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Biochemistry

Properties of permease dimer, a fusion protein containing two lactose permease molecules from *Escherichia coli*

(active transport/bioenergetics/oligomerization/negative dominance/complementation)

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ABSTRACT **An engineered fusion protein containing two tandem lactose permease molecules (permease dimer) exhibits high transport activity and is used to test the phenomenon of negative dominance. Introduction of the mutation Glu-325 → Cys into either the first or the second half of the dimer results in a 50% decrease in activity, whereas introduction of the mutation into both halves of the dimer abolishes transport. Lactose transport by permease dimer is completely inactivated by *N*-ethylmaleimide; however, 40–45% activity is retained after *N*-ethylmaleimide treatment when either the first or the second half of the dimer is replaced with a mutant devoid of cysteine residues. The observations demonstrate that both halves of the fusion protein are equally active and suggest that each half may function independently. To test the possibility that oligomerization between dimers might account for the findings, a permease dimer was constructed that contains two different deletion mutants that complement functionally when expressed as untethered molecules. Because this construct does not catalyze lactose transport to any extent whatsoever, it is unlikely that the two halves of the dimer interact or that there is an oligomeric interaction between dimers. The approach is consistent with the contention that the functional unit of lactose permease is a monomer.**

Lactose permease of *Escherichia coli* is a hydrophobic, polytopic membrane protein that catalyzes the coupled stoichiometric translocation of β-galactosides and H^+ (i.e., 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 (for reviews, see refs. 1–3). On the basis of circular dichroic studies and hydropathy analysis of the primary amino acid sequence (4), a secondary structure was proposed in which the permease has a short hydrophilic N terminus, 12 α-helical hydrophobic domains that traverse the membrane in zig-zag fashion connected by hydrophilic loops, and a 17-residue hydrophilic C-terminal tail. 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 has been obtained from laser Raman spectroscopy (5), limited proteolysis (6, 7), immunological studies (8–16), and chemical modification (17). Exclusive support for the 12-helix motif has been obtained from analyses of an extensive series of lactose permease–alkaline phosphatase (*lacY–phoA*) fusions (18).

On the basis of the observation that the H^+ electrochemical gradient across the membrane ($\Delta\bar{\mu}_{H^+}$) alters the distribution of the permease between two distinct kinetic pathways, it was proposed that the permease might exist in two forms, monomer and dimer, and that $\Delta\bar{\mu}_{H^+}$ might cause dimerization (19). Subsequently, electron inactivation analysis (20) and the demonstration that the permease is dimeric in dodecyl octaethylene glycol monoether (21) lent support to the idea that dimerization may be important for function. On the other hand, the permease is monomeric in dodecyl maltoside (22) or hexamethylphosphorus triamide (23), and the rotational diffusion coefficient determined from fluorescence anisotropy with eosinylmaleimide-labeled permease indicates that the polypeptide reconstitutes as a monomer and the monomeric state is maintained in the presence of $\Delta\bar{\mu}_{H^+}$ (24). Furthermore, freeze-fracture electron microscopic studies on proteoliposomes reconstituted with purified lactose permease demonstrate that the protein reconstitutes as a monomer, and no change is observed in size or distribution of the permease when the proteoliposomes are energized (25). Importantly, it was also shown that the initial rate of $\Delta\bar{\mu}_{H^+}$-driven lactose transport in proteoliposomes reconstituted at very low protein-to-lipid ratios varies linearly with the ratio of permease to phospholipid. In summary, the bulk of the evidence indicates that lactose permease is functional as a monomer.

More recently, it was demonstrated (26) that certain paired in-frame deletion mutants are able to complement functionally. Although cells expressing the deletions individually do not catalyze active transport, cells simultaneously expressing specific pairs of deletions catalyze transport up to 60% as well as cells expressing wild-type permease, and it is clear that the phenomenon does not occur at the DNA level. Remarkably, complementation is observed only with pairs of permease molecules containing large deletions and not with missense mutations or point deletions. Although the mechanism of complementation is unclear, it may be related to the phenomenon whereby independently expressed N- and C-terminal fragments of the permease interact to form a functional complex (27). In any case, the observation that certain pairs of deletion mutants exhibit complementation rekindled concern regarding the oligomerization state of wild-type permease.

In this communication, a fusion protein is described that contains two lactose permease molecules covalently linked in tandem (permease dimer). Permease dimer is inserted into the membrane in a functional state, and each half of the dimer is equally active. Evidence consistent with the conclusion that the two halves of the dimer function independently is presented, thereby supporting the argument that lactose permease is functional as a monomer.

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Abbreviations: Cys-less permease, functional lactose permease devoid of cysteine residues; NEM, *N*-ethylmaleimide; $\Delta\bar{\mu}_{H^+}$, H^+ electrochemical gradient across the membrane. Site-directed mutants are designated as follows: the one-letter amino acid code is used followed by a number indicating the position of the residue in wild-type lactose permease; the sequence is followed by a second letter denoting the amino acid replacement at this position.

