WA, for stimulating discussions.

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Monoclonal Antibodies against the *lac* Carrier Protein from *Escherichia coli*. 1. Functional Studies†

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ABSTRACT: The effects of various monoclonal antibodies against purified *lac* carrier protein on carrier-mediated lactose transport were studied in right-side-out membrane vesicles and in proteoliposomes reconstituted with purified *lac* carrier protein. Out of more than 60 monoclonal antibodies tested, only one antibody, designated 4B1, inhibits transport. Furthermore, the nature of the inhibition is highly specific in that the antibody inhibits only those transport reactions that involve net proton translocation (i.e., active transport, carrier-mediated influx and efflux under nonenergized conditions, and lactone-induced proton influx). In contrast, the antibody has little effect on equilibrium exchange and no effect on generation of the proton electrochemical gradient or on the ability of the carrier to bind a high-affinity ligand. Clearly, therefore, the antibody alters the relationship between lactose and proton translocation at the level of the *lac* carrier protein. When entrance counterflow is studied with external [1-^{14}C]lactose at saturating and subsaturating concentrations, it is apparent that antibody 4B1 mimics the effects of deuterium oxide [Viitanen, P., Garcia, M. L., Foster, D. L., Kaczorowski, G. J., & Kaback, H. R. (1983) *Biochemistry 22*, 2531]. That is, the antibody has no effect on the rate or extent of counterflow when external lactose is saturating but stimulates the efficiency of counterflow when external lactose is below the apparent K_m. It seems likely, therefore, that the antibody either inhibits the rate of deprotonation or alters the equilibrium between protonated and deprotonated forms of the carrier. Monovalent Fab fragments prepared from antibody 4B1 inhibit transport in a manner that is similar qualitatively to that of the intact antibody. However, intact 4B1 is approximately twice as effective as the Fab fragments on a molar basis, suggesting that the intact molecule binds bivalently while the Fab fragments bind 1:1. Support for this conclusion is provided by binding experiments with radiolabeled 4B1 and 4B1 Fab fragments presented in the following paper [Herzlinger, D., Viitanen, P., Carrasco, N., & Kaback, H. R. (1984) *Biochemistry* (following paper in this issue)].

β-Galactoside transport across the plasma membrane of *Escherichia coli* is mediated by the *lac* carrier protein (i.e., *lac* permease), an intrinsic protein encoded by the *lac y* gene [cf. Kaback (1983) for a recent review]. The protein catalyzes the coupled translocation of substrate with protons in a symport (cotransport) reaction. Thus, in the presence of a proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$,[1] interior negative and alkaline), downhill transport of protons in response to $\Delta\bar{\mu}_{H^+}$ drives uphill transport of substrate (i.e., active transport). Alternatively, downhill transport of substrate under nonenergized conditions drives the uphill transport of protons with generation of $\Delta\bar{\mu}_{H^+}$.

By use of a strain of *E. coli* with multiple copies of the *lac y* gene, a highly specific photoaffinity label for the *lac* carrier protein, and reconstitution of transport activity in proteoliposomes, the *lac* carrier protein has been purified to homogeneity (Newman et al., 1981; Foster et al., 1982). Proteoliposomes reconstituted with this single polypeptide species catalyze all of the transport activities observed in right-side-out

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[1] Abbreviations: $\Delta\bar{\mu}_{H^+}$, the proton electrochemical gradient; RSO, right-side-out; octyl glucoside, octyl β-D-glucopyranoside; $\Delta\psi$, membrane potential; PMS, phenazine methosulfate; *p*CMBS, *p*-(chloromercuri)-benzenesulfonate; NPG, *p*-nitrophenyl α-D-galactopyranoside.

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(RSO) membrane 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).

The product of the *lac y* gene is a 46.5-kdalton polypeptide containing 417 amino acid residues of known sequence (Büchel et al., 1980). A preliminary secondary structure model has been proposed (Foster et al., 1983) on the basis of circular dichroic measurements indicating that the protein has an exceptionally high α-helical content and also on the hydropathic profile of the protein along its primary sequence. The model suggests that the protein may consist of 12–13 α-helical segments that traverse the bilayer in a zig-zag fashion. Proteolysis experiments with RSO and inside-out vesicles containing photoaffinity-labeled *lac* carrier protein demonstrate directly that the polypeptide spans the membrane (Goldkorn et al., 1983). Furthermore, site-directed polyclonal antibodies against the C-terminus and the polypeptide segment connecting the first two N-terminal transmembrane α-helical stretches (loop 2) indicate that the C-terminus is accessible from the cytoplasmic surface of the membrane (Seckler et al., 1983; Carrasco et al., 1984a,b) while loop 2 appears to be inaccessible from either surface (Carrasco et al., 1984a). Finally, kinetic studies (Robertson et al., 1980; Ghazi & Shechter, 1981; Viitanen et al., 1984), experiments with group-specific reagents (Padan et al., 1979; Cohn et al., 1981), the demonstration that certain *lac y* mutations are dominant (Mieschendahl et al., 1981), and radiation inactivation studies (Goldkorn et al., 1984) have led to the suggestion that a large conformational alteration such as oligomerization may be important for *lac* carrier function.

In an effort to develop highly specific structure/function probes for dissecting the topology and mechanism of the *lac* carrier protein, we have recently described the preparation and preliminary characterization of monoclonal antibodies directed against the purified polypeptide (Carrasco et al., 1982, 1983a,b). Antibody secreted by hybridoma 4B1, an IgG2a immunoglobulin, was found to inhibit active transport of lactose with no effect on $\Delta\bar{\mu}_{H^+}$ or the ability of the *lac* carrier protein to bind a high-affinity ligand. In this paper, a detailed analysis of the functional properties of this monoclonal antibody and its monovalent Fab fragments is reported. Binding studies with radiolabeled 4B1 and 4B1 Fab fragments are described in the following paper (Herzlinger et al., 1984).

Experimental Procedures

Materials

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

Methods

Growth of Bacteria and Preparation of Membrane Vesicles. *E. coli* ML 308-225 ($i^-z^-y^+a^+$) was grown on minimal medium A (Davis & Mingioli, 1959) containing 1% disodium succinate (hexahydrate). *E. coli* T206 which harbors a recombinant plasmid with the *lac y* gene was grown and induced as described (Teather et al., 1980). Right-side-out (RSO) membrane vesicles were prepared as described (Kaback, 1971; Short et al., 1975).

Purification and Reconstitution of lac Carrier Protein. The *lac* carrier protein was purified from T206 membranes (Newman et al., 1981; Foster et al., 1982). Purified carrier was reconstituted by octyl β-D-glucopyranoside (octyl glucoside) dilution, and the proteoliposomes were harvested by centrifugation (Garcia et al., 1983). The pellets were resuspended in 50 mM potassium phosphate (pH 7.5) containing 1.0 mM dithiothreitol to 90 μg of *lac* carrier protein and 37.5 mg of *E. coli* phospholipid/mL and stored at −180 °C until use.

Preparation of Immunological Reagents. Hybridomas secreting monoclonal antibodies against purified *lac* carrier protein were prepared as described by Carrasco et al. (1982), and approximately 5×10^6 cells were injected intraperitoneally into pristane-primed Balb/c mice. Antibodies were purified from the ascites fluid of tumor-bearing mice by protein A–Sepharose affinity chromatography (Ey et al., 1979). Purified IgG was concentrated to at least 2 mg/mL by evaporation under vacuum, dialyzed against 1000 volumes of 50 mM potassium phosphate (pH 7.5) with two changes, and stored in liquid nitrogen. Monovalent Fab fragments were prepared from purified IgG by papain digestion (Mishell & Shiigi, 1980), and the F_c portion was removed by chromatography on protein A–Sepharose (Carrasco et al., 1982). Protein was determined as described (Lowry et al., 1951) with bovine serum albumin as standard, and the purity of the preparations was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Laemmli, 1970).

Transport Assays. (*1*) *Membrane Vesicles.* Respiration-driven accumulation of [1-^{14}C]lactose was measured under oxygen with reduced phenazine methosulfate (PMS) as electron donor (Kaback, 1974). Carrier-mediated influx and efflux down a concentration gradient in the absence of $\Delta\bar{\mu}_{H^+}$, lactose-induced proton influx, and entrance counterflow were measured as described (Kaczorowski & Kaback, 1979; Kaczorowski et al., 1979; Patel et al., 1982).

(*2*) *Proteoliposomes.* All transport measurements with proteoliposomes were conducted with preparations subjected to freeze–thaw/sonication (Foster et al., 1982; Garcia et al., 1983). Membrane potential ($\Delta\psi$) driven [1-^{14}C]lactose accumulation, carrier-mediated influx and efflux down a concentration gradient in the absence of $\Delta\bar{\mu}_{H^+}$, lactose-induced proton influx, and counterflow were assayed as described (Foster et al., 1982; Garcia et al., 1983; Viitanen et al., 1983, 1984).

Results

Functional Screening of Monoclonal Antibodies. As reported previously (Carrasco et al., 1982), a number of hybridomas that secrete monoclonal antibodies with a high degree of specificity for the *lac* carrier protein have been isolated. In order to correlate the immunochemical properties of the antibodies with function, supernatants from the hybridoma cultures were screened initially for effects on lactose transport in RSO vesicles, and preliminary results suggested that a significant percentage of the monoclonal antibodies inhibit active transport of lactose. However, after the hybridomonas were recloned and the antibodies were purified from five different clones, only one antibody, 4B1, exhibited potent inhibitory activity, suggesting that this antibody is particularly unique. Subsequently, supernatants from 60 different hybridoma cultures were tested for inhibition of carrier-mediated lactose efflux from proteoliposomes reconstituted with purified *lac* carrier protein (data not shown). Supernatant from hybridoma 4B1 is the only sample that produced significant inhibition. Therefore, this antibody was selected for detailed studies.

Effect of 4B1 on $\Delta\bar{\mu}_{H^+}$-Driven Lactose Accumulation. Antibody 4B1 markedly inhibits respiration-driven lactose accumulation in RSO vesicles prepared from *E. coli* ML 308-225 (Figure 1). In the absence of electron donors, lactose uptake proceeds slowly and a relatively small amount of di-

