles preferentially relative to RSO vesicles, thus localizing the COOH terminus of the permease to the cytoplasmic surface of the membrane. Surprisingly, however, anti-COOH-terminal Fab fragments not only bind to proteoliposomes containing the purified carrier, but they do so with essentially the same stoichiometry as 4B1 Fab fragments. Thus, the COOH terminus of the permease in the reconstituted system is on the same surface of the membrane as the 4B1 epitope (i.e., on the wrong surface of the proteoliposomes). In other words, although we have considered the possibility that a portion of the reconstituted carrier molecules might be scrambled, the data strongly indicate that the *lac* carrier undergoes intramolecular dislocation of the COOH terminus during reconstitution.

Remarkably, despite the COOH-terminal dislocation, reconstituted *lac* carrier exhibits full activity, as evidenced by many criteria. First, the reconstituted system catalyzes each translocation reaction characteristic of the β-galactoside transport system with turnover numbers and apparent K_m values comparable to those observed in RSO vesicles (1, 6). Second, detailed kinetic studies show that the reconstituted system exhibits properties analogous to those observed in RSO vesicles (6, 7). Finally, digestion of the COOH terminus with carboxypeptidases A and B has no demonstrable effect on any of the translocation reactions tested.

In conclusion, therefore, it seems reasonable to suggest that the functionality of reconstituted *lac* carrier is not an adequate criterion in and of itself to judge the fidelity of reconstitution. In addition, it seems apparent that localization of a single domain in this transmembrane protein does not constitute a sufficiently rigorous test for native conformation. The potential relevance of these conclusions to other reconstituted membrane proteins is self-evident. Finally, it should be mentioned that in addition to anti-COOH terminus, site-directed polyclonal antibodies against other portions of the *lac* carrier have been generated. Experiments with anti-loop 7 Fab fragments demonstrate that this portion of the permease is present on the cytoplasmic surface of the membrane but inaccessible in proteoliposomes, thus providing further evidence that a significant number of molecules are not scrambled in the reconstituted system.

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