Monoclonal Antibodies against the *lac* Carrier Protein from *Escherichia coli*. 2. Binding Studies with Membrane Vesicles and Proteoliposomes Reconstituted with Purified *lac* Carrier Protein†

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ABSTRACT: Monoclonal antibodies 4B1 and 5F7 bind to distinct, nonoverlapping epitopes in the *lac* carrier protein. By use of immunofluorescence microscopy and radiolabeled monoclonal antibodies and Fab fragments, it is shown that both 4B1 and 5F7 bind to spheroplasts and to right-side-out vesicles, but only to a small extent to inside-out vesicles. Clearly, therefore, the *lac* carrier protein has an asymmetric orientation within the cytoplasmic membrane of *Escherichia coli*, and both epitopes are located on the periplasmic surface. In right-side-out vesicles, radiolabeled 4B1 binds with a stoichiometry of 1 mol of antibody per 2 mol of *lac* carrier protein, while radiolabeled 4B1 Fab fragments bind 1:1. Importantly, the intact antibody and its Fab fragments bind to proteoliposomes reconstituted with purified *lac* carrier protein with a stoichiometry very similar to that observed in right-side-out membrane vesicles. Thus, it seems highly likely that the orientation of the *lac* carrier protein in the reconstituted system is similar to that in the bacterial cytoplasmic membrane, at least with respect to 4B1 epitope.

In the preceding paper (Carrasco et al., 1984a), an analysis of the functional properites of monoclonal antibody 4B1 (Carrasco et al., 1982) is presented. This IgG2a immunoglobulin is unusual in that it is the only monoclonal antibody of a large number tested that inhibits lactose transport. Furthermore, antibody 4B1 inhibits only those reactions involving net proton transport with little or no effect on equilibrium exchange, binding of a high-affinity ligand, or generation of the proton electrochemical gradient. Therefore, the epitope for 4B1 in the *lac* carrier protein represents an important site in the molecule. In this paper, we describe a parallel series of experiments in which the binding properties of radiolabeled 4B1 and 4B1 Fab fragments are studied.

Experimental Procedures

Materials

Unless specified otherwise, all materials were reagent grade and obtained from commercial sources.

Methods

Growth of Cells. Escherichia coli ML 308-225 ($i^-z^-{}^+a^+$) and ML 30 ($i^+z^+y^+a^+$) were grown on minimal medium A (Davis & Mingioli, 1959) containing 1% disodium succinate (hexahydrate). *E. coli* T206, which harbors a recombinant plasmid with the *lay y* gene, was grown and induced as described (Teather et al., 1980).

Preparation of Spheroplasts and Membrane Vesicles. Spheroplasts were prepared by a method based on those of Birdsell and Cota-Robels (1967) and Osborn et al. (1972) which ruptures the outer membrane, thereby exposing the cytoplasmic membrane to the medium. Cells (ca. 6 g wet weight) were resuspended in 100 mL of 10 mM Tris-HCl[1] (pH 8.0) containing 0.75 M sucrose and incubated at 20 °C for 7 min. Lysozyme was added to a final concentration of 0.1 mg/mL, and the incubation was continued for 5 min. The suspension was then diluted with 2 volumes of 1.5 mM ethylenediaminetetraacetate (K^+ salt) containing 0.1 mg/mL lysozyme and incubated at 20 °C for an additional 45 min. The spheroplasts were harvested by centrifugation ($10000g_{max}$) for 15 min and washed once in 100 mM potassium phosphate (pH 6.6) containing 0.5 M sucrose.

Right-side-out (RSO) membrane vesicles were prepared by osmotic lysis (Kaback, 1971; Short et al., 1975), and inside-out (ISO) vesicles were prepared by passage of cells through a French pressure cell at low shear forces (5000 psi; Reenstra et al., 1980).

Purification and Reconstitution of lac Carrier Protein. The *lac* carrier protein was purified from T206 membranes as described (Newman et al., 1981; Foster et al., 1982). Purified carrier was reconstituted into proteoliposomes by octyl β-D-glucopyranoside (octyl glucoside) dilution (Newman et al., 1981; Foster et al., 1982; Garcia et al., 1983).

p-Nitro[2-^{3}H]phenyl α-D-Galactopyranoside (NPG) Binding and Photoaffinity Labeling. In order to determine the amount of *lac* carrier protein present in RSO and ISO vesicles from *E. coli* ML 308-225 and T206, binding of [^{3}H]NPG was measured under nonenergized conditions by flow dialysis as described by Rudnick et al. (1976). Alternatively, photoaffinity labeling was carried out under anaerobic reducing conditions (Kaczorowski et al., 1980). By these means, the specific content of *lac* carrier protein was 0.43 nmol/mg of membrane protein and 1.22 nmol/mg of membrane protein, respectively, in the preparations of ML 308-225 and T206 membrane vesicles used in these experiments.[2] No significant difference was observed between RSO and ISO vesicles. Similar experiments were performed with proteoliposomes reconstituted with various amounts of *lac* carrier protein, and the results obtained from binding and photoaffinity assays yielded an excellent correlation with the amount of protein present as judged by an amido black determination of protein content (Newman et al., 1981).