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MATERIALS AND METHODS

Materials. [1-^{14}C]Lactose was purchased from Amersham. Oligonucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer. Site-directed rabbit polyclonal antiserum against a dodecapeptide corresponding to the C terminus of lactose permease (10) was prepared by BabCo (Richmond, CA). *N*-Ethylmaleimide (NEM) was from Sigma. All other materials were reagent grade and obtained from commercial sources.

Bacterial Strains and Plasmids. *E. coli* HB101 [*hsdS20* (r^-_B, m^-_B), *recA13*, *ara-14*, *proA2*, *lacY1*, *galK2*, *rpsL20*(Sm^r), *xyl-5*, *mtl-1*, *supE44*, λ^-/F$^-$] (28) was used as carrier for the plasmids described. *E. coli* T184 [*lacI*$^+$*O*$^+$*Z*$^-$*Y*$^-$*(A)*, *rpsL*, *met*$^-$, *thr*$^-$, *recA*, *hsdM*, *hsdR/F'*, *lacI*^{q}*O*$^+$*Z*^{D118}*(Y*$^+$*A*$^+$*)*] (29) harboring plasmid pT7-5/*lacY* with given mutations was used for expression from the *lac* promoter by induction with isopropyl β-D-thiogalactopyranoside. As indicated, both strains are defective in DNA recombination, and as expected, no deletions or other alterations of the plasmid DNA were observed in the dimer constructs as judged by restriction analysis. A cassette *lacY* gene (EMBL-X56095) containing the *lac* promoter/operator was used for all *lacY* gene manipulations.

Oligonucleotide-Directed Site-Specific Mutagenesis. The cassette *lacY* gene was inserted into plasmid pT7-5 and used as template for mutagenesis. Introduction of unique *Sac* I sites at the 5′ and 3′ ends of the *lacY* gene was achieved by PCR mutagenesis (30) using the following mutagenic primers (the *Sac* I site is shown in boldface type): 5′ sense primer: 5′-GTAATAAGGAAAGGATCC**GAGCTC**ATGTAC-TATTTGAAAAACACAAC-3′; 3′ antisense primer: 5′-GCTTATCATCGATAAGCTTA**GAGCTC**AGCGACT-TCATTCACCTGA-3′.

DNA Sequencing. Double-stranded plasmid DNA prepared by Magic Minipreps (Promega) was sequenced by using the dideoxynucleotide chain-termination method (31) and synthetic sequencing primers after alkaline denaturation (32).

Active Transport. Active transport was measured in *E. coli* T184 (*Z*$^-$ *Y*$^-$) transformed with each plasmid described. Fully grown overnight cultures of cells were diluted 10-fold and grown aerobically for 2 hr at 37°C in the presence of streptomycin at 10 μg/ml and ampicillin at 100 μg/ml. To induce expression of lactose permease, 0.5 mM isopropyl β-D-thiogalactopyranoside (final concentration) was added, and growth was continued for 2 hr unless indicated otherwise. Cells were harvested by centrifugation, washed with 100 mM potassium phosphate, pH 7.5/10 mM $MgSO_4$, resuspended to an OD_{420} of 10 (≈0.7 mg of protein per ml), and assayed by rapid filtration (33).

Preparation of Membranes. Crude membrane fractions from *E. coli* T184 harboring plasmids with given mutations were prepared by osmotic shock and sonication (34).

Immunological Analyses. Membrane fractions were subjected to SDS/PAGE as described (35). Proteins were electroblotted, and the immunoblots were probed with a site-directed polyclonal antibody against the C terminus of lactose permease (15).

Protein Determinations. Protein was assayed in the presence of SDS by a modified Lowry method (36).

RESULTS

Construction of Permease Dimers. The gene encoding permease dimer was created by the 3′ to 5′ in-frame fusion of two *lacY* genes through a *Sac* I restriction site (Fig. 1). Initially, two precursor plasmids were constructed with a new unique *Sac* I site engineered either before the termination codon at the 3′ end of the gene (plasmid p3′) or before the initiator methionine at the 5′ end (plasmid p5′), using PCR mutagenesis as described. The *lacY* gene was mobilized from both plasmids by digesting p3′ with *Bam*HI and *Sac* I and p5′ with *Sac* I and *Hin*dIII (see Fig. 1). Finally, in a three-piece ligation, the two *lacY* genes were ligated together via the *Sac* I site and into plasmid pT7-5 with the *lac* promoter/operator that had been treated with *Bam*HI and *Hin*dIII. Although the *Sac* I site introduces two new amino acid residues (glutamate and leucine), interference with the transport mechanism is unