Site-Directed Mutagenesis of Cysteine-148 in the *Lac* Permease of *Escherichia coli*: Effect on Transport, Binding, and Sulfhydryl Inactivation

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ABSTRACT: By subjecting the *lac y* gene of *Escherichia coli* to oligonucleotide-directed, site-specific mutagenesis, Cys_{148} in the *lac* permease has been replaced with a Gly residue [Trumble, W. R., Viitanen, P. V., Sarkar, H. K., Poonian, M. S., & Kaback, H. R. (1984) *Biochem. Biophys. Res. Commun. 119*, 860]. Recombinant plasmids bearing wild-type or mutated *lac y* were constructed and used to transform *E. coli* T184. Steady-state levels of lactose accumulation, the apparent K_m for lactose under energized conditions, and the K_D for *p*-nitrophenyl α-D-galactopyranoside are comparable in right-side-out vesicles containing wild-type or mutant permease. In contrast, the V_{max} for lactose transport in vesicles containing mutant permease is significantly decreased. Although antibody binding studies reveal that vesicles from the mutant contain almost as much permease as wild-type vesicles, surprisingly only about one-fourth of the altered molecules bind *p*-nitrophenyl α-D-galactopyranoside with high affinity. Mutant permease is less sensitive to inactivation by *N*-ethylmaleimide, although the alkylating agent is still capable of completely inhibiting transport activity. Importantly, β-galactosyl 1-thio-β-D-galactopyranoside affords complete protection of wild-type permease against *N*-ethylmaleimide but has no protective effect whatsoever in the mutant. The rate of inactivation of wild-type and mutant permeases by *N*-ethylmaleimide is increased at alkaline pH and by the presence of a proton electrochemical gradient (interior negative and alkaline), and these phenomena are exaggerated in vesicles containing mutant permease. Finally, *p*-(chloromercuri)benzenesulfonate, which completely displaces bound *p*-nitrophenyl α-D-galactopyranoside from wild-type permease, does not affect binding in the mutant. The findings indicate that Cys_{148} is not obligatory for lactose/proton symport and that there is (are) another (other) Cys residue(s) in the permease that may be essential for activity. The other Cys residue(s) is (are) less reactive than Cys_{148}, is (are) not protected against alkylation by β-galactosyl 1-thio-β-D-galactopyranoside, and is (are) not involved in binding of substrate.

The *lac* permease of *Escherichia coli* is an intrinsic membrane protein encoded by the *lac y* gene that catalyzes symport of β-galactosides with protons [cf. Kaback (1981, 1983, 1985a), Hengge & Boos (1983), and Overath & Wright (1983) for reviews]. Physiologically, the permease is responsible for accumulating lactose against a concentration gradient, and the immediate driving force for the process is a proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$,[1] interior negative and/or alkaline), as first postulated by Mitchell (1963). The mechanism by which the *lac* permease transduces $\Delta\bar{\mu}_{H^+}$ into a chemical gradient of β-galactosides is unknown; however, the process is fully reversible. That is, downhill movement of β-galactosides along a concentration gradient drives uphill translocation of protons with the generation of $\Delta\bar{\mu}_{H^+}$.

The *lac y* gene has been cloned into a multicopy recombinant plasmid (Teather et al., 1978), allowing amplification of the *lac* permease (Teather et al., 1980) as well as elucidation of its amino acid sequence (Büchel et al., 1980). Furthermore, the permease has been purified to homogeneity and reconstituted into proteoliposomes in a fully functional state (Newman et al., 1981; Foster et al., 1982; Garcia et al., 1983; Viitanen et al., 1983, 1984, 1985; Matsushita et al., 1983; Costello et al., 1984; Wright & Overath, 1984). Since proteoliposomes reconstituted with purified *lac* permease catalyze all of the reactions typical of β-galactoside transport with turnover numbers and apparent K_m's comparable to those of bacterial membrane vesicles (Viitanen et al., 1984), it is highly likely that the product of the *lac y* gene is the only polypeptide species required for lactose transport.

Although oligomerization may be important to permease function (Mieschendahl et al., 1981; Goldkorn et al., 1984), the product of the *lac y* gene is a single polypeptide containing 417 amino acid residues. Circular dichroic measurements and an analysis of sequential hydropathic character suggest that the molecule is organized into 12 hydrophobic, α-helical segments that span the bilayer in a zig-zag fashion connected by more hydrophilic, charged segments (Foster et al., 1983). Although by no means proven, preliminary support for the general model has been obtained. Proteolysis experiments with right-side-out (RSO) and inside-out cytoplasmic membrane vesicles in which the permease was photoaffinity labeled demonstrate directly that the protein spans the bilayer (Goldkorn et al., 1983). In addition, binding studies with monoclonal antibodies (Mabs) directed against the purified protein and with site-directed polyclonal antibodies demonstrate that the permease is inserted asymmetrically into the membrane with the carboxyl-terminus and hydrophilic segments 5 and 7 exposed on the cytoplasmic surface (Seckler et al., 1983; Carrasco et al., 1984b) and epitopes for two independent Mabs exposed on the periplasmic (i.e., external)

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[1] Abbreviations: $\Delta\bar{\mu}_{H^+}$, proton electrochemical gradient; RSO, right side out; Mab, monoclonal antibody; NEM, *N*-ethylmaleimide; TDG, β-galactosyl 1-thio-β-D-galactopyranoside; NPG, *p*-nitrophenyl α-D-galactopyranoside; ss, single stranded; kbp, kilobase pair(s); RF, replicative form; IPTG, isopropyl 1-thio-β-D-galactopyranoside; x-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; PMS, phenazine methosulfate; pCMBS, *p*-(chloromercuri)benzenesulfonate; DTT, dithiothreitol.

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surface (Carrasco et al., 1982, 1984a,b; Herzlinger et al., 1984, 1985).