Preparation of Immunological Reagents. Monoclonal antibodies and Fab fragments were prepared as described

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[1] Abbreviations: RSO, right-side-out; ISO, inside-out; octyl glucoside, octyl β-D-glucopyranoside; NPG, *p*-nitrophenyl α-D-galactopyranoside; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

[2] Although *E. coli* T206 vesicles can be prepared with 5–6 times the specific content of *lac* carrier protein as ML 308-225 vesicles (Teather et al., 1980; Patel et al., 1982), the T206 preparations used here contained only about a 3-fold excess.

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(Carrasco et al., 1982, 1984a).

Iodination. IgG and Fab fragment preparations were radiolabeled with ^{125}I by glucose oxidase/lactoperoxidase-catalyzed iodination utilizing Enzymobeads (Bio-Rad). Freshly purified protein (50–100 μg) in 75 μL of 200 mM potassium phosphate (pH 7.5) was mixed with 50 μL of Enzymobead reagent, 1 mCi of $Na^{125}I$ (Amersham; 300–600 mCi/mL), and 25 μL of 1% β-D-glucose, and the samples were incubated at 25 °C for 7 min. Reactions were terminated, and free iodide was separated from ^{125}I protein by passage over an 8-mL Sephadex G-25 column that had been equilibrated with 50 mM potassium phosphate (pH 7.5) containing 1% bovine serum albumin (BSA). The specific activities of the ^{125}I proteins ranged from 1 to 3 μCi/μg, and greater than 90% of the radioactivity was precipitated by trichloroacetic acid (Cl_3CCOOH). The specificity of the ^{125}I proteins for the *lac* carrier protein was routinely assayed by direct immunoblotting (Towbin et al., 1979; Carrasco et al., 1982). For binding assays, radiolabeled IgG and Fab fragments were diluted with unlabeled IgG or Fab fragments in 50 mM potassium phosphate (pH 7.5) containing 1% BSA to specific activities of approximately 0.05 μCi/μg.

Binding of ^{125}I-IgG and ^{125}I-Fab Fragments. RSO and ISO membrane vesicles were thawed rapidly and incubated in 50 mM potassium phosphate (pH 7.5) containing 5% BSA for 1 h at 25 °C. An aliquot of the vesicle suspension containing 46 μg of membrane protein was then incubated with a given amount of ^{125}I-IgG or ^{125}I-Fab fragments in a final volume of 50 μL for a given period of time at 25 °C. Vesicles were flocculated by addition of 1 mL of 50 mM potassium phosphate (pH 7.5) containing 250 μg of poly(L-lysine) (Sigma; M_r 140 000), and after 10 s, the samples were filtered through 0.22-μm nitrocellulose filters (GSTF; Millipore Filter Corp.) and washed twice with 3 mL of 50 mM potassium phosphate (pH 7.5) containing 5% BSA for 1 h at room temperature in order to reduce nonspecific binding. Radioactivity retained on the filters was assayed in a Beckman gamma 300 counter.

Binding studies with proteoliposomes were conducted as follows: Proteoliposomes containing 90–850 μg of purified *lac* carrier protein/mL and 37.5 mg of *E. coli* phospholipids/mL were thawed rapidly and diluted to a given protein concentration with 50 mM potassium phosphate (pH 7.5) containing 5% BSA. Following a 1–2-h incubation at room temperature, aliquots of the suspension (50 μL) were incubated with given amounts of ^{125}I-IgG or ^{125}I-Fab fragments for 1–2 h at 25 °C. The samples were then diluted with 3.5 mL of 50 mM potassium phosphate (pH 7.5) and immediately filtered under vacuum on 0.22-μm GSTF filters that had been treated with 5% BSA as described above. The filters were washed with another 3.5 mL of buffer, and the radioactivity retained was assayed in a Beckman gamma 300 counter.

Indirect Immunofluorescence Microscopy. Freshly prepared spheroplasts (0.5 mg of protein) were suspended in 100 mM potassium phosphate (pH 7.0) containing 0.5 M sucrose and 5% BSA and incubated at 25 °C for 1 h. Purified antibody (100 μg) was added to an approximate 30-fold molar excess relative to *lac* carrier protein, and the incubation was continued at 25 °C for 1 h with constant gentle agitation. Spheroplasts were harvested by centrifugation ($10000g_{max}$) for 15 min, washed once in 30 mL of 10 mM potassium phosphate (pH 7.0) containing 0.5 M sucrose, and resuspended in 400 μL of the same buffer. Fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG (Miles-Yeda) was added to a final concentration of 0.3 mg/mL, and the samples were incubated at 25 °C for 45 min. The spheroplasts were then harvested, washed, resuspended to 0.5 mL (final volume) as described above, and viewed with epifluorescent optics in a Leitz microscope.

