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A conformational change in the lactose permease of *Escherichia coli* is induced by ligand binding or membrane potential

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Abstract

Lactose transport in membrane vesicles containing lactose permease with a single Cys residue in place of Val 315 is inactivated by *N*-ethylmaleimide in a manner that is stimulated by substrate or by a H^+ electrochemical gradient ($\Delta\bar{\mu}_{H^+}$; Sahin-Tóth M, Kaback HR, 1993, *Protein Sci 2*:1024–1033). The findings are confirmed and extended in this communication. Purified, reconstituted Val 315 → Cys permease reacts with *N*-ethylmaleimide or hydrophobic fluorescent maleimides but not with a membrane impermeant thiol reagent, and β-galactosides specifically stimulate the rate of labeling. Furthermore, the reactivity of purified Val 315 → Cys permease is enhanced by imposition of a membrane potential ($\Delta\Psi$, interior negative). The results indicate that either ligand binding or $\Delta\Psi$ induces a conformational change in the permease that brings the N-terminus of helix X into an environment that is more accessible from the lipid phase.

Keywords: bioenergetics; Cys modification; membrane proteins; membrane transport; site-directed mutagenesis

The lactose permease of *Escherichia coli* is a hydrophobic, polytopic cytoplasmic membrane protein that catalyzes the coupled translocation of β-galactosides and H^+ with a stoichiometry of unity (e.g., symport or cotransport). Encoded by the *lacY* gene, the permease has been solubilized from the membrane, purified, reconstituted into proteoliposomes, and shown to be solely responsible for β-galactoside transport in monomeric form (reviewed by Kaback, 1989, 1992; Kaback et al., 1993; Sahin-Tóth et al., 1994). Based on circular dichroism and hydropathy analysis of the primary amino acid sequence, a secondary-structure model was proposed in which the protein consists of a short hydrophilic N-terminus, 12 hydrophobic domains in α-helical conformation that traverse the membrane in zig-zag fashion connected by hydrophilic loops, and a 17-amino acid residue hydrophilic C-terminal tail (Foster et al., 1983). Evidence supporting the general features of the model and demonstrating that both the N and C termini (hydrophilic domains 1 and 13, respectively), as well as hydrophilic domains 5 and 7, are on the cytoplasmic face of the membrane was obtained from spectroscopic techniques, limited proteolysis, immunological studies, and chemical modification (see Kaback, 1992). Moreover, the 12-helix model has received unequivocal support from the analysis of an extensive series of lac permease–alkaline phosphatase (*lacY–phoA*) fusions (Calamia & Manoil, 1990). Most recently, the use of site-directed fluorescence labeling has led to a model describing helix packing in the C-terminal half of the permease (Jung et al., 1993; Kaback et al., 1993).

As part of an extensive, ongoing study with a functional permease mutant devoid of Cys residues (van Iwaarden et al., 1991), putative helices IX and X were subjected to Cys-scanning mutagenesis (Sahin-Tóth & Kaback, 1993). Out of 46 amino acid residues that were replaced individually with Cys, only Arg 302 (Menick et al., 1987; Matzke et al., 1992) in helix IX and His 322 (Padan et al., 1985; Püttner et al., 1986, 1989; King & Wilson, 1989a, 1989b, 1990) and Glu 325 (Carrasco et al., 1986, 1989) in helix X were found to be important for active lactose transport. Furthermore, studies with *N*-ethylmaleimide show that only a few Cys-replacement mutants are inactivated significantly. Remarkably, however, the rate of inactivation of V315C[1] permease (cf. Fig. 1) is dramatically enhanced in the presence of

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Abbreviations: lac, lactose; $\Delta\bar{\mu}_{H^+}$, H^+ electrochemical gradient; NEM, *N*-ethylmaleimide; $\Delta\Psi$, membrane potential; PM, *N*-(1-pyrene) maleimide; CPM, 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin; TDG, β,D-galactopyranosyl 1-thio-β,D-galactopyranoside; stilbenemaleimide, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonate; OG, octyl-β,D-glucopyranoside; DTT, dithiothreitol.

[1] Site-directed mutants are designated as follows: the 1-letter amino acid code is used followed by a number indicating the position of the residue in wild-type lac permease. The sequence is followed by a second letter denoting the amino acid replacement at this position.