On the basis of substrate protection against *N*-ethylmaleimide (NEM) inactivation of β-galactoside transport, Fox & Kennedy (1965) postulated that there is an essential sulfhydryl group in the *lac* permease located at or near the active site, and Beyreuther et al. (1981) subsequently identified Cys_{148} as the reactive thiol. In order to address this problem directly, we have recently employed oligonucleotide-directed, site-specific mutagenesis (Zoller & Smith, 1983) on the *lac y* gene to substitute a glycyl residue for Cys_{148} (Trumble et al., 1984). Cells bearing the mutated *lac y* gene transport lactose to steady-state levels of accumulation that are comparable to those observed in cells bearing wild-type *lac y*, and initial rates of transport are reduced only about 4-fold. Furthermore, transport activity is less sensitive to inactivation by NEM, and β-galactosyl 1-thio-β-D-galactopyranoside (TDG) affords no protection against inactivation. The observations suggest that although Cys_{148} is essential for substrate protection against sulfhydryl inactivation, it is not obligatory for lactose/proton symport and that another sulfhydryl group elsewhere within the permease may be required for activity.

The findings presented in this paper confirm and extend the conclusions of Trumble et al. (1984) and highlight the importance of additional sulfhydryl group(s) elsewhere in the permease molecule.

Experimental Procedures

Materials

T_4 DNA ligase, DNA polymerase I (Klenow fragment), bacterial alkaline phosphatase, all restriction enzymes, and dideoxy sequencing materials were purchased from Bethesda Research Laboratories. [1-^{14}C]Lactose, [γ-^{32}P]ATP, [α-^{32}P]dATP, and T_4 polynucleotide kinase were obtained from Amersham/Searle; [U-^{14}C]proline was purchased from New England Nuclear. Nitrocellulose (0.1-μm pore size) and Elutip-d columns were acquired from Schleicher & Schuell. *p*-Nitro[6-^{3}H]phenyl α-D-galactopyranoside (NPG) was synthesized by Yu-Ying Liu (Isotope Synthesis Group, Hoffmann-La Roche Inc.) under the direction of Arnold Liebman. All other materials were reagent grade obtained from commercial sources.

Methods

Oligonucleotide-Directed, Site-Specific Mutagenesis. Cys_{148} in the *lac* permease was converted to Gly by using oligonucleotide-directed, site-specific mutagenesis (Zoller & Smith, 1983) as described (Trumble et al., 1984; Sarkar et al., 1985). The template was single-stranded (ss) M13mp9 DNA containing the antisense strand of a 2.3 kilobase pair (kbp), *lac y* gene containing *Eco*RI restriction fragment as an insert (Büchel et al., 1980). The mutagenic primer was a 21-base synthetic oligonucleotide (5′-pCCAGCCAACACC*GCCAAACAT-3′) complementary to the ss *lac y* template with the exception of a single mismatch (designated by the asterisk) designed to convert the Cys codon (TGT) encoding residue 148 of the *lac* permease to a Gly codon (GGT). Phage harboring the mutation were identified initially by dot-blot hybridization (Zoller & Smith, 1983) using ^{32}P-labeled mutagenic primer as a probe. Cloning of the mutated 2.3 kbp fragment into plasmid pACYC184 and subsequent transformation of *E. coli* T184 with the recombinant plasmid pG148 were performed as described (Trumble et al., 1984; Sarkar et al., 1985).

*Hin*cII restriction enzyme analysis was used to demonstrate that the orientation of the mutated 2.3 kbp fragment in pG148 is identical with that of the parental plasmid pGM21 (Büchel et al., 1980). To verify that the mutant strain (G148) harbors the mutation, pG148 was isolated (Birnboim, 1983) and restricted with endonuclease *Eco*RI. The 2.3 kbp fragment was purified by agarose gel electrophoresis (Schmitt & Cohen, 1983) and ligated into *Eco*RI-linearized M13mp19 replicative form (RF) DNA, and a small portion of the ligation mixture was used to transfect *E. coli* JM103 (Messing, 1983). Phage harboring the insert were identified as white plaques in the presence of isopropyl 1-thio-β-D-galactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (x-Gal), and ss template phage DNA was prepared as described (Messing, 1983). The DNA was then sequenced as described by Sanger et al. (1977) and Sanger & Coulson (1978) using a synthetic 24-base oligonucleotide (5′-CCAGT*CAGAAACAAACTA*ATTAT-3′) that is complementary to bases 493 through 517 of the antisense strand of the *lac y* gene with the exception of two mismatches (designated by asterisks).

Growth of Cells and Preparation of Membrane Vesicles. *E. coli* T206 (Teather et al., 1980) and G148 (Trumble et al., 1984) were grown and induced with IPTG as described (Teather et al., 1980). RSO membrane vesicles prepared as described (Kaback, 1971, 1974) were resuspended to a final concentration of 3 mg of protein/mL in 100 mM potassium phosphate (pH 6.6). Aliquots were frozen and stored in liquid nitrogen for subsequent use.

Transport Assays. Transport of [1-^{14}C]lactose or [U-^{14}C]proline by RSO membrane vesicles was measured in the presence of oxygen and reduced phenazine methosulfate (PMS) using rapid filtration as described (Kaback, 1971, 1974). Concentrations and specific activities of radioactive substrates are given in the figures.

Binding of Mab 4B1. Binding of ^{125}I-labeled 4B1 to membrane vesicles was assayed by filtration as described (Herzlinger et al., 1984) except that the vesicles were not flocculated with poly(L-lysine).

Binding of [^{3}H]NPG. Binding of [^{3}H]NPG to membrane vesicles was assayed under nonenergized conditions using flow dialysis as described (Rudnick et al., 1976). The upper chamber contained 0.2 mL of membrane vesicles (6 mg of protein) in 100 mM potassium phosphate (pH 7.5). The same buffer was pumped through the lower chamber at 3.5 mL/min, and 1.5-mL fractions were collected. Assays were initiated by the addition of given concentrations of [^{3}H]NPG to the upper chamber. After equilibration was achieved, TDG or *p*-(chloromercuri)benzenesulfonate (pCMBS) was added to the upper chamber as indicated. Specific binding of NPG was quantitated from the increase in the dialyzable concentration of [^{3}H]NPG after the addition of saturating concentrations of TDG. K_D and B_{max} values for NPG binding were calculated according to Scatchard (1949) from flow dialysis experiments using [^{3}H]NPG concentrations ranging from 1.9 to 57.6 μM.