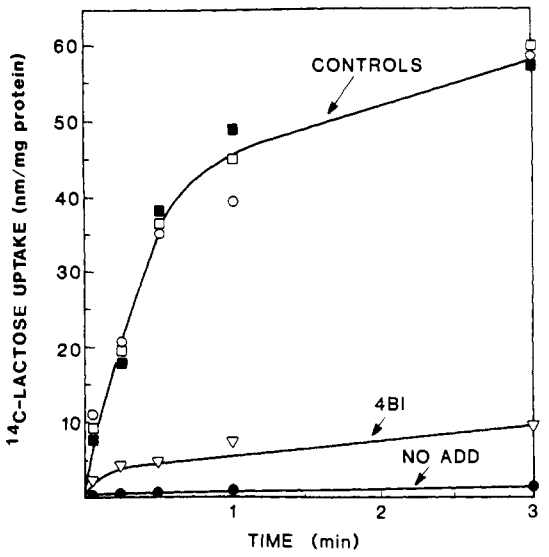

FIGURE 1: Effect of monoclonal antibodies on respiration-driven transport in RSO membrane vesicles. *E. coli* ML 308-225 membrane vesicles were incubated at room temperature for 30 min without (○, ●) or with a 3-fold molar excess of antibodies 4B1 (▽), 5F7 (■), or 4A10R (□) relative to *lac* carrier protein. Aliquots containing 50 μg of membrane protein (50 μL) were assayed for lactose uptake at indicated times by using 0.4 mM [1-^{14}C]lactose (29.9 mCi/mmol) in the presence (▽, ■, □, ○) or absence (●) of reduced PMS as described (Kaback, 1974).

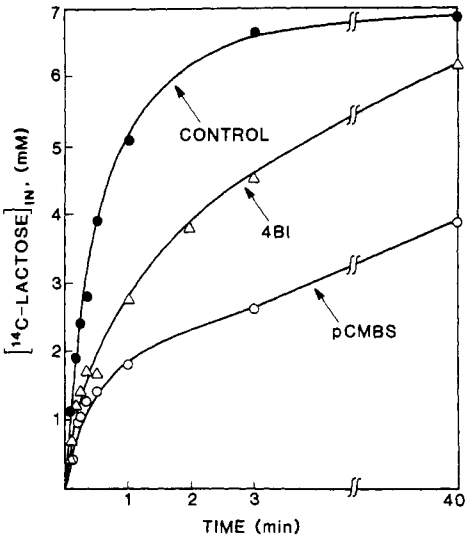

FIGURE 2: Effect of 4B1 on carrier-mediated lactose influx under nonenergized conditions in RSO vesicles. *E. coli* T206 vesicles were incubated with a 3-fold molar excess of 4B1 relative to *lac* carrier (△), 1 mM pCMBS (○), or 50 mM potassium phosphate (pH 7.5) (●) for 30 min at room temperature. The vesicles were harvested by centrifugation at 40000g_{max} for 20 min and resuspended in 50 mM potassium phosphate (pH 6.6) containing 10 mM $MgSO_4$ at a protein concentration of 33 mg/mL. Valinomycin and nigericin were added to final concentrations of 20 and 0.2 μM, respectively, and aliquots (10 μL) were diluted into 75 μL of 50 mM potassium phosphate (pH 6.6)/10 mM $MgSO_4$ containing 7.0 mM [1-^{14}C]lactose (4.93 mCi/mmol) at 25 °C. Reactions were terminated with 5 μL of 200 mM $HgCl_2$ and diluted with 4 mL of 100 mM potassium phosphate (pH 5.5) containing 100 mM LiCl. The samples were filtered immediately on Amicon nitrocellulose filters (0.45 μm) and washed twice with the same salt solution. Radioactivity retained on the filters was determined by liquid scintillation spectrometry. Antibodies 5F7 and 4A10R yielded data similar to those shown for the control.

saccharide is accumulated over the time course of the experiment. When $\Delta\bar{\mu}_{H^+}$ (interior negative and alkaline) is generated by means of reduced PMS oxidation (Ramos et al., 1976; Ramos & Kaback, 1977a,b; Felle et al., 1980), marked stimulation of both the initial rate of lactose transport and the steady-state level of accumulation is observed. Strikingly, addition of 4B1 at a 3-fold molar excess of antibody to *lac* carrier protein causes drastic inhibition of $\Delta\bar{\mu}_{H^+}$-driven transport, while antibodies 5F7 and 4A10R, which were obtained from the same fusion, have no effect whatsoever. As shown previously (Carrasco et al., 1982), similar effects were obtained with $\Delta\psi$-driven lactose accumulation in proteoliposomes reconstituted with purified *lac* carrier protein.

Although not shown, inhibition of active transport by 4B1 is constant from pH 5.5 to pH 7.5, and incubation of the vesicles with 4B1 at concentrations that induce maximum inhibition of transport has no effect on the ability of the vesicles to generate $\Delta\bar{\mu}_{H^+}$, as judged by flow dialysis experiments with [^{3}H]tetraphenylphosphonium and [^{14}C]acetate (Carrasco et al., 1982; data not shown). Also, despite potent inhibition of lactose transport, the antibody has no effect on *p*-nitro[2-^{3}H]phenyl α-D-galactopyranoside (NPG) binding under nonenergized conditions when assayed either by flow dialysis (Rudnick et al., 1976) or by photoaffinity labeling (Kaczorowski et al., 1980).

Effect of 4B1 on Lactose/Proton Symport in the Absence of $\Delta\bar{\mu}_{H^+}$. In RSO membrane vesicles (Kaczorowski et al., 1979; Garcia et al., 1982; Patel et al., 1982) and proteoliposomes containing *lac* carrier protein (Foster et al., 1982; Garcia et al., 1983; Viitanen et al., 1983, 1984), carrier-mediated lactose translocation down a concentration gradient leads to the generation of $\Delta\bar{\mu}_{H^+}$, the polarity of which reflects the direction of the lactose concentration gradient. The experiments presented in Figure 2 represent time courses of lactose influx in RSO vesicles from *E. coli* T206 treated with valinomycin and nigericin in order to abolish the rate-limiting $\Delta\bar{\mu}_{H^+}$ generated during lactose/proton symport under these conditions (Patel et al., 1982). In the control samples, influx is relatively rapid initially, decreases, and ultimately reaches a steady state in about 5 min, at which time the intravesicular pool is in equilibrium with the external medium. Treatment of the vesicles with *p*-(chloromercuri)benzenesulfonate (*p*CMBS) markedly inhibits the rate of influx, but eventually, the intravesicular space equilibrates with the medium by means of passive diffusion (Patel et al., 1982). In the presence of antibody 4B1 at a stoichiometry of 3 (4B1:*lac* carrier protein), the rate of influx is inhibited 60–70% relative to the control with the rate in the presence of *p*CMBS taken as 100% inhibition. Within 1 h, however, the vesicles equilibrate with the external medium. Notably, antibodies purified from hybridomas 5F7 and 4A10R have no discernible effect (not shown).

In a corollary series of experiments, proton translocation was measured under similar conditions (Figure 3). When lactose is added to the vesicles, transient alkalinization of the medium is observed, and the pH tracing reaches maximum displacement in about 20 s and returns to the base line after approximately 5 min [cf. Patel et al. (1982) in addition]. As shown, this process is markedly retarded when the vesicles are treated with antibody 4B1 (antibody:*lac* carrier protein ≃ 2.5). On the other hand, antibodies 5F7 or 4A10R have no effect.

Although data are not shown, the same experiments were carried out with vesicles from *E. coli* ML 308-225, and similar results were obtained at a 2.5 molar excess of antibody to *lac* carrier. In contrast, however, although lactose influx under nonenergized conditions and lactose-induced proton influx are readily observed in proteoliposomes reconstituted with purified *lac* carrier protein (Foster et al., 1982; Viitanen et al., 1984), no significant inhibition is observed with antibody 4B1.

Lactose efflux from RSO vesicles (Kaczorowski & Kaback, 1979) and proteoliposomes (Garcia et al., 1983; Viitanen et

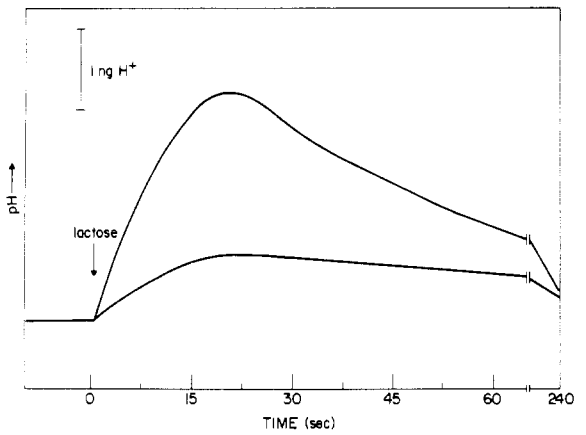

FIGURE 3: Effect of monoclonal antibodies on lactose-induced proton influx in RSO membrane vesicles. A 1.5-mL suspension of *E. coli* ML 308-225 membrane vesicles (1 mg of protein/mL) in 150 mM KCl and 10 mM $MgSO_4$ was placed in a closed electrode vessel that was continuously flushed with a stream of water-saturated nitrogen and maintained at 25 °C. Valinomycin was added to final concentrations of 20 μM. The reaction was initiated by addition of lactose to a final concentration of 10 mM, and the pH of the solution was registered continuously. Upper trace: no addition or a 2.5-fold molar excess of antibodies 4A10R or 5F7 relative to *lac* carrier. Lower trace: 2.5-fold molar excess of 4B1. The pH change was calibrated at the end of each experiment by the addition of 10 μL of 1 mM HCl.