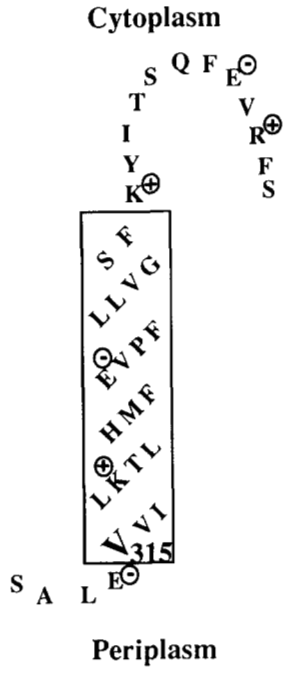

Fig. 1. Secondary-structure model of putative transmembrane domain X and adjacent hydrophilic loops of lac permease (Foster et al., 1983). Val 315 is highlighted.

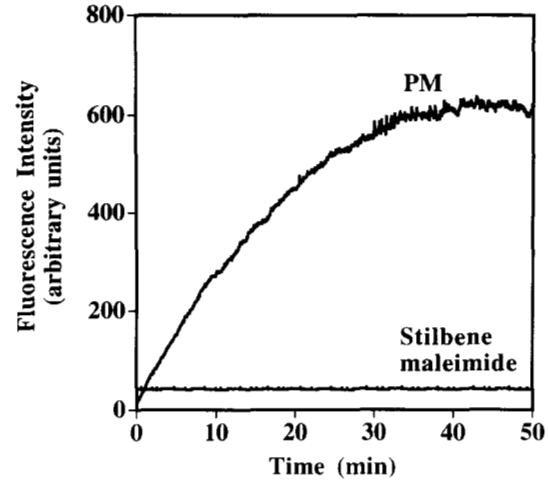

Fig. 2. Time course of reaction of PM and stilbenemaleimide with V315C proteoliposomes. Two milliliters of 50 mM KPi (pH 7.5) containing 1 μM PM or 10 μM stilbenemaleimide was placed in a cuvette, 54 μg V315C protein reconstituted into proteoliposomes was added, and fluorescence was recorded with continuous stirring at 25 °C. With PM, fluorescence emission was measured at 379 nm with excitation at 344 nm. The data were corrected for controls obtained with reconstituted C-less permease. With the control, the increase in fluorescence did not exceed 20% of the labeling rate obtained with V315C proteoliposomes. Stilbenemaleimide fluorescence was measured at an emission wavelength of 411 nm with excitation at 322 nm.

β,D-galactopyranosyl 1-thio-β,D-galactopyranoside or an H^+ electrochemical gradient, thereby raising the possibility that ligand binding or $\Delta\tilde{\mu}_{H^+}$ may induce a similar conformational alteration in the permease. Jung et al. (1994) have demonstrated that E269C or H322C permease also undergoes a marked increase in reactivity with *N*-(1-pyrene)maleimide, and other studies using a similar approach (J. Wu & H.R. Kaback, in prep.) indicate that ligand binding may induce a widespread conformational change in the permease. Because the observations with V315C permease in right-side-out membrane vesicles suggest that either ligand binding or $\Delta\tilde{\mu}_{H^+}$ may induce the same conformational change, we have focused initially on V315C permease in order to develop the methodology to study this postulate in more detail.

The experiments presented here utilize purified, reconstituted V315C permease, and the findings demonstrate that either ligand binding or imposition of $\Delta\tilde{\mu}_{H^+}$ leads to an increase in the reactivity of a Cys residue at position 315 to hydrophobic maleimides.

Results

Reactivity of V315C permease with fluorescent maleimides

A variety of aromatic maleimides become fluorescent only after reaction with thiols (Haugland, 1992), and this property can be used to study the reactivity of Cys residues in lac permease fluorimetrically (Jung et al., 1994). In the experiments shown in Figure 2, proteoliposomes reconstituted with purified V315C permease were added to a cuvette containing a fluorescent maleimide to initiate the reaction. With PM, a hydrophobic thiol reagent, an increase in fluorescence intensity at 379 nm (excitation at 344 nm) is observed during incubation with the reconstituted mutant protein, and the reaction is complete in about 30 min. Although data are not presented, very similar behavior is observed with 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin, another hydrophobic probe. In marked contrast, V315C permease exhibits no reactivity whatsoever with the membrane impermeant fluorescent probe 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonate. The results are consistent with the interpretation that Cys 315 is not exposed to the aqueous phase.

Effect of ligand on the reactivity of V315C permease

In order to test the influence of substrate on the reactivity of V315C permease with PM, the high-affinity substrate TDG was added to the cuvette during incubation of proteoliposomes containing V315C permease with PM. In 3 independent experiments with different protein preparations, addition of 5 mM TDG caused a 4–6-fold increase in labeling rate (Fig. 3). When the experiment is repeated at different TDG concentrations, it is apparent that the effect saturates with increasing concentrations of TDG and that the concentration of the analog that produces half-maximal stimulation is about 0.8 mM (Fig. 4). TDG also enhances the reactivity of V315C permease with PM after solubilization in dodecylmaltoside, thereby suggesting that the conformation of the solubilized protein is similar to that of the reconstituted permease. The same experiments were also performed with stilbenemaleimide, and addition of TDG at concentrations up to 10 mM has no effect whatsoever (data not shown).

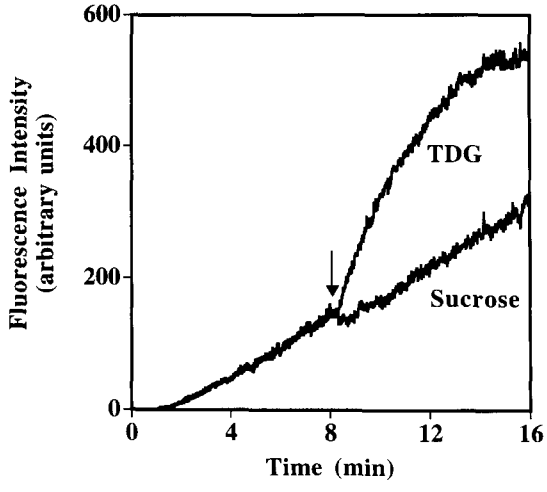

Fig. 3. Influence of TDG or sucrose on the time course of labeling of V315C proteoliposomes with PM. Two milliliters of 50 mM KPi (pH 7.5) containing 1 μM PM was placed in a cuvette and fluorescence was recorded (excitation wavelength, 344 nm; emission wavelength, 379 nm) with continuous stirring at 25 °C. After 1 min, proteoliposomes containing purified V315C permease (54 μg protein; protein/phospholipid ratio 1:10, w/w) were added and the measurements were continued. As indicated by the arrow, either TDG or sucrose was added to a final concentration of 10 mM or 100 mM, respectively. With TDG, the rate of labeling increases markedly; with sucrose, no change is observed. The data were corrected for controls carried out with proteoliposomes reconstituted with C-less permease. With C-less proteoliposomes, an increase in fluorescence is not observed when TDG was added.

Substrate specificity

Addition of 100 mM sucrose to reaction mixtures containing V315C proteoliposomes has no effect on the rate of fluorescence increase observed with PM (Fig. 3). The data presented in Table 1 summarize results obtained with a few other sugars. In addition to TDG, lactose at a relatively high concentration (50 mM) stimulates the rate of labeling by a factor of 2. In contradistinction, no effect is observed with 100 mM sucrose or glucose. The results indicate that the phenomenon described is specific for β-galactosides.

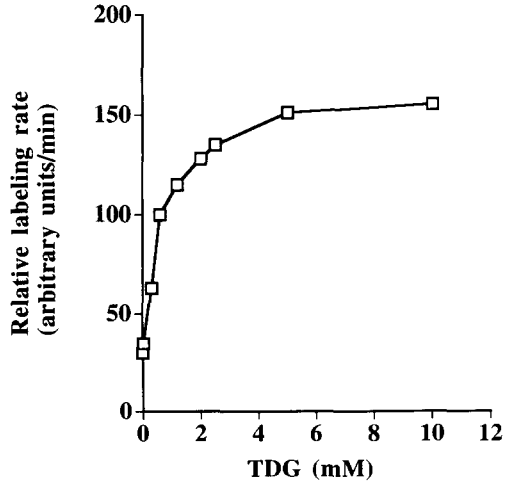

Fig. 4. Substrate dependence of V315C permease reactivity with PM. Experiments were carried out as described in Figure 2 for PM, except that TDG was added at given concentrations before addition of proteoliposomes and fluorescence was monitored for 2 min. Relative labeling rates were determined from the linear portion of the fluorescence increase.