Treatment of Vesicles with NEM. RSO membrane vesicles were treated with NEM in the presence or absence of $\Delta\bar{\mu}_{H^+}$ essentially as described by Cohn et al. (1981). Vesicles were washed and resuspended to about 1.5 mg of protein/mL in 50 mM potassium phosphate at an appropriate pH containing 10 mM $MgSO_4$. For experiments performed in the absence of $\Delta\bar{\mu}_{H^+}$, NEM was added to given concentrations, and at given times, reactions were terminated by the addition of 5 mM dithiothreitol (DTT). For experiments conducted in the presence of $\Delta\bar{\mu}_{H^+}$, vesicles were first energized by addition of 20 mM ascorbate and 0.1 mM PMS for 30 s under an atmosphere of water-saturated oxygen prior to addition of NEM. Reactions were terminated by addition of 5 mM DTT. NEM

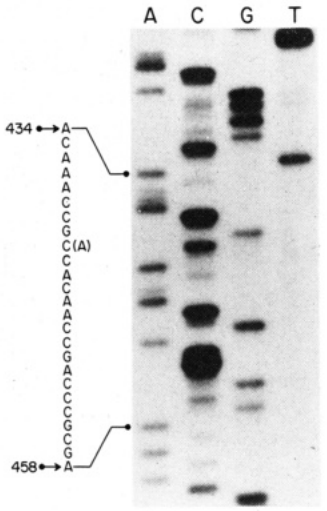

FIGURE 1: Dideoxy sequencing of recombinant M13mp19 ss DNA containing mutant ($Cys_{148} \rightarrow Gly$) *lac y*. Experimental procedures were carried out as described. The sequence in the vicinity of the mutation is shown at the left.

treatment was conducted at 25 °C, and the vesicles were assayed for [1-^{14}C]lactose transport immediately thereafter. Control samples received either no NEM or NEM was added after addition of 5 mM DTT. In either case, identical rates of lactose transport were observed.

Protein Determinations. Protein was measured as described by Lowry et al. (1951) with bovine serum albumin as a standard.

RESULTS

$Cys_{148} \rightarrow Gly$. (A) Verification of the Mutation by DNA Sequencing. *E. coli* T206 and G148 are derived from a chromosomal *lac* y^- strain, *E. coli* T184, by transformation with plasmid DNA. *E. coli* T206 carries plasmid pGM21 containing wild-type *lac y* (Teather et al., 1978, 1980), while *E. coli* G148 harbors plasmid pG148 containing a *lac y* gene modified by site-directed mutagenesis (Trumble et al., 1984). Thus, pG148 should be identical with pGM21 with the exception of a single base change at nucleotide 442 in *lac y* (A → C) that changes a Cys codon (TGT) to a Gly codon (GGT). To verify the mutation, the *lac y* gene from pG148 was cloned into RF M13mp19 DNA, and ss phage DNA harboring the antisense strand of *lac y* was isolated (cf. Experimental Procedures). The ss template DNA was then sequenced (Sanger et al., 1977; Sanger & Coulson, 1978) by using a primer complementary to a region of *lac y* 50 bases downstream from the mutation. Due to the nature of dideoxy sequencing, the sequence shown (Figure 1) corresponds to that of the sense strand of *lac y*. The autoradiogram clearly reveals a single base substitution (A → C) at position 442. Although the remainder of *lac y* in pG148 has not been sequenced completely, no other differences between wild-type and mutant *lac y* DNA have been observed with synthetic sequencing primers complementary to three or four other regions of the gene (data not shown).

(B) Effect on Transport. The steady-state level of lactose accumulation in *E. coli* G148 is comparable to that observed in *E. coli* T206, and the initial rate of transport is reduced only about 4-fold in the mutant (Trumble et al., 1984). As shown in Figure 2A, these properties are retained by RSO membrane vesicles. In the absence of electron donors, initial rates of lactose transport and steady-state levels of accumulation are negligible in both vesicle preparations. When ascorbate/PMS is added, T206 and G148 vesicles accumulate lactose to similar

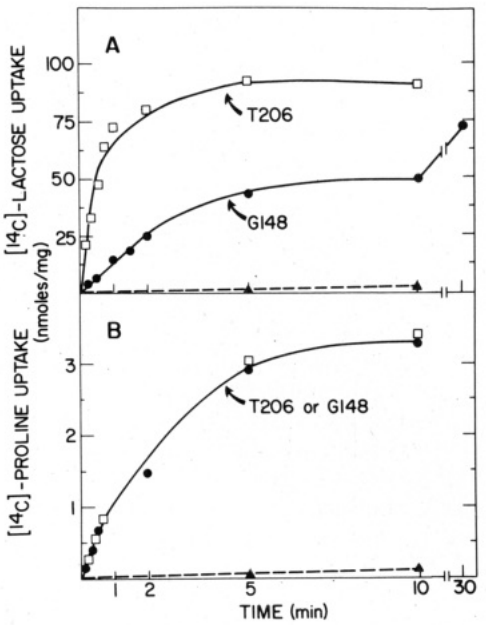

FIGURE 2: Time course of lactose (A) and proline (B) transport in T206 and G148 membrane vesicles. (A) Aliquots (50 μL) of T206 (□) or G148 (●) vesicles containing 70 μg of protein in 50 mM potassium phosphate (pH 6.6)/10 mM $MgSO_4$ were assayed for [1-^{14}C]lactose transport at 25 °C in the presence of 20 mM ascorbate and 1 mM PMS under oxygen. Reactions were initiated by the addition of [1-^{14}C]lactose (19 mCi/mmol) to a final concentration of 0.4 mM and terminated by the addition of 3 mL of 0.1 M potassum phosphate (pH 5.5) containing 0.1 M lithium chloride. Samples were then filtered immediately on nitrocellulose filters (0.45 μm) and washed once with the same solution. Radioactivity retained on the filters was determined by liquid scintillation spectrometry. (▲) Transport in the absence of ascorbate/PMS. (B) T206 (□) and G148 (●) membrane vesicles were incubated with ascorbate and PMS as described above, and [U-^{14}C]proline (286 mCi/mol) was added to 6.94 μM final concentration to initiate the reactions. (▲) Transport in the absence of ascorbate and PMS.

steady states, although the rate of transport in G148 vesicles is significantly lower than that observed in T206 vesicles. In contrast, both vesicle preparations transport proline at identical rates and to identical steady-state levels of accumulation (Figure 2B).