Table I: Competitive Binding of Monoclonal Antibodies 4B1 and 5F7 to Proteoliposomes Reconstituted with Purified *lac* Carrier[a]

primary antibody incubation	IgG bound during secondary antibody incubation (%)	
	^{125}I-4B1	^{125}I-5F7
4B1		100
5F7	95	

[a] Proteoliposomes containing 90 μg of purified *lac* carrier protein and 37.5 mg of *E. coli* phospholipid/mL were thawed and incubated in 50 mM potassium phosphate (pH 7.5) containing 5% BSA for 1 h at room temperature. The samples were then diluted with 50 mM potassium phosphate (pH 7.5) containing 1% BSA and a 5-fold molar excess of unlabeled IgG relative to *lac* carrier protein (50 μL final volume containing 0.93 μg of *lac* carrier protein) and incubated at 25 °C for 2 h (primary antibody incubation). Saturating amounts of a given competing ^{125}I-labeled IgG were added, and the incubations were continued at 25 °C for another 2 h (secondary antibody incubation). Proteoliposomes were collected, washed by filtration, and assayed for bound radioactivity as described under Experimental Procedures. Nonspecific binding was determined by incubating the proteoliposomes in a 5-fold molar excess of unlabeled IgG prior to incubation with the same labeled antibody. Values for 100% binding were determined by incubation of proteoliposomes with saturating amounts of labeled antibody without a primary antibody incubation.

Results

Monoclonal Antibodies 4B1 and 5F7 Recognize Distinct Epitopes in the lac Carrier Protein. In this study, monoclonal antibodies are used to quantitate the amount of *lac* carrier protein in membrane vesicles and to assess the orientation of the *lac* carrier in vesicles and proteoliposomes reconstituted with purified carrier. Initially, immunocompetition experiments were performed to determine whether or not antibodies 4B1 and 5F7 recognize unique, nonoverlapping structural domains (i.e., epitopes) in the carrier. Thus, proteoliposomes reconstituted with purified *lac* carrier protein were preincubated with a 5-fold molar excess of a specified unlabeled IgG and subsequently exposed to ^{125}I-labeled 4B1 or 5F7 at concentrations stoichiometric to the amount of *lac* carrier protein present (Table I). To establish values for 100% binding, preincubation with unlabeled IgG was omitted, and the proteoliposomes were incubated with ^{125}I-IgGs only. As controls for background binding, proteoliposomes were incubated with an excess of the same unlabeled IgG as that used in the subsequent incubation with labeled IgGs. Clearly, neither of the monoclonal antibodies used in the initial incubation inhibits the binding of the second ^{125}I-IgG, demonstrating that antibodies 4B1 and 5F7 recognize distinct epitopes in the *lac* carrier protein. Competitive binding experiments were also carried out with RSO membrane vesicles, and no immunocompetition between 4B1 and 5F7 was observed (data not shown).

Binding of Antibodies 4B1 and 5F7 to Spheroplasts As Determined by Immunofluorescence Microscopy. Binding of antibodies 4B1 and 5F7 to the periplasmic surface of the cell membrane was determined qualitatively by immunofluorescence microscopy with spheroplasts prepared from *E. coli* ML 308-225. Freshly prepared spheroplasts were incubated successively with excess monoclonal antibody and FITC-labeled goat anti-mouse IgG as described. Binding of antibody 4B1 is readily detected by this means (Figure 1B), although the assertion is not easily documented. That is, all of the spheroplasts visualized by phase contrast microscopy (Figure 1A) do not appear to be fluorescent (Figure 1B); however, the

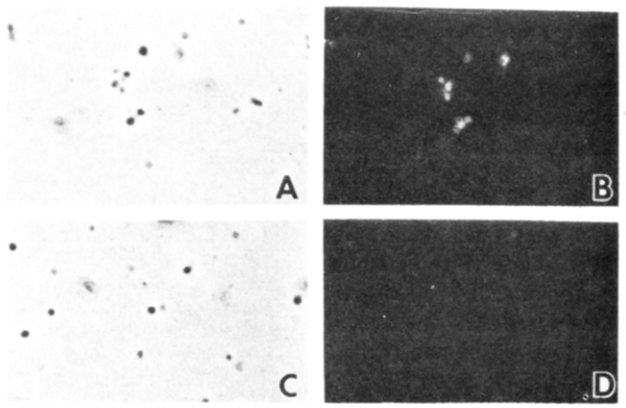

FIGURE 1: Visualization of monoclonal antibody binding to spheroplasts by indirect immunofluorescence microscopy. The photomicrograhs shown were obtained with antibody 4B1 and spheroplasts freshly prepared from *E. coli* ML 308-225 (panel A and B) and uninduced ML 30 (panel C and D) as described under Experimental Procedures by using FITC–conjugated goat anti-mouse IgG to visualize bound antibody. Panels A and C are phase contrast micrographs; panel B and D are corresponding fluorescence micrographs. Magnification is 550×. Similar results were obtained with monoclonal antibody 5F7 (not shown).