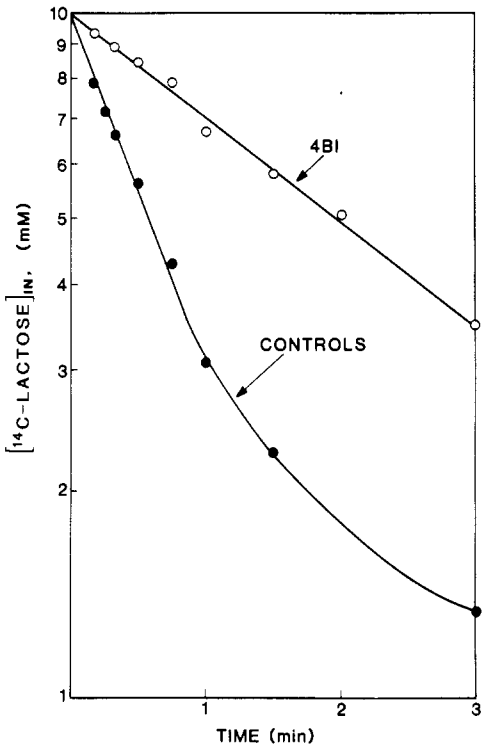

FIGURE 4: Effect of antibody 4B1 on lactose efflux in proteoliposomes reconstituted with purified *lac* carrier. Proteoliposomes containing 90 μg of *lac* carrier protein/mL and 37.5 mg of *E. coli* phospholipids/mL in 50 mM potassium phosphate (pH 7.5)/1 mM dithiothreitol were loaded with 10 mM [1-^{14}C]lactose (11.8 mCi/mmol) in the presence of 20 μM valinomycin by incubation at room temperature for 45 min. The samples were then incubated for 1 h with a 2.5-fold molar excess of monoclonal antibody 4B1 (○) or in the absence of the antibody (●). Aliquots (1 μL) were rapidly diluted into 200 μL of 50 mM potassium phosphate (pH 5.5) at 25 °C. At the times indicated, the samples were diluted with 3 mL of ice-cold 50 mM potassium phosphate (pH 7.5) and immediately filtered. The filters (type GSTF, 0.2 μm, Millipore Filter Corp.) were washed once with the same cold buffer. Zero time values were determined in triplicate by dilution of equilibrated proteoliposomes directly into ice-cold 50 mM KP_i (pH 7.5) followed by immediate filtration. Radioactivity was determined by liquid scintillation spectrometry. Incubation with antibodies 5F7 or 4A10R give results identical with those observed without antibody.

al., 1983) is a carrier-mediated process that involves proton symport. Thus, when proteoliposomes reconstituted with purified *lac* carrier are equilibrated with 10 mM [1-^{14}C]-lactose, treated with valinomycin, and diluted rapidly into media devoid of lactose, the rate of efflux remains constant until the internal concentration approaches the apparent K_m for efflux [Figure 4, closed symbols; cf. Garcia et al. (1983) in addition]. At this point, the rate slows, and the function deviates from linearity. When the proteoliposomes are treated with antibody 4B1 at a 2.5-fold molar excess over *lac* carrier, the rate of efflux is slowed, as reflected by the increase in $t_{1/2}$ from about 30 s in the control to 2 min in the sample treated with 4B1 (Figure 4, open symbols). Additionally, it should be emphasized that antibodies 5F7 and 4A10R do not alter the rate of efflux, and very similar results are obtained with RSO vesicles from both *E. coli* ML 308-225 and *E. coli* T206.

Effect of 4B1 on Exchange. Exchange of internal [1-^{14}C]lactose with equimolar unlabeled lactose in the external medium is very fast relative to efflux in RSO vesicles (Kaczorowski & Kaback, 1979), as well as proteoliposomes (Garcia et al., 1983; Viitanen et al., 1983). Importantly, moreover, in contrast to efflux, the exchange reaction does not involve net translocation of protons, since it is independent of pH and unaffected by imposition of $\Delta\bar{\mu}_{H^+}$ of either polarity, by the presence of ionophores, or by substitution of deuterium for protium. In the experiments presented in Figure 5, proteoliposomes reconstituted with purified *lac* carrier protein were equilibrated with 10 mM [1-^{14}C]lactose, incubated without or with antibody 4B1, and then diluted into media containing equimolar concentrations of unlabeled lactose. In close agreement with previous observations (Garcia et al., 1983; Viitanen et al., 1983), the untreated control samples exhibit a very rapid rate of efflux with a $t_{1/2}$ approximating 5 s. In contrast to the reactions described above, however, which involve net proton translocation, the rate of exchange is only minimally inhibited by antibody 4B1. Although not shown, the same results were obtained with RSO vesicles from ML 308-225 and T206.

Effect of 4B1 on Lactose Counterflow. As demonstrated previously (Kaczorowski & Kaback, 1979; Garcia et al., 1983; Viitanen et al., 1983), entrance counterflow can be used to assess the frequency with which the carrier returns from the outer to the inner surface of the membrane in the loaded vs. the unloaded form. With respect to the proposed mechanism (Figure 6A, inset), unlabeled internal lactose is translocated with a proton to the outer surface of the membrane where it is released prior to the symported proton. Depending on the external concentration of labeled lactose, the resulting C-H (protonated) form of the carrier can partition between two pathways: (1) binding of the labeled external lactose and return of the loaded carrier, resulting in counterflow (i.e., exchange of internal-unlabeled lactose for external-labeled lactose); (2) deprotonation and return of the unloaded carrier, resulting in efflux.

With saturating external [1-^{14}C]lactose concentrations, the magnitude of the overshoot observed during counterflow is independent of pH, the coupling efficiency is 1:1 (Kaczorowski & Kaback, 1979; Garcia et al., 1983), and replacing the protium in the medium with deuterium has no effect on the efficiency of the process (Viitanen et al., 1983). Therefore, when external lactose is saturating, the vast majority of the carrier is presumably turning over in the protonated state (i.e., pathway 1 is favored), and antibody 4B1, since it has little effect on exchange, should have little effect on the efficiency

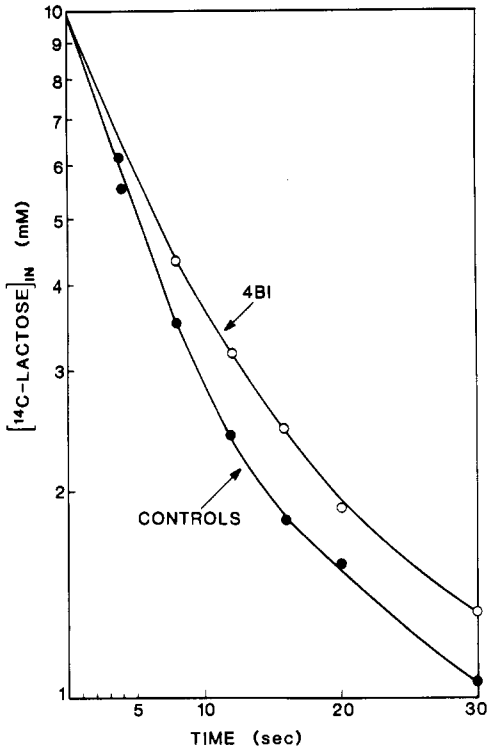

FIGURE 5: Effect of antibody 4B1 on equilibrium exchange in proteoliposomes reconstituted with *lac* carrier protein. All the experimental conditions were the same as described in Figure 4, except that proteoliposomes equilibrated with [1-^{14}C]lactose were rapidly diluted into 200 μL of 50 mM potassium phosphate (pH 7.5) containing 10 mM unlabeled lactose. (●) No addition; (○) incubation with a 2.5-fold molar excess of 4B1. Results identical with those shown for the control were also obtained with antibodies 5F7 and 4A10R.

of counterflow. The prediction is borne out by the experiments described in Figure 6A, where entrance counterflow was measured in the absence and presence of an excess of 4B1 with saturating external [1-^{14}C]lactose concentrations. Both the rate of counterflow and the extent of the overshoot are similar with and without 4B1. On the other hand, the antibody slows the rate at which the overshoot decays, since it inhibits efflux (cf. Figure 4).

When counterflow is monitored with external [1-^{14}C]lactose below saturation, the magnitude of the overshoot is inversely proportional to pH, the coupling efficiency is diminished (Kaczorowski & Kaback, 1979; Garcia et al., 1983), and deuterium enhances the efficiency of the process (Viitanen et al., 1983). Under these circumstances, lactose is released on the surface of the membrane, but binding of labeled substrate occurs relatively infrequently, allowing deprotonation and return of the unloaded carrier which is reflected by a decrease in counterflow (i.e., pathway 2 is favored). However, deuterium dissociates more slowly from the carrier than protium and favors binding of external [1-^{14}C]lactose (Viitanen et al., 1983). Consequently, the frequency with which the carrier returns to the inner surface of the membrane in the loaded vs. the unloaded form is enhanced in the presence of deuterium oxide. Like deuterium, antibody 4B1 inhibits efflux much more markedly than it inhibits exchange (compare Figures 4 and 5). Thus, it might be expected that the antibody would stimulate counterflow under subsaturating conditions (Figure 6B). The experiments were performed exactly as described in Figure 6A, except that the external [1-^{14}C]lactose concentration was almost 8-fold lower than the apparent K_m for counterflow. In the control samples (closed symbols), the magnitude of the overshoot is reduced from about 12 mM internal lactose at the peak to less than 2 mM; however, in the presence of antibody 4B1, it is increased almost 2-fold (compare closed and open symbols).

Monovalent Fab Fragments. Since IgG molecules are bivalent and large relative to the *lac* carrier protein (i.e., 150 vs. 46.5 kdaltons), monovalent Fab fragments from 4B1, 5F7, and 4A10R were prepared and tested for their ability to inhibit