To determine whether the diminished rate of lactose transport in G148 vesicles reflects a decreased V_{max} or an increase in the apparent K_m, kinetic analyses were performed (Figure 3). As determined from the slopes of the functions in the Eadie–Hofstee representation, very similar apparent K_m values of 0.48 and 0.46 mM are obtained for T206 and G148 membrane vesicles, respectively. On the other hand, the extrapolated ordinate values, which yield V_{max}, are markedly different (note the differences in the abscissa and ordinate values in Figure 3). The V_{max} value observed for G148 vesicles [22 nmol min^{-1} (mg of protein)$^{-1}$] is about one-fifteenth of that observed for T206 vesicles [336 nmol min^{-1} (mg of protein)$^{-1}$]. It should be emphasized that this discrepancy in V_{max} is much larger than that observed in intact cells (data not shown).

Simplistically, a decrease in V_{max} can result either from a lower turnover number for the mutant permease or from a decrease in the number of permease molecules in the membrane of G148. Binding studies with Mab 4B1, which is directed against an externally disposed epitope in the permease (Carrasco et al., 1982, 1984a,b; Herzlinger et al., 1984), were used to examine the latter possibility (Figure 4). Clearly, similar titration curves are obtained with T206 and G148 membrane vesicles. Binding of ^{125}I-labeled 4B1 increases linearly to a maximum (20 pmol of bivalent IgG/31 μg of

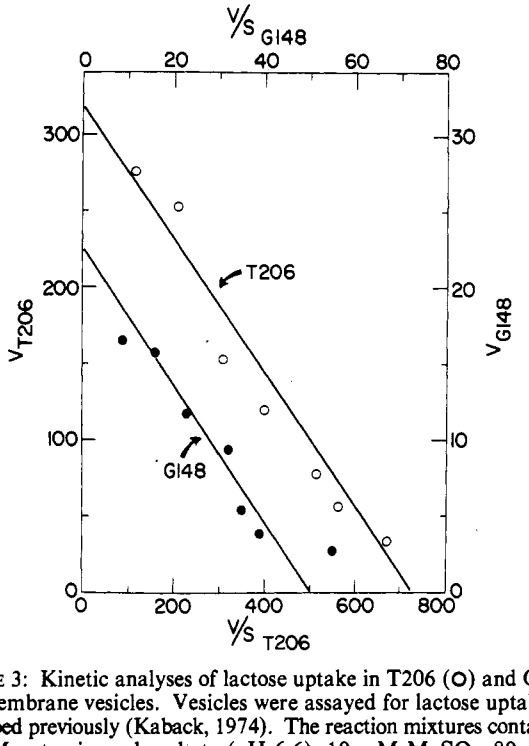

FIGURE 3: Kinetic analyses of lactose uptake in T206 (○) and G148 (●) membrane vesicles. Vesicles were assayed for lactose uptake as described previously (Kaback, 1974). The reaction mixtures contained 50 mM potassium phosphate (pH 6.6), 10 mM $MgSO_4$, 80 μg of membrane protein, 1 mM PMS, 20 mM potassium ascorbate, and concentrations of [1-^{14}C]lactose (59 mCi/mmol) ranging from 0.05 to 1.9 mM. Initial velocities were calculated from the early linear portions of the uptake curves at each lactose concentration. Data are presented as Eddie–Hofstee plots where the intercept with the y axis represents V_{max} and the slope of the function yields the apparent K_m. Because of the disparity in V_{max} between T206 and G148 vesicles, the axes for T206 are 10-fold greater than those for G148.

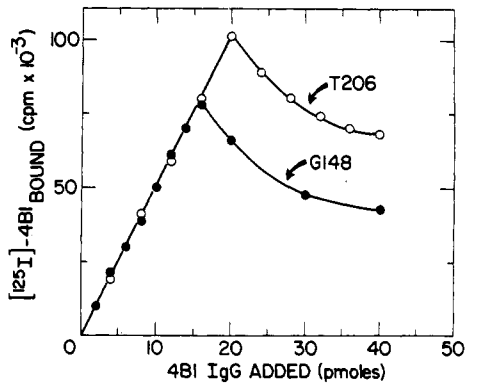

FIGURE 4: Binding of Mab 4B1 to RSO vesicles from T206 and G148. Membrane vesicles (31 μg of membrane protein/sample) from T206 (○) and G148 (●) were incubated at room temperature in 50 mM potassium phosphate (pH 7.5) containing 5% bovine serum albumin for 1 h. Given amounts of ^{125}I-labeled 4B1 IgG were added, and binding was assayed as described under Experimental Procedures. Values were corrected for nonspecific binding by incubating the vesicles in a 10-fold molar excess of unlabeled 4B1 IgG prior to incubation with ^{125}I-labeled 4B1.

T206 membrane protein; 16 pmol of IgG/31 μg of G148 membrane protein) and then decreases due to displacement of ^{125}I-labeled 4B1 by unlabeled IgG which has a higher affinity (Herzlinger et al., 1984). Furthermore, the values obtained are similar to those reported previously and indicate that the *lac* permease constitutes about 6% of the membrane protein in T206, as well as G148 (Herzlinger et al., 1984).