Table 1. *Substrate specificity of the enhancement of reactivity of V315C proteoliposomes with PM*[a]

Substrate	Concentration (mM)	Relative labeling rate (arbitrary units/min)	Stimulation
No addition	–	30	–
TDG	10	151	5-Fold
Lactose	50	63	2-Fold
Sucrose	100	28	No stimulation
Glucose	100	35	No stimulation

[a] The experiments were carried out as described in Figure 2. Different sugars were added at given concentrations before each measurement and fluorescence was monitored for 22 min. Relative labeling rates were determined from the linear portion of the fluorescence increase.

Effect of TDG or $\Delta\Psi$ on reactivity of V315C permease with [^{14}C]NEM

The experiments described above were carried out with proteoliposomes reconstituted at a relatively high protein to phospholipid ratio (1:10, w/w) because PM partitions into the bilayer and reacts with the primary amino group of phosphatidylethanolamine. Although the reaction occurs at a relatively slow rate, when excess phospholipid is present, reaction with a single Cys residue in the permease is obscured. However, at high protein to phospholipid ratios, proteoliposomes are relatively permeant and will not sustain a $\Delta\bar{\mu}_{H^+}$ (Matsushita et al., 1983). Therefore, in order to test the postulate that either ligand binding or $\Delta\bar{\mu}_{H^+}$ induces increased reactivity of V315C permease, proteoliposomes were reconstituted at a much lower protein to phospholipid ratio (1:380, w/w), and reactivity with [^{14}C]NEM was tested under various conditions. As shown in Figure 5A, V315C proteoliposomes react with [^{14}C]NEM in essentially a linear fashion for about 10 min, and by 1 h, a maximum level of incorporation of 0.8 ± 0.09 mol NEM/mol V315C permease is attained. In the presence of 10 mM TDG, a 2-fold increase in the rate of labeling is observed. Although the effect is less than that observed with PM, the difference may be due to the smaller size of NEM, which could result in a higher accessibility of Cys 315 to the reagent, particularly in the absence of substrate. In any case, the maximum labeling ratio observed at 1 h is not altered by TDG. It is noteworthy that these experiments were carried out in the presence of valinomycin and nigericin to avoid generation of $\Delta\bar{\mu}_{H^+}$ (interior positive and acidic) resulting from downhill transport of TDG (cf. Kaback, 1989). When the ionophores are omitted, TDG exerts no significant stimulatory effect on [^{14}C]NEM labeling (Fig. 5A). Furthermore, addition of valinomycin and nigericin in the absence of TDG does not affect V315C permease reactivity (data not shown). Finally, when V315C proteoliposomes prepared in potassium phosphate and containing valinomycin are diluted into equimolar sodium phos-