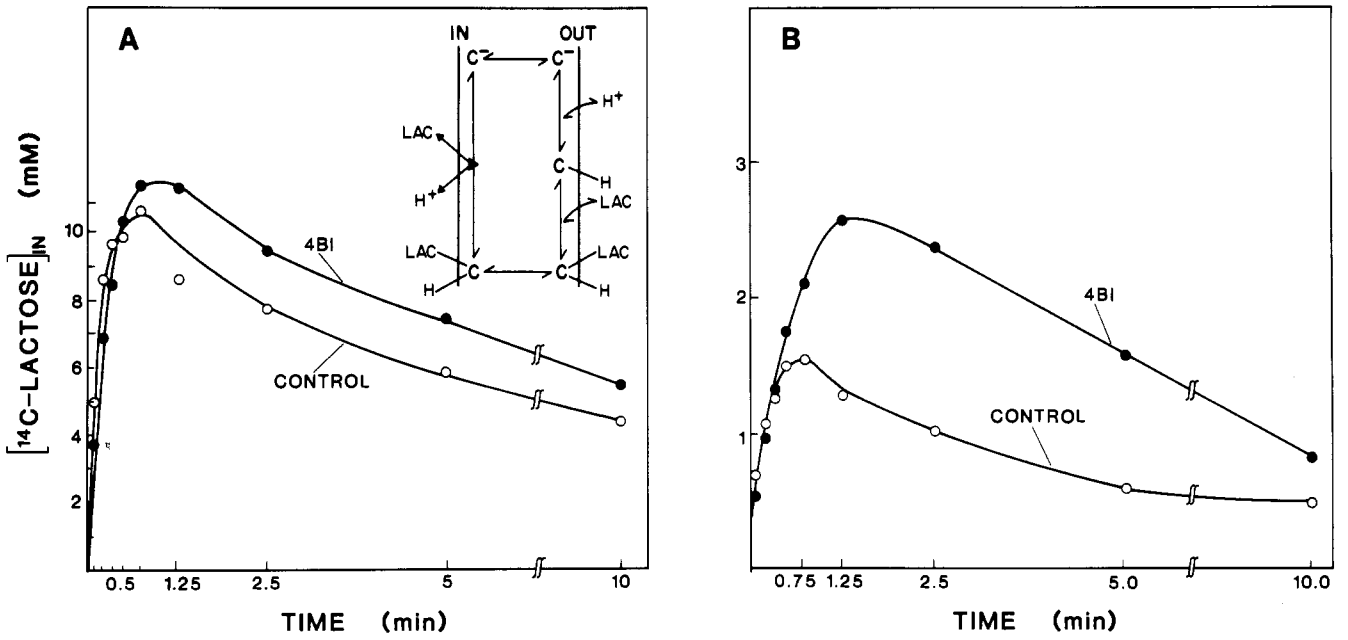

FIGURE 6: Effect of antibody 4B1 on counterflow at saturating (panel A) and subsaturating (panel B) external lactose concentrations. Aliquots (150 μL) of proteoliposomes reconstituted with purified *lac* carrier protein were equilibrated with 12 mM lactose in the presence of 20 μM valinomycin (Viitanen et al., 1983). Samples were then incubated without (○) or with a 2.5-fold molar excess of 4B1 (●) for an additional 45 min. Counterflow was initiated by diluting a 1-μL aliquot into 100 μL of 50 mM potassium phosphate (pH 7.5) containing 1.6 mM [1-^{14}C]lactose (7.4 mCi/mmol) (panel A) or 75 μM [^{14}C]lactose (59 mCi/mmol) (panel B). At given times, 3 mL of ice-cold 50 mM potassium phosphate (pH 7.5) was added, and the samples were filtered immediately as described in Viitanen et al. (1983). (Panel A, Inset) Schematic representation of reactions involved in lactose efflux, exchange, and counterflow. C represents the *lac* carrier protein. The order of substrate binding at the inner surface of the membrane is not implied. From Kaczorowski & Kaback (1979).

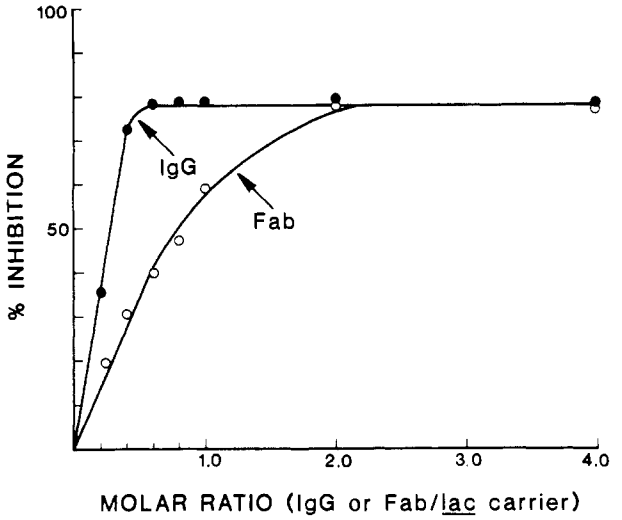

FIGURE 7: Inhibition of the initial rate of lactose efflux by 4B1 IgG and 4B1 Fab fragments. Proteoliposomes containing 90 μg of purified *lac* carrier protein/mL and 37.5 mg of *E. coli* phospholipid/mL in 50 mM potassium phosphate (pH 7.5) containing 1 mM dithiothreitol were treated with 20 μM valinomycin and loaded with 10 mM [1-^{14}C]lactose (12 mCi/mmol) as described (Viitanen et al., 1983). Aliquots were incubated for 1 h at room temperature with either intact 4B1 IgG (●) or 4B1 Fab fragments (○) at given molar ratios (abscissa). Efflux was conducted as described in Figure 4, and initial rates were determined from the linear portion of the time courses.

the various translocation reactions described. Although data will not be presented, the results are very similar to those obtained with the intact antibodies. 4B1 Fab fragments inhibit $\Delta\bar{\mu}_{H^+}$-driven active transport, facilitated influx and efflux under nonenergized conditions, and lactose-induced proton influx in RSO vesicles with little or no effect on exchange or [^{3}H]NPG binding, and Fab fragments from 5F7 and 4A10R have no effect on any of these parameters. On the other hand, there is an interesting quantitative difference in the behavior of intact 4B1 and that of its Fab fragments (Figure 7). In the experiment shown, initial rates of [1-^{14}C]lactose efflux from proteoliposomes reconstituted with purified *lac* carrier protein were measured in the presence of increasing concentrations of intact 4B1 and 4B1 Fab fragments, and percent inhibition is plotted as a function of the molar ratio of 4B1 or 4B1 Fab to *lac* carrier protein. Clearly, on a molar basis, the intact antibody with two combining sites per molecule inhibits more effectively than the monovalent Fab fragments. Thus, half-maximal inhibition with intact 4B1 is observed at a molar ratio (4B1:*lac* carrier protein) of about 0.25, while the same degree of inhibition occurs at a molar ratio of 0.5–0.6 with the Fab fragments. Although not shown, a similar relationship between intact 4B1 and 4B1 Fab fragments was observed with respect to inhibition of $\Delta\psi$-driven lactose accumulation in the proteoliposome system.

Discussion

These results describe a detailed functional characterization of antibody 4B1, a monoclonal antibody directed against the *lac* carrier protein that inhibits transport via the β-galactoside transport system of *E. coli*. Antibody 4B1 and a number of other monoclonal antibodies were prepared by somatic cell fusion of mouse myeloma cells with splenocytes from a mouse immunized with purified *lac* carrier protein (Carrasco et al., 1982). A number of clones were shown to produce antibodies that react with the purified protein as demonstrated by solid-phase radioimmunoassay, and all of the hybridomas chosen for expansion react with the major polypeptide in the purified *lac* carrier preparation that migrates at M_r 33K and with an aggregate that migrates at M_r 65K. Furthermore, essentially identical results are obtained with RSO vesicles containing the *lac* carrier, as evidenced by immunoblotting subsequent to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, providing even stronger evidence for the high degree of specificity of the antibodies for the *lac* carrier protein.