apparent heterogeneity of staining is due to the location of spheroplasts in different focal planes. Thus, all of the spheroplasts are fluorescent when the preparation is examined through focus, and similar results are obtained with antibody 5F7 (not shown). On the other hand, little, if any, fluorescent staining is observed when spheroplasts prepared from uninduced *E. coli* ML 30, which do not express the *lac* carrier protein, are incubated with 4B1 or 5F7 prior to FITC–goat antimouse IgG (Figure 1C,D). Similarly, no significant staining is observed when ML 308-225 spheroplasts are incubated with a commercially available mouse IgG2a, followed by FITC–goat antimouse IgG (not shown). The results suggest that antibodies 4B1 and 5F7 recognize periplasmically disposed epitopes in the *lac* carrier protein.

Direct Binding Assays. In order to quantitate binding of 4B1 and 5F7 to the *lac* carrier protein in a membranous environment, a rapid filtration assay employing ^{125}I-labeled IgG was developed that includes the following features: (i) "blocking" nonspecific interactions of the ^{125}I-IgG with BSA; (ii) titration with ^{125}I-labeled monoclonal antibodies; (iii) separation of bound and free ^{125}I-labeled antibody by filtration.

The data presented in Figure 2 were obtained with proteoliposomes containing three different amounts of purified *lac* carrier protein and the same preparation of ^{125}I-4B1. With each sample of proteoliposomes, binding of ^{125}I-4B1 exhibits a maximum at a concentration of antibody that is proportional to the amount of *lac* carrier protein present (arrows) and then decreases slightly as increasing amounts of ^{125}I-4B1 at constant specific activity are added. In the experiments shown, 20.9, 10.5, and 5.2 pmol of *lac* carrier protein, respectively, bind about 10, 5, and 2.5 pmol of bivalent 4B1 IgG at saturation (arrows).

The efficacy of the technique and the unusual shape of the binding curves are related to two factors: (i) the high affinity of 4B1 for its epitope (the K_d is less than 10^{-8} M; unpublished observations) and (ii) the somewhat lower affinity of the iodinated antibody relative to that of the unmodified molecule. In the presence of excess antigen (i.e., *lac* carrier protein), labeled antibody and unlabeled antibody bind independently until all of the binding sites are occupied (arrows). Further addition of antibody past saturation results in a "paradoxical" decrease in binding which is presumably due to competition between labeled and unlabeled antibody molecules for a limited

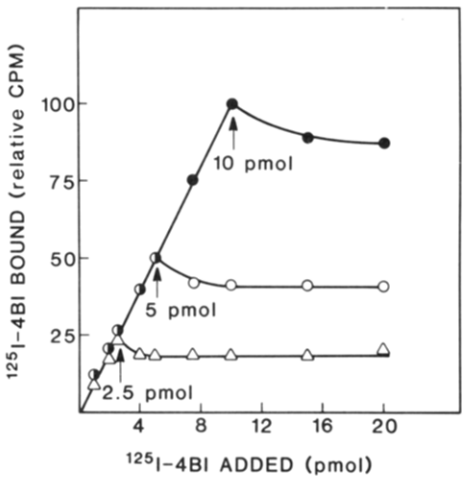

FIGURE 2: Binding of ^{125}I-4B1 to proteoliposomes reconstituted with purified *lac* carrier protein. Proteoliposomes containing 20.9 (●), 10.5 (○), or 5.2 (△) pmol of *lac* carrier protein/sample were assayed for binding of ^{125}I-4B1 as described under Experimental Procedures. Values given were corrected for nonspecific binding which was determined by incubation of proteoliposomes in a 10-fold molar excess of unlabeled 4B1 relative to *lac* carrier protein prior to incubation with ^{125}I-4B1.