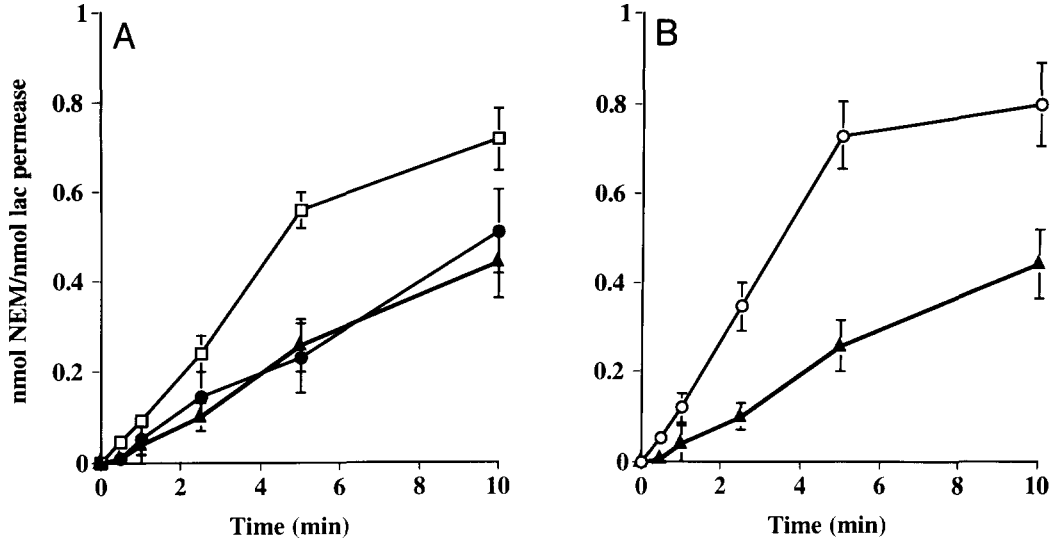

Fig. 5. Time course of reaction of V315C proteoliposomes with NEM. **A:** Influence of substrate. Proteoliposomes (50 μL) reconstituted with purified V315C permease (70 μg protein/mL in 50 mM KP_i [pH 7.5]) were diluted to a final volume of 100 μL in the same buffer. [^{14}C]NEM (39 mCi/mmol) was then added to a final concentration of 5 μM. The samples were incubated at 25 °C under the following conditions: in the absence of ligand (▲); in the presence of 10 mM TDG (●); in the presence of 20 μM valinomycin, 0.5 μM nigericin, and 10 mM TDG (□). After given periods of time, the reaction was stopped by addition of 1 mM DTT. The samples were filtered as described in the Materials and methods. Addition of valinomycin and nigericin in the absence of TDG does not stimulate the reactivity of V315C permease (data not shown). **B:** Influence of $\Delta\Psi$ (interior negative). A 20-μL aliquot of V315C proteoliposomes (175 μg protein/mL in 50 mM KP_i [pH 7.5]) containing 20 μM valinomycin was diluted 200-fold into 50 mM NaP_i (pH 7.5) containing 5 μM [^{14}C]NEM (39 mCi/mmol) and incubated at 25 °C (○). At the times indicated, the reactions were terminated by addition of 1 mM DTT. For measurements in the absence of $\Delta\Psi$ (▲), proteoliposomes were diluted into 50 mM KP_i (pH 7.5) as described above. All results were corrected for controls obtained with C-less proteoliposomes.

phate so as to generate a potassium diffusion potential (interior negative; cf. Viitanen et al., 1986), a 3-fold increase in the rate of [^{14}C]NEM labeling is observed with no effect on the maximum labeling stoichiometry (Fig. 5B). There is no effect on the labeling rate when the proteoliposomes are diluted into equimolar potassium phosphate (i.e., under conditions where no $\Delta\Psi$ is generated) (Fig. 5B).

Discussion

The data presented here confirm and extend previous studies on V315C permease (Sahin-Tóth & Kaback, 1993) and provide the basis for further studies in which the effects of ligand binding or $\Delta\bar{\mu}_{H^+}$ on the reactivity of single-Cys replacements in lac permease can be tested. Replacement of Val 315, presumably the N-terminal residue at the periplasmic end of helix X, with Cys in C-less permease has no effect on transport in intact cells, but treatment of this single-Cys mutant in right-side-out membrane vesicles with NEM leads to a relatively slow rate of inactivation. Most interestingly, either addition of TDG or generation of $\Delta\bar{\mu}_{H^+}$ enhances the rate of inactivation of lactose transport by the alkylating agent. Furthermore, although the effect of $\Delta\bar{\mu}_{H^+}$ is abolished by carbonylcyanide-*m*-chlorophenylhydrazone, the effect of TDG is unchanged in the presence of the protonophore in right-side-out membrane vesicles, which are significantly more ion permeable than proteoliposomes (see Garcia et al., 1983). Based on these observations, it was suggested that ligand binding or $\Delta\bar{\mu}_{H^+}$ may cause the permease to assume the same conformation.