Hybridoma culture supernatants were screened initially for effects on lactose transport in RSO membrane vesicles, and inhibition ranging from 20 to 90% was observed, depending on the hybridoma supernatant tested. Antibody 4B1 exhibited the most pronounced effect (Carrasco et al., 1982). Subsequently, antibodies were purified from five hybridoma clones and retested for inhibitory activity, and 4B1 was the only antibody observed to give potent inhibition. Further studies demonstrate that antibody secreted by hybridoma 4B1 is the only one of over 60 tested that inhibits lactose transport. It is readily apparent, therefore, that this antibody has unique properties.

As shown previously (Carrasco et al., 1982), antibody 4B1 inhibits active transport of lactose in proteoliposomes reconstituted with purified *lac* carrier protein and in RSO vesicles. Furthermore, the amount of antibody needed for a given degree of inhibition is proportional to the amount of *lac* carrier in the membrane. Thus, in T206 vesicles with 5–6 times the amount of *lac* carrier as ML 308-225 vesicles, about 5 times more 4B1 is required to achieve 50% inhibition of the initial rate of lactose transport. Notably, antibody-induced inhibition occurs within seconds, an observation that is consistent with the external localization of the 4B1 epitope (Herzlinger et al., 1984).

Remarkably, antibody 4B1 specifically inhibits each transport reaction that involves net translocation of protons ($\Delta\bar{\mu}_{H^+}$-driven lactose accumulation, carrier-mediated influx and efflux down a concentration gradient in the absence of $\Delta\bar{\mu}_{H^+}$, and lactose-induced proton influx) with little or no effect on exchange, entrance counterflow under saturating conditions or binding of [^{3}H]NPG. Clearly, therefore, the antibody interferes with the "coupling" between lactose and proton translocation at the level of the carrier. Although it is impossible to provide a satisfactory explanation for the phenomenon on a molecular level, some insight into the mechanism of 4B1 inhibition is obtained from the counterflow experiments presented in Figure 6, where it is demonstrated that the antibody simulates the effects of deuterium oxide (Viitanen et al., 1983). When counterflow is measured with external [1-^{14}C]lactose at saturating concentrations, antibody 4B1 has no effect on the rate of counterflow or the magnitude of the overshoot; the coupling efficiency is 1:1 in the absence or presence of antibody. In accordance with the model (Figure 6, inset), high external lactose concentrations prevent deprotonation of the carrier (the C-H form), and it recycles across the membrane in the fully loaded state, catalyzing 1:1 exchange of internal unlabeled lactose with external [1-^{14}C]-lactose. When the external lactose concentration is well below the apparent K_m, however, 4B1 increases the coupling efficiency for counterflow. Under subsaturating conditions, the C-H form of the carrier partitions between two pathways, one involving loss of the proton which results in net efflux and the other involving rebinding of lactose prior to loss of the proton which results in exchange (i.e., counterflow). The latter pathway appears to be favored in the presence of antibody 4B1 possibly because the antibody, like deuterium, decreases the rate of deprotonation and favors binding of external [1-^{14}C]lactose. Consequently, the frequency with which the carrier returns to the inner surface of the membrane in the

loaded vs. the unloaded form is enhanced, resulting in stimulation of counterflow.

Within the limits of experimental error, antibody 4B1 exerts similar effects in RSO vesicles from *E. coli* ML 308-225 and T206 and in proteoliposomes reconstituted with purified *lac* carrier protein. This is not surprising since the reconstituted system catalyzes each of the translocation reactions typical of the β-galactoside transport system with turnover numbers and apparent K_m values comparable to those observed in RSO vesicles and exhibits kinetic properties analogous to those observed in membrane vesicles (Kaback, 1983; Viitanen et al., 1984). Despite the similarities, however, the antibody does not inhibit lactose influx under nonenergized conditions or lactose-induced proton influx in the proteoliposome system, although it does so in RSO membrane vesicles. Since both of these reactions have been demonstrated in the reconstituted system (Foster et al., 1982; Viitanen et al., 1984) and 4B1 binds to the *lac* carrier protein in proteoliposomes with the same stoichiometry as that observed in RSO vesicles (Herzlinger et al., 1984), an explanation for the discrepancy is not readily apparent at the present time.

Qualitatively, monovalent Fab fragments prepared from 4B1 behave in a manner identical with that of the intact antibody. An interesting quantitative difference is documented in Figure 7, however, where it is demonstrated that the intact antibody is approximately twice as effective as the Fab fragments on a molar basis relative to the amount of *lac* carrier protein present. A reasonable, preliminary interpretation of the findings is that a single intact antibody molecule, which is bivalent, has the capacity to bind two molecules of *lac* carrier protein, while the monovalent Fab fragments bind 1:1. Direct support for this notion is presented in the following paper (Herzlinger et al., 1984) where binding of radiolabeled 4B1 and its Fab fragments is examined.

Registry No. D-Lactose, 63-42-3; hydrogen ion, 12408-02-5; lactose permease, 9068-45-5.

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