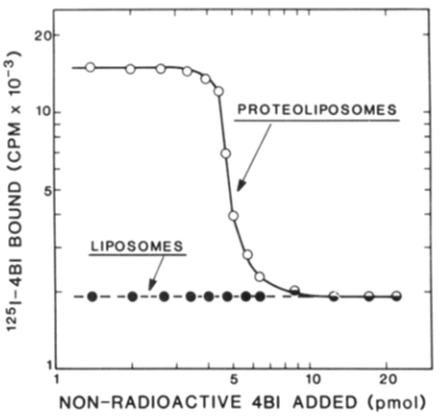

FIGURE 3: Effect of unlabeled 4B1 on binding of ^{125}I-4B1. Proteoliposomes containing 10.5 pmol of purified *lac* carrier protein and 200 μg of *E. coli* phospholipid/sample (○) were incubated with given amounts of unlabeled antibody 4B1 (50 μL final volume). After a 2-h incubation at 25 °C, a trace amount of ^{125}I-4B1 was added about 0.15 pmol), and incubation was continued for 1 h, at which time the samples were filtered, washed, and assayed for bound radioactivity as described under Experimental Procedures. Identical experiments were carried out with liposomes (200 μg of phospholipid/sample) formed from *E. coli* phospholipids in the absence of protein (●).

number of binding sites. The suggestion is supported by the observation that the maximum level of ^{125}I-4B1 binding occurs at a concentration that consistently exhibits the same propotionality to the amount of *lac* carrier protein (i.e., at the maximum, the stoichiometry between bound 4B1 and *lac* carrier protein is 0.5 consistently), whereas the extent of paradoxical binding varies from one preparation of ^{125}I-4B1 to another.

In order to pursue this interpretation and further document the validity of the binding assays, an independent experimental design was employed (Figure 3; note the log/log plot). Proteoliposomes containing 10.5 pmol of purified *lac* carrier protein were preincubated with increasing amounts of unlabeled 4B1, and subsequently, a trace amount of ^{125}I-4B1 was added. As shown, binding of ^{125}I-4B1 is not affected by preincubation with up to 4.6 pmol of unlabeled 4B1 but decreases precipitously between 4.6 and 5.8 pmol, approaching the level observed for nonspecific binding (i.e., liposomes prepared in the absence of *lac* carrier protein; Figure 3, broken

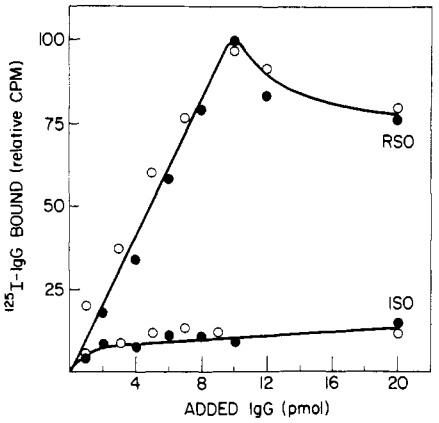

FIGURE 4: Topological distribution of the epitopes for 4B1 and 5F7 on the periplasmic and cytoplasmic surfaces of the membrane. Right-side-out (RSO) and inside-out (ISO) membrane vesicles prepared from *E. coli* ML 308-225 (46 μg of membrane protein/sample containing approximately 20 pmol of *lac* carrier protein) were incubated in 50 mM potassium phosphate (pH 7.5) containing 5% BSA for 1 h at 25 °C. Given amounts of ^{125}I-4B1 (○) or ^{125}I-5F7 (●) were added, and the incubations were continued for 2 h at 25 °C. Samples were then flocculated with poly(L-lysine), filtered, and assayed for bound radioactivity as described under Experimental Procedures. The data shown were corrected for nonspecific binding which was determined by incubation of vesicles with a 10-fold molar excess of the appropriate unlabeled IgG (relative to *lac* carrier protein) prior to incubation with ^{125}I-4B1 or ^{125}I-5F7.

line). Therefore, exposure of 10.5 pmol of *lac* carrier protein to 4.6–5.8 pmol of relatively high-affinity unlabeled 4B1 is sufficient to saturate the epitope, thus blocking subsequent binding of the labeled probe which has a lower affinity.

Topological Localization of 4B1 and 5F7 Epitopes. Osmotically intact membrane vesicles that have either the same polarity (RSO; Kaback, 1971, 1974; Short et al., 1975; Owen & Kaback, 1978, 1979a,b) or the opposite polarity (ISO; Hertzberg & Hinkle, 1974; Rosen & McClees, 1974; Reenstra et al., 1980) as the intact cell membrane are readily prepared from *E. coli*. When binding of radiolabeled monoclonal antibodies in such preparations is studied, the distribution of epitopes in the *lac* carrier protein on the periplasmic and cytoplasmic surfaces of the membrane can be determined. Since RSO and ISO vesicles are prepared by different techniques (i.e., osmotic lysis vs. shearing at low pressure), binding (Rudnick et al., 1976) and photoaffinity-labeling experiments (Kaczorowski et al., 1980) with [^{3}H]NPG were performed initially in order to determine the specific content of *lac* carrier protein in the two preparations. Although data are not shown, the amount of *lac* carier relative to membrane protein is essentially identical.

When RSO vesicles (46 μg of membrane protein containing about 20 pmol of *lac* carrier) are exposed to given amounts of radiolabeled 4B1 or 5F7, the general shape of the binding isotherm is similar to that observed with proteoliposomes reconstituted with purified *lac* carrier protein (Figure 4). Binding increases linearly below saturation, achieves a maximum at about 10 pmol of antibody, and then decreases gradually. Importantly, below saturation, specific binding to the *lac* carrier protein represents about 50–75% of input radioactivity, indicating that at least half of the antibody molecules are functional after purification and iodination. Controls for these experiments, which include incubation of radiolabeled antibodies with uninduced ML 30 vesicles and incubation of ML 308-225 vesicles with a 10–20-fold excess of unlabeled antibody prior to addition of labeled antibody, bind only about 5% of input radioactivity.

Dramatically, binding of 4B1 or 5F7 to ISO vesicles con-

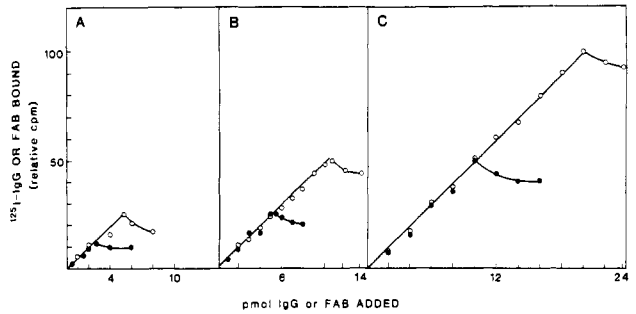

FIGURE 5: Binding of 4B1 IgG and Fab fragments to RSO membrane vesicles and proteoliposomes reconstituted with purified *lac* carrier protein. RSO membrane vesicles prepared from *E. coli* ML 308-225 (panel A) and *E. coli* T206 (panel B) and proteoliposomes reconstituted with purified *lac* carrier protein (panel C) were incubated in 50 mM potassium phosphate (pH 7.5) containing 5% BSA for 1 h at 25 °C. Aliquots (50 μL) of each suspension containing 5 (11.5 μg of ML 308-225 membrane protein), 10 (8 μg of T206 membrane protein), and 20 pmol of *lac* carrier protein, respectively, were then incubated with given amounts of ^{125}I-4B1 IgG (●) or ^{125}I-4B1 Fab fragments (○) for 2 h at 25 °C. The samples were filtered and assayed for bound radioactivity as described under Experimental Procedures. The data shown were corrected for nonspecific binding by incubation of each sample with a 10-fold molar excess of the appropriate unlabeled immunological reagent (relative to *lac* carrier protein) prior to incubation with ^{125}I-4B1 IgG or ^{125}I-4B1 Fab fragments.

taining the same amount of *lac* carrier protein exhibits saturation at only 10–20% of the value observed with RSO vesicles (i.e., 1–2 pmol of IgG; Figure 4). Additionally, antibody 4B1, which inhibits lactose efflux in RSO vesicles (Carrasco et al., 1984a), has no effect whatsoever on efflux in ISO vesicles (data not shown). The experiments, taken together, confirm the periplasmic localization of the epitopes for antibodies 4B1 and 5F7, as determined by immunofluorescence microscopy, and provide strong evidence for the contention (Carrasco et al., 1982; Foster et al., 1983; Goldkorn et al., 1983; Kaback, 1983; Seckler et al., 1983) that the *lac* carrier protein is asymmetrically oriented within the membrane.

Binding of Intact Antibody vs. Fab Fragments. Evidence presented in the preceding paper (Carrasco et al., 1984a) indicates that intact 4B1 molecules inhibit efflux from proteoliposomes about twice as effectively as 4B1 Fab fragments on a mole for mole basis relative to the amount of *lac* carrier protein. Thus, comparative binding assays were carried out with intact 4B1 and with Fab fragments prepared from 4B1 by papain digestion (Figure 5). As shown, with ML 308-225 membrane vesicles (panel A), T206 membrane vesicles[2] (panel B), and proteoliposomes reconstituted with purified *lac* carrier protein (panel C) containing about 5, 10, and 20 pmol of *lac* carrier protein/sample, respectively, binding of 4B1 Fab fragments saturates at a value about twice that observed with intact 4B1. Furthermore, it is apparent that binding of 4B1 Fab fragments at saturation quantitatively reflects the amount of *lac* carrier protein in the preparations. Parenthetically, it is also noteworthy that 4B1 Fab fragments do not bind significantly to ISO vesicles prepared from either ML 308-225 or T206 (data not shown).