In this study, site-directed labeling with fluorescent maleimides or NEM has been employed in a defined system using purified V315C permease reconstituted into phospholipid vesicles (i.e., proteoliposomes). The reactivity of Cys at position 315 with the hydrophobic maleimides NEM, PM, and CPM, coupled with the lack of reactivity with the membrane impermeant probe stilbenemaleimide suggest that Cys 315 is relatively inaccessible to the aqueous phase. However, the lack of reaction with stilbenemaleimide may also be explained by repulsion of the negatively charged probe by Glu 314 (cf. Fig. 1).

In full agreement with the results obtained by Sahin-Tóth and Kaback (1993) with right-side-out membrane vesicles, the rate of reaction of purified V315C permease with hydrophobic fluorescent maleimides is clearly enhanced in the presence of the high-affinity ligand TDG. Moreover, the effect appears to be related qualitatively at least to the affinity of the permease for the ligands tested. Thus, the concentration of TDG that produces half-maximal stimulation is about 0.8 mM, while lactose concentrations of 50 mM are needed to observe a significant effect. Although the "apparent K_D" for TDG obtained from these experiments is relatively high (i.e., the K_D for TDG is 20 μM; Wright et al., 1983), it is difficult to accurately quantitate the effective concentrations of ligand with respect to Cys reactivity because the hydrophobic nature of PM makes it impossible to saturate the velocity of its reaction with Cys residues in the permease. However, sucrose and glucose, which have little or no affinity for the permease, are without effect, indicating that the phenomenon is specific for substrates of the permease. Finally, replacement of Glu 314 with Ala has no ef-

fect on the increased rate of inactivation of V315C permease with NEM in the presence of TDG (S. Frillingos, M. Sahin-Tóth, & H.R. Kaback, unpubl.). Therefore, it is unlikely that the increased reactivity of V315C permease in the presence of ligand is related to deprotonation of Glu 314.

As discussed, for technical reasons it is not possible to study the effect of $\Delta\bar{\mu}_{H^+}$ on the reactivity of single-Cys replacement mutants by site-directed fluorescence labeling. Therefore, we have utilized [^{14}C]NEM to study the reactivity of V315C permease in proteoliposomes reconstituted at a low protein to phospholipid ratio where an imposed $\Delta\Psi$ can be maintained for a significant period of time. When a potassium diffusion potential (interior negative) is imposed, a 3-fold increase in the rate of reactivity of V315C permease with NEM is observed, an effect similar to that observed with TDG in the same preparations. Importantly, the TDG effect is observed only in the presence of valinomycin and nigericin, which dissipates the $\Delta\bar{\mu}_{H^+}$ (interior positive and acid) generated by the β-galactoside/H^+ symport (Patel et al., 1982; Viitanen et al., 1984). This observation is particularly interesting because it suggests that generation of a reverse $\Delta\bar{\mu}_{H^+}$ may nullify the effect of ligand binding, thereby implying that binding energy and $\Delta\bar{\mu}_{H^+}$ may be equivalent. This conclusion, albeit preliminary, is consistent with the postulate that ligand binding or $\Delta\bar{\mu}_{H^+}$ may induce the same conformational change in the permease. In the context of these studies, it will be informative to extend the approach described here to other single-Cys replacement mutants that exhibit alterations in site-directed fluorescent labeling in the presence of ligand (Jung et al., 1994; J. Wu & H.R. Kaback, in prep.).

Materials and methods

Materials

[^{14}C]NEM was purchased from Amersham (Arlington Heights, Illinois), NEM and TDG from Sigma Chemical Company (St. Louis, Missouri), and PM, CPM, and stilbenemaleimide from Molecular Probes (Eugene, Oregon). Immobilized monomeric avidin was obtained from Pierce (Rockford, Illinois) and *E. coli* phospholipids from Avanti (Alabaster, Alabama). Glass fiber filters (Type GF; 25 mm) were from Nucleopore (Cambridge, Massachusetts). All other materials were reagent grade and obtained from commercial sources.

Bacterial strains and plasmids

E. coli T184 (*lacI*$^+$*O*$^+$*Z*$^-$*Y*$^-$(*A*), *rpsL*, *met*$^-$, *thr*$^-$, *recA*, *hsdM*, *hsdR/F'*, *lacI*^{q}*O*$^+$*Z*^{D118}(*Y*$^+$*A*$^+$)) (Teather et al., 1980) harboring plasmid pT7-5/C-less *lacY* L6XB (cassette *lacY* gene [EMBL-X56095] devoid of Cys codons [C-less; van Iwaarden et al., 1991] with a biotin acceptor domain in the middle cytoplasmic loop [Consler et al., 1993]) encoding V315C permease (Sahin-Tóth & Kaback, 1993) and plasmid pGP1-2 was used for overexpression of the mutant protein via the T7 promoter (Tabor & Richardson, 1985).