NPG is a potent competitive inhibitor of lactose transport that binds to the *lac* permease with a K_D corresponding to its K_i (Rudnick et al., 1976). Furthermore, comparative binding studies with this ligand and Mab 4B1 indicate that 1 mol of

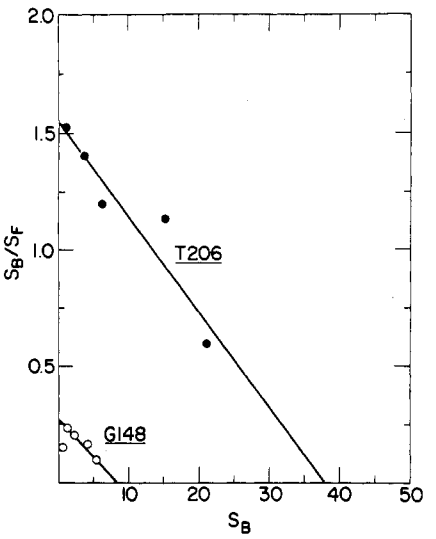

FIGURE 5: NPG binding in T206 and G148 membrane vesicles. Binding of [^{3}H]NPG was assayed by flow dialysis under nonenergized conditions [cf. Experimental Procedures and Rudnick et al. (1976)]. The upper chamber contained 0.2 mL of T206 or G148 membrane vesicles (26 mg of protein/mL) suspended in 100 mM potassium phosphate (pH 7.5), and the [^{3}H]NPG concentration varied from 1.9 to 57.8 μM. After equilibrium was achieved, 20 mM TDG was added to the upper chamber, and the amount of NPG bound was calculated from the increase in radioactivity in the dialysate. Data are plotted according to Scatchard (1949).

Table I: Effect of pH on NEM Inactivation of Lactose Transport[a]

membrane vesicles	rate of NEM inactivation [$t_{1/2}$ (min)] at pH		
	5.5	6.6	7.5
T206	5.1	3.1	1.1
G148	30.0	11.0	1.2
ML 308-225[b]	4.8	3.2	1.1

[a] RSO membrane vesicles were washed with 50 mM potassium phosphate/10 mM $MgSO_4$ at the indicated pH and resuspended in the same buffer at 1–2 mg/mL of protein. The vesicles were then exposed to NEM (0.5 mM with T206 and ML 308-225 vesicles; 1.0 mM with G148 vesicles). Reactions were terminated at various times by addition of 5 mM DTT. Initial rates of [1-^{14}C]lactose uptake in the presence of oxygen and ascorbate/PMS were measured immediately, and $t_{1/2}$ values were calculated from semilogarithmic plots (cf. Figure 7). [b] Values taken from Cohn et al. (1981).

NPG is bound/mol of permease (Herzlinger et al., 1984). Scatchard analyses of NPG binding experiments with T206 and G148 vesicles yield K_D values of 24.5 and 29.6 μM, respectively (Figure 5). At saturation, about 1.23 nmol of NPG is bound/mg of membrane protein with T206 vesicles, a value that is entirely consistent with the amount of permease present, as determined by Mab 4B1 binding. In contrast, however, the amount of NPG bound to G148 vesicles at saturation is only about 0.26 nmol/mg of membrane protein, a value one-fourth to one-fifth of that determined by NPG binding, and no evidence for a component with a higher K_D is observed. As discussed below, the reason for the discrepancy is not understood.

Effect of the Mutation on Sulfhydryl Inactivation and Substrate Protection. Replacement of Cys_{148} with Gly does not make the *lac* permease completely refractory to sulfhydryl reagents, and NEM inactivation of lactose transport in *E. coli* G148, albeit slow, can be carried out to completion (Trumble et al., 1984; cf. Figure 8). The data presented in Table I summarize the effect of pH on the half-time ($t_{1/2}$) for NEM inactivation of lactose transport in T206 and G148 vesicles; data for ML 308-225 membrane vesicles are taken from Cohn

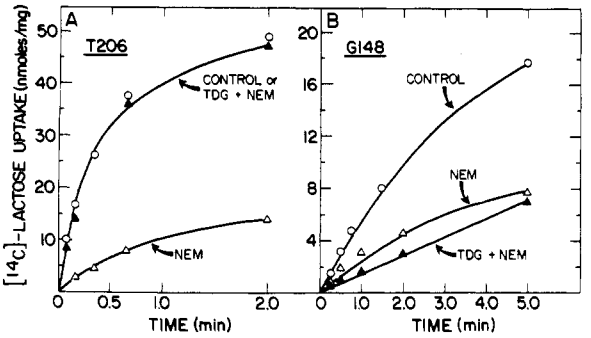

FIGURE 6: Effect of TDG on NEM inactivation of lactose transport in T206 and G148 vesicles. (A) T206 membrane vesicles were suspended in 50 mM potassium phosphate (pH 7.5) and treated for 1 min at 25 °C with 1 mM NEM in the presence (▲) or absence (△) of 20 mM TDG. Reactions were stopped by addition of 5 mM DTT. The vesicles were then extensively washed and resuspended in 50 mM potassium phosphate (pH 6.6) and assayed for lactose transport as described in Figure 2. Control samples (○) were treated with 5 mM DTT prior to addition of NEM. (B) Experimental procedures were identical with those described in (A) except that G148 vesicles were used, and treatment with NEM was carried out for 3 min.

et al. (1981) and are presented for comparison. In each case, NEM inactivation is a pseudo-first-order process at all pH values tested (not shown; cf. Figure 8). Clearly, the pH dependence for NEM inactivation in vesicles from T206 is similar to that observed for ML 308-225 vesicles, with the rate of inactivation increasing about 5-fold from pH 5.5 to pH 7.5. In contrast, the pH dependence of NEM inactivation in G148 vesicles is highly exaggerated, increasing at least 25-fold over the same pH range.[2] Interestingly, the effect is due primarily to slower rates of inactivation at more acidic pH values. Thus, at pH 5.5, the rate of NEM inactivation in G148 vesicles is about one-sixth of that observed in T206 or ML 308-225 vesicles; at pH 6.6, it is about one-fourth; and at pH 7.5, the rate of NEM inactivation is similar in all three vesicle preparations.

Although lactose transport in *E. coli* G148 is inactivated by NEM at a relatively slow rate, TDG affords no protection whatsoever against inactivation (Trumble et al., 1984), and this result is also obtained with isolated membrane vesicles (Figure 6). When T206 vesicles are treated with 1.0 mM NEM for 1 min, the initial rate of lactose transport is decreased to about 10% of that observed in untreated vesicles (panel A). When NEM treatment is carried out in the presence of TDG, however, complete protection against inactivation is observed. In contrast, when similar experiments are carried out with G148 vesicles, NEM treatment for 3 min inactivates the initial rate of lactose transport by only 45%, and slight enhancement of inactivation is observed when NEM treatment is carried out in the presence of TDG (panel B). The results taken as a whole are consistent with the original conclusion of Fox & Kennedy (1965), who suggested that there is a sulfhydryl group in the *lac* permease that is essential for substrate protection against inactivation by NEM. Since G148 vesicles catalyze active transport with an unaltered apparent K_m for lactose and an unaltered K_D for NPG, however, it seems unlikely that the sulfhydryl group at position 148 is required for binding.