Discussion

As shown previously (Carrasco et al., 1982, 1984a), 4B1 is the only antibody of a large number of monoclonal antibodies directed against purified *lac* carrier protein that inhibits transport. Furthermore, the antibody appears to inhibit in a highly unique manner in that it blocks those translocation reactions involving proton/lactose symport with little or no effect on exchange, generation of the proton electrochemical gradient, or binding of NPG. The studies presented here

utilize radiolabeled antibody 4B1 and antibody 5F7, a noninhibiting antibody from the same fusion (Carrasco et al., 1982), to characterize the binding properties of these antibodies in RSO and ISO membrane vesicles and proteoliposomes reconstituted with purified *lac* carrier protein.

Competitive binding experiments provide a clear demonstration that antibodies 4B1 and 5F7 bind to specific, nonoverlapping structural domains in the *lac* carrier protein. Therefore, both antibody molecules can bind simultaneously to the *lac* carrier protein, a finding that is somewhat surprising given the mass of the IgG molecule (150 kdaltons) relative to that of the carrier (46.5 kdaltons) and the putative secondary structure of the latter which indicates that as much as 85% of the carrier may be embedded in the membrane (Foster et al., 1983). In other words, some steric inhibition at least might be expected. On the other hand, given the elongated nature of the IgG molecule and the terminal location of the antigen combining sites, considerable distortion might be possible, thus allowing simultaneous occupation of more than one epitope within a relatively small surface area.

Initial experiments with 4B1 demonstrating that inhibition of active transport in RSO vesicles occurs within seconds provided a preliminary indication that the epitope for 4B1 is present in a periplasmic site within the *lac* carrier protein (Carrasco et al., 1982). The present studies utilizing immunofluorescence microscopy and radiolabeled monoclonal antibodies and Fab fragments demonstrate that both 4B1 and 5F7 bind to spheroplasts and to RSO membrane vesicles, but only to a small extent to ISO vesicles. It is apparent, therefore, that the *lac* carrier protein has an asymmetric orientation within the membrane, despite its ability to catalyze lactose/proton symport in both directions across the membrane. It is also noteworthy that this conclusion rules out oligomeric models of the *lac* carrier protein in which the subunits have an antiparallel orientation.

Preliminary studies in the preceding paper (Carrasco et al., 1984a) led to the suggestion that intact antibody 4B1 binds the *lac* carrier protein with a stoichiometry of 1:2 while the monovalent Fab fragments bind 1:1. This conclusion receives strong support from the binding studies presented in this paper (cf. Figure 5). With RSO vesicle from *E. coli* ML 308-225 and T206, as well as proteoliposomes reconstituted with purified *lac* carrier protein, radiolabeled 4B1 binds with a stoichiometry of 1 mol of antibody per 2 mol of *lac* carrier protein, while radiolabeled 4B1 Fab fragments bind 1:1. On a superficial level, it might be concluded that the observations favor the notion that the *lac* carrier protein may have a dimeric structure in the membrane under certain conditions (Kaback, 1983; Goldkorn et al., 1984). In this context, however, it must be emphasized that antibody-induced dimerization is a possibility that cannot be ruled out. In any event, it is evident that binding studies with radiolabeled Fab fragments, in particular, provide an excellent quantitative measure of the amount of *lac* carrier protein in the membrane.

Proteoliposomes reconstituted with purified *lac* carrier protein catalyze all of the transport activities typical of RSO vesicles (Newman et al., 1981; Foster et al., 1982; Garcia et al., 1983; Viitanen et al., 1983, 1984; Matsushita et al., 1983) with comparable turnover numbers and apparent K_m values (Kaback, 1983; Viitanen et al., 1984). On the basis of these observations, it seems reasonable to conclude that purified *lac* carrier protein reconstitutes with a high degree of fidelity. That is, the orientation of the protein in the reconsituted system must be similar to that in the bacterial cytoplasmic membrane. Importantly, the conclusion is entirely consistent with the results presented here. Binding experiments with RSO and ISO membrane vesicles show that the epitope for antibody 4B1 is present virtually exclusively on the periplasmic surface of the membrane and the antibody binds to the reconstituted proteoliposomes with a stoichiometry very close to that observed in RSO vesicles. Clearly, therefore, with respect to the 4B1 epitope at least, the *lac* carrier protein has the same orientation in the reconstituted system as it has in the native membrane.