Protein purification and reconstitution

Twelve liters of cells were cultivated at 30 °C and heat-shocked for 20 min. Membranes were prepared as described (Viitanen et al., 1986) and extracted with 1.25% octyl-β,D-glucopyranoside. The mutant protein was purified by affinity chromatography on immobilized monomeric avidin (Consler et al., 1993). The resin was equilibrated with 50 mM KP_i (pH 7.5), 150 mM KCl, 1.25% OG (w/v), 1 mM dithiothreitol, 20 mM lactose, and 0.25 mg/mL of acetone/ether-washed *E. coli* phospholipids (column buffer). After application of the sample, the column was washed thoroughly with column buffer. Bound lac permease was then eluted with 8 mM *d*-biotin in column buffer. Purified samples were analyzed by SDS/polyacrylamide gel electrophoresis (Laemmli, 1970) followed by silver staining. Fractions containing purified permease were pooled and the protein was reconstituted into acetone/ether-washed *E. coli* phospholipids by detergent dilution (Viitanen et al., 1986). The protein/phospholipid ratio was adjusted to 1:380 (w/w) for experiments in which a potassium diffusion potential was generated. In all other studies, a protein/phospholipid ratio of 1:10 (w/w) was used. Proteoliposomes were centrifuged (150,000 g_{max}) and resuspended in 50 mM KP_i (pH 7.5), followed by 2 cycles of freeze–thaw sonication.

Labeling with fluorescent maleimides

The single Cys residue in V315C permease was modified with fluorescent maleimides by incubation of the purified, reconstituted permease (54 μg) with a given maleimide in 50 mM KP_i (pH 7.5) in a total volume of 2.0 mL at 25 °C. Further additions were made as indicated. PM was used at a final concentration of 1 μM, and the reaction was measured by monitoring the increase of fluorescence at 379 nm (excitation at 344 nm) using an Aminco-SLM Spectrofluorometer Type 8000C equipped with a thermostated cell. The results were corrected by subtracting the fluorescence obtained with proteoliposomes containing purified C-less permease at the same protein/phospholipid ratio. With stilbenemaleimide (10 μM final concentration), the progress of the reaction was monitored at an emission wavelength of 411 nm (excitation 322 nm).

Labeling with [^{14}C]NEM

For experiments in which the effect of TDG was tested, an aliquot of proteoliposomes (50 μL) reconstituted with purified V315C permease (70 μg protein/mL in 50 mM KP_i [pH 7.5]) was diluted to a final volume of 100 μL in the same buffer. [^{14}C]NEM (39 mCi/mmol) was then added to a final concentration of 5 μM. At given times, the reactions were terminated by addition of 1 mM DTT, and the samples were immediately mixed with 0.9 mL acetone, vortexed, and filtered through glass fiber filters (type GF 25 mm). The tubes were rinsed 3 times with 1 mL of acetone. Radioactivity retained on the filters was measured by liquid scintillation spectrometry. Where indicated, 10 mM TDG was added in the presence or absence of 20 μM valinomycin and 0.5 μM nigericin.

For experiments in which a potassium diffusion potential (interior negative) was imposed, a 20-μL aliquot of proteoliposomes (175 μg protein/mL in 50 mM KP_i [pH 7.5]) containing 20 μM valinomycin was diluted 200-fold into 50 mM NaP_i (pH 7.5) containing 5 μM [^{14}C]NEM (39 mCi/mmol) at 25 °C. At the times indicated, the reactions were terminated by addition of 1 mM DTT and 8 mL of acetone, the sample was vortexed and filtered through glass fiber filters (type GF 25 mm).

The tubes were rinsed 3 times with 2 mL acetone, and radioactivity was assayed as described.

Protein determination

Protein was determined using the amino black method of Schaffner and Weissmann (1973).

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