Further evidence supporting this conclusion is provided by binding experiments with NPG (Figure 7). In the upper tracing, results of a flow dialysis experiment with T206 membrane vesicles under nonenergized conditions are pres-

[2] It should be noted that the concentration of NEM used with G148 vesicles was 1.0 mM, as opposed to 0.5 mM with T206 vesicles.

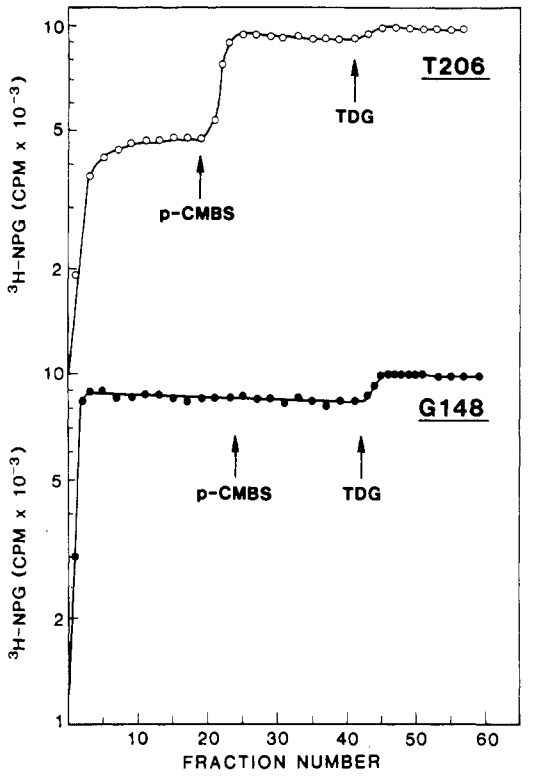

FIGURE 7: Effect of pCMBS on binding of [^{3}H]NPG. Flow dialysis was carried out with 0.2 mL of T206 (○) or G148 (●) membrane vesicles (26 mg/mL protein) suspended in 100 mM potassium phosphate (pH 7.5) as described under Experimental Procedures. [^{3}H]NPG (9.46 Ci/mmol) was added at fraction 1 to a final concentration of 14.5 μM. As indicated by arrows, pCMBS and TDG were added to the upper chamber at final concentrations of 1 and 20 mM, respectively.

ented, and it is apparent that bound NPG is displaced by either pCMBS or TDG [in the experiment shown, a submaximal concentration of pCMBS was used in order to observe the effect of TDG, although either compound releases bound ligand completely [not shown; cf. Rudnick et al. (1976)]]. The analogous experiment with G148 vesicles is also shown, and it is clear that pCMBS does not affect release of bound NPG (Figure 7, lower tracing). Addition of TDG, on the other hand, leads to release of ligand, but the absolute amount released is only about one-third of that observed in T206 vesicles (cf. above).

Effect of $\Delta\bar{\mu}_{H^+}$ *on NEM Inactivation.* As demonstrated by Cohn et al. (1981), the *lac* permease in ML 308-225 vesicles exhibits enhanced sensitivity to inactivation by various maleimides in the presence of $\Delta\bar{\mu}_{H^+}$ (interior negative and/or alkaline). When T206 vesicles are treated with NEM under nonenergized or energized conditions (i.e., in the absence or presence of $\Delta\bar{\mu}_{H^+}$) and then assayed for initial rates of lactose transport, as reported previously, the rate of inactivation increases almost 2-fold in the presence of $\Delta\bar{\mu}_{H^+}$ (Figure 8A; $t_{1/2}$ decreases from 1.6 to 0.85 min). The effect is enhanced in G148 vesicles where there is a 3.5-fold increase in the rate of NEM inactivation in the presence of $\Delta\bar{\mu}_{H^+}$ (Figure 8B; $t_{1/2}$ decreases from 11.1 to 3.2 min).

Additional data summarizing the effect of pH on $\Delta\bar{\mu}_{H^+}$-enhanced NEM inactivation of *lac* permease activity in T206 and G148 vesicles are given in Table II with values for ML 308-225 vesicles for comparison (Cohn et al., 1981). The pH profiles for T206 and ML 308-225 vesicles, which contain wild-type permease, are virtually identical with maximal stimulation of NEM inactivation by $\Delta\bar{\mu}_{H^+}$ at pH 6.6. Vesicles

Table II: Effect of $\Delta\bar{\mu}_{H^+}$ on NEM Inactivation of Lactose Transport as a Function of pH[a]

membrane vesicles	inhibn ratio $[t_{1/2}(-\Delta\bar{\mu}_{H^+})/t_{1/2}(+\Delta\bar{\mu}_{H^+})]$ at pH		
	5.5	6.6	7.5
T206	1.1	1.9	1.7
G148	4.5	3.7	2.4
ML 308-225[b]	1.1	2.5	1.7

[a] RSO membrane vesicles were washed and resuspended as described in Table I. Vesicles were then treated with NEM (0.5 mM with T206 and ML 308-225 vesicles; 1.0 mM with G148 vesicles) in the absence of $\Delta\bar{\mu}_{H^+}$ as described in Table I. To determine the effect of $\Delta\bar{\mu}_{H^+}$ on the rate of NEM inactivation, the same vesicles were incubated in the presence of oxygen and 20 mM ascorbate/0.1 mM PMS for 30 s prior to and during treatment with NEM. After the reactions were terminated with 5 mM DTT, initial rates of [1-^{14}C]lactose uptake were measured immediately in the presence of oxygen ascorbate/PMS. Values given are the ratio of half-times ($t_{1/2}$) for NEM inactivation observed in the absence vs. the presence of $\Delta\bar{\mu}_{H^+}$. [b] Values taken from Cohn et al. (1981).

from G148, on the other hand, exhibit significantly more marked responses to $\Delta\bar{\mu}_{H^+}$ at all pHs tested, and the greatest stimulation is observed at pH 5.5 (4.1-fold) where the wild-type permease exhibits essentially no stimulation of NEM inactivation by $\Delta\bar{\mu}_{H^+}$.

Discussion

The findings presented in this paper confirm and extend previous studies by Trumble et al. (1984) utilizing oligonucleotide-directed, site-specific mutagenesis of the *lac y* gene of *E. coli* to demonstrate that a sulfhydryl function at Cys_{148} in the *lac* permease is not obligatory for lactose/proton symport. Thus, RSO membrane vesicles from a mutant in which Gly is substituted for Cys_{148} in the permease accumulate lactose to a steady-state level comparable to that of vesicles containing wild-type permease, although the initial rate of transport is significantly reduced. The overall conclusion is important, as sulfhydryl groups have received considerable attention as critical residues in permease function since 1965, when Fox & Kennedy (1965) first demonstrated that NEM inactivation of lactose transport is protected by certain permease substrates. Having documented the presence of the appropriate alteration at nucleotide 442 in *lac y* and described the functional properties of the permease superficially, the intent was to characterize the altered system more fully in RSO vesicles in order to understand what role, if any, Cys_{148} plays in substrate binding and/or translocation.

Kinetic analyses reveal that the diminished rate of transport in G148 vesicles is the consequence of a 15-fold decrease in the V_{max} with no significant change in the apparent K_m for lactose. Furthermore, binding studies with NPG demonstrate that the K_D for NPG is essentially the same in G148 and T206 vesicles. It is unlikely, therefore, that Cys_{148} is directly involved in binding. Rather, it seems probable that alkylation of Cys_{148} leads to loss of binding and transport due to a steric effect and/or a conformational alteration with long-range secondary effects.

Binding studies with Mab 4B1 demonstrate that similar amounts of permease are incorporated into T206 and G148 membranes, and although not shown, almost identical results were obtained with Mab 4A10R (Herzlinger et al., 1985) and a site-directed polyclonal antibody directed against the carboxyl terminus of the permease (Carrasco et al., 1984). Therefore, the diminished V_{max} observed in G148 vesicles cannot be attributed to a decrease in the specific content of permease in the membrane.

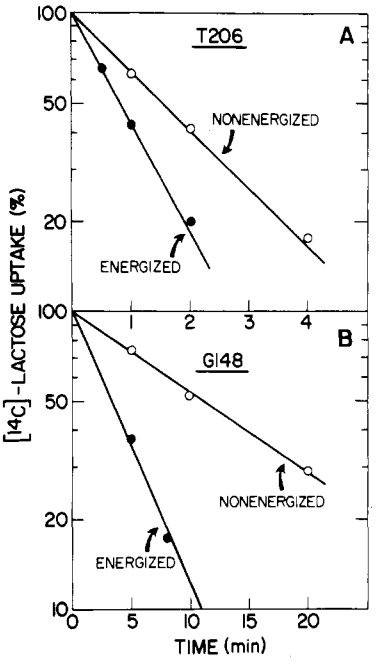

FIGURE 8: Time course of NEM inactivation of lactose transport in T206 (A) and G148 (B) membrane vesicles in the absence and presence of $\Delta\bar{\mu}_{H^+}$. T206 (A) or G148 (B) membrane vesicles were washed and suspended at 1–2 mg of protein/mL in 50 mM potassium phosphate (pH 6.6)/10 mM $MgSO_4$. For inhibition in the absence of $\Delta\bar{\mu}_{H^+}$ (○), NEM was added to a final concentration of 1.0 mM, and the sample was incubated at 25 °C. Aliquots were withdrawn, and 5 mM DTT was added at the times indicated. The samples were then assayed for initial rates of transport using 0.4 mM [1-^{14}C]lactose (19 mCi/mmol) as described under Experimental Procedures. For NEM inhibition in the presence of $\Delta\bar{\mu}_{H^+}$ (●), the vesicles were incubated with 20 mM ascorbate and 0.1 mM PMS under oxygen for 30 s before the addition of 0.5 mM NEM (final concentration). The reaction was stopped at given times by addition of 5 mM DTT, and 50-μL aliquots were assayed immediately for initial rates of lactose transport without further addition of ascorbate/PMS. Results are given as a percentage of the initial rates observed in control samples to which 5 mM DTT was added prior to NEM.

Although G148 vesicles exhibit a normal K_D for NPG and antibody binding studies demonstrate clearly that the mutant vesicles contain almost as many permease molecules as T206 vesicles, surprisingly, the mutant permease binds only about one-fourth as much NPG as wild-type permease at saturating NPG concentrations. It is likely, therefore, that about three-fourths of the permease molecules in the mutant are nonfunctional. One possible explanation for the discrepancy is that substitution of Gly for Cys at position 148 makes the permease sufficiently unstable that a significant number of molecules are rendered nonfunctional. In this context, it is noteworthy that Gly residues are "helix breaking" (Chou & Fasman, 1974). Moreover, recent studies (Kaback, 1985b) with a mutant containing Ser at residue 148 of the permease rather than Gly transport lactose at initial rates that are similar to those of *E. coli* T206. In any event, if the V_{max} for lactose transport in G148 vesicles is corrected for the amount of nonfunctional permease, the difference in activity between T206 and G148 vesicles approximates that observed in the intact cells (i.e., 3–4-fold, rather than 15-fold).

Preliminary studies (Trumble et al., 1984) with intact cells indicate that the altered permease in G148, although less sensitive to NEM, is inactivated by alkylation with the sulfhydryl reagent over longer periods of exposure, thus suggesting that a Cys residue other than that at position 148 may be essential for symport. Further evidence in support of this conclusion is presented here. Clearly, inactivation of the

wild-type permease by NEM in T206 and ML 308-225 vesicles is virtually identical, increasing about 5-fold in rate from pH 5.5 to pH 7.5 and about 2-fold in response to $\Delta\bar{\mu}_{H^+}$ under optimal conditions (i.e., at pH 6.6). In contrast, the rate of inactivation of the mutant permease is markedly diminished at acidic pH values (i.e., at pH 5.5 and 6.6). Furthermore, the effect of $\Delta\bar{\mu}_{H^+}$ on inactivation of the mutant permease is enhanced, increasing the rates as much as 4.1-fold at pH 5.5. Given these observations, it seems likely that in addition to playing a critical role in symport, the "other" Cys residue(s) is (are) responsible for enhanced reactivity to maleimides in the presence of $\Delta\bar{\mu}_{H^+}$ (Cohn et al., 1981), an effect that is partially obscured when Cys_{148} is present.