Efforts to localize and identify the 4B1 epitope within the *lac* carrier protein are in progress. When the purified protein is fragmented with cyanogen bromide, 4B1 binding activity is retained, and a number of cyanogen bromide fragments can be resolved and identified by reverse-phase high-performace liquid chromatography. Thus far, however, none of the fragments bind 4B1. In addition, polypeptides containing 10–15 amino acid residues have been synthesized that correspond to the C-terminus, the N-terminus, and portions of the hydrophilic segments connecting putative transmembrane segments 1 and 2 (loop 2), 4 and 5 (loop 5), and 6 and 7 (loop 7) (cf. Foster et al., 1983). Although these peptides are being used primarily for preparation of site-directed polyclonal antibodies (Carrasco et al., 1983), they have also been tested for 4B1 binding activity by solid-phase radioimmunoassay and by immmunocompetition. In each instance, the results have been negative. In any event, the experiments presented here and in the preceding paper (Carrasco et al., 1984a) provide a strong indication that further characterization of antibody 4B1 and some of the other monoclonal antibodies (Carrasco et al., 1982) combined with detailed structure/function studies may provide important insight into the structure and mechanism of the *lac* carrier protein.

Added in Proof

Recent experiments (Carrasco et al., 1984b) with antibodies directed against a dodecapeptide corresponding in sequence to the C-terminus of the *lac* carrier protein confirm the conclusion of Seckler et al. (1983) that this portion of the permease is located on the cytoplasmic surface of the membrane. In the reconstituted system, however, anti-C-terminal IgG binds stoichiometrically in the same manner as antibody 4B1 (i.e., in the proteoliposomes, both the 4B1 epitope and the ultimate C-terminus of the carrier are present on the exterior surface). Furthermore, when reconstituted proteoliposomes are digested with carboxypeptidases A and B, binding of anti-C-terminal IgG decreases by >80%, while binding of antibody 4B1 and various transport activities remain essentially unchanged. It is suggested, therefore, that during reconstitution, the *lac* carrier protein undergoes intramolecular dislocation of the C-terminus with no significant effect on its catalytic activity.

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Topography of the Photosynthetic Apparatus of *Chloroflexus aurantiacus*†

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ABSTRACT: The isolation and identification of reaction center, antenna bacteriochlorophyll *a* (Bchl *a*), and chlorosome antenna Bchl *c* polypeptides of *Chloroflexus aurantiacus* have been described. Subsequently, the localization of the chlorosome- and pigment-specific polypeptides was probed in two subcellular fractions. Reaction centers, located in the cytoplasmic membrane, were isolated according to the method of Pierson and Thornber [Pierson, B. K., & Thornber, J. P. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 80–84]. The reaction center preparation contained a major polypeptide with an apparent molecular weight of 26 000. The antenna Bchl *a* (B808–866) complex, also a cytoplasmic membrane constituent, was isolated by hydrophobic interaction chromatography on phenyl-Sepharose. In fractions with the highest purity and still native absorption properties, only one polypeptide of M_r 5300 was found. Purified chlorosomes contained three major proteins (M_r 18 000, 11 000, and 3700), and a fourth polypeptide (M_r 5800) was present in minor quantities. The M_r 3700 polypeptide was found to be associated with the antenna Bchl *c*. The two larger chlorosome proteins were accessible to proteinase K proteolyses and could additionally be modified with the membrane surface specific photolabel 3-azido-2,7-naphthalenedisulfonate. From the resistance against proteolytic cleavage and the lack of photolabeling, we surmised that the antenna Bchl *c* polypeptide (M_r 3700) is located in the chlorosome interior. Upon chemical cross-linking with dimethyl 3,3′-dithiobis(propionimidate), only dimers of the M_r 3700 protein were formed in high yield. According to our calculations, this dimer was comprised of 2 copies of the M_r 3700 protein and 10–16 molecules of Bchl *c*. The cross-linking pattern of the B808–866 antenna Bchl *a* protein (M_r 5300) indicated a quaternary structure of a trimer, which should be part of a penta- or hexameric in vivo aggregate. Under the experimental conditions used, the M_r 5300 antenna protein was never photolabeled and together with the reaction center protein resisted any proteolytic attack, indicating a more sequestered localization in the cytoplasmic membrane.

The photosynthetic apparatus of the thermophilic, facultative aerobic green bacterium *Chloroflexus aurantiacus* is located in two cytologically distinct compartments. This arrangement and the presence of two bacteriochlorophyll (Bchl)[1] types, Bchl *a* and species-dependent Bchl *c*, *d*, or *e*, are characteristic for all green bacteria (*Chlorobiaceaece*). In *C. aurantiacus*, the photochemical reaction centers and a Bchl *a* containing light-harvesting complex with absorption maxima at 866 and 808 nm are located in the cytoplasmic membrane (CM).

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[1] Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; LDAO, lauryldimethylamine oxide; NaDodSO$_4$–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; Bchl, bacteriochlorophyll; DTBP, dimethyl 3,3′-dithiobis(propionimidate); CM, cytoplasmic membrane; Bpheo, bacteriopheophytin; RC, reaction center; ANDS, 3-azido-2,7-naphthalenedisulfonate; DSP, dithiobis(succinimidyl propionate).

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