While the mutant permease in G148 vesicles is inactivated by NEM, albeit at a slow rate relative to the wild type, no protection whatsoever is observed in the presence of TDG. Rather, when NEM inactivation is carried out in the presence of the thiogalactoside, slightly enhanced rates of inactivation are routinely observed with G148 vesicles. These results are in striking contrast to those obtained with T206 vesicles where TDG almost completely protects the permease against inactivation by NEM. Taken together, the observations are consistent with the following conclusions: (i) wild-type *lac* permease is inactivated by NEM principally through alkylation of Cys_{148}; (ii) Cys_{148} is not obligatory for either substrate binding or translocation but is protected from alkylation by TDG; and (iii) the sulfhydryl group responsible for NEM inactivation of lactose transport in G148 is less reactive than Cys_{148}, is not protected from alkylation by TDG, and, under certain conditions, may not react with NEM in the wild-type permease (i.e., TDG affords complete protection against NEM inactivation of the wild-type permease). Finally, it is unlikely that this sulfhydryl group is involved in binding, as pCMBS does not displace bound NPG from vesicles containing the mutant permease. Attempts to identify the essential Cys residue(s) in G148 by oligonucleotide-directed, site-specific mutagenesis are currently in progress.

Added in Proof

Recent experiments (Menick et al., 1985) utilizing site-directed mutagenesis suggest that Cys_{154} plays an important role in lactose/proton symport. Thus, permease with Gly in place of Cys_{154} exhibits essentially no transport activity, while substitution of Cys_{154} with Ser also causes marked, though less complete, loss of activity.

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Registry No. Lactose permease, 9068-45-5.

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Interaction of Myotoxin *a* with Artificial Membranes: Raman Spectroscopic Investigation†

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ABSTRACT: Myotoxin *a* from the venom of *Crotalus viridis viridis* (prairie rattlesnake) is a small protein which is responsible for myonecrosis. It is a basic protein with 42 amino acid residues of known sequence. Three disulfide bonds give it a highly compact structure. Microscopic examination of the toxin's effects reveals that the most pronounced and earliest visible damage occurs intracellularly, in the sarcoplasmic reticulum membrane system of skeletal muscle. A better understanding of its mechanism of action is therefore of particular interest. The interaction of myotoxin *a* with artificial membranes (multibilamellar phospholipid dispersions) was investigated by using dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylserine (DMPS). Two regions of the Raman spectrum were examined for information: the C–H stretching region between 2800 and 3000 cm^{-1} and the C–C stretching region between 1000 and 1300 cm^{-1}. The effects of myotoxin *a* on the thermotropic phase behavior of the artificial membranes were determined. This was done by monitoring three structurally sensitive Raman intensity ratios, $I_{2932/2880}$, $I_{2880/2850}$, and $I_{1088/1126}$. It was found that myotoxin *a* destabilized the ordered structure of the gel phase of phospholipid bilayers. This effect was seen with both DMPC and DMPS. The pretransition of DMPC was perturbed by myotoxin *a*, while the main gel to liquid-crystal phase transition temperature was decreased. The effect of myotoxin *a* on the phase behavior of DMPS was found to be pH dependent with the least effect observed at low pH values. These results suggest the involvement of negatively charged phosphate groups of phospholipids in the interaction of myotoxin *a* with artificial membranes.

Myotoxin *a*, a low molecular weight basic protein from the venom of *Crotalus viridis viridis*, is a well-characterized myonecrotic factor found widely among rattlesnake venoms (Ownby et al., 1976; Pool et al., 1981; Tu, 1982). It is representative of the small myotoxins, whose biological activity appears to be quite distinct from the large myotoxins (Gutierrez et al., 1984). Vacuolization of the sarcoplasmic reticulum is the first observable effect small myotoxins have on muscle tissue. Recent electron microscopic evidence shows that horseradish peroxidase conjugated myotoxin *a* is located at the sarcoplasmic reticulum membrane (Tu & Morita, 1983). The mechanism of the toxin's action is of considerable interest since the first site of tissue damage is at an intracellular location. The effect of myotoxin *a* on the Ca^{2+}-ATPase of sarcoplasmic reticulum is of particular interest.

Phospholipids in aqueous systems have characteristic phase behaviors which depend on the nature of the polar head groups, as well as chain length and degree of unsaturation of the acyl chains. They exist in a densely packed array in the gel phase below the main transition temperature (T_m).[1] This is a highly ordered state with maximal lateral packing between the acyl chains from neighboring phospholipids. Above the T_m, lipids are in a less ordered state known as the liquid-crystal phase. Under these conditions, lateral interactions between acyl chains are reduced and hydrocarbon chain packing decreases.

A number of Raman spectral parameters, including frequency and intensity, have been shown to be sensitive to the structural dynamics of phospholipids in aqueous dispersion. The hydrocarbon skeletal C–C stretching region provides information about the conformation of the acyl chains (Lippert & Peticolas, 1972), while the C–H region reflects the packing of the hydrocarbon chains (Lavialle & Levin, 1980). Numerous studies have been reported on the effect of various perturbants [including cholesterol (Bush et al., 1980), proteins (Curatolo et al., 1978), peptides (Verma & Wallach, 1976), and ions and ionophores (Hank & Ho, 1980; Mendelson et al., 1982)] on the phase transition characteristics of phospholipid dispersions.

We report here studies on the effects of myotoxin *a* on the thermotropic phase behavior of multibilamellar dispersions of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylserine (DMPS). These effects were observed by

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[1] Abbreviations: DMPC, dimyristoylphosphatidylcholine; DMPS, dimyristoylphosphatidylserine; *I*, Raman peak intensity; T_m or T_{m_2}, primary gel to liquid-crystal phase transition; T_{m_1}, pretransition or head-group transition seen with DMPC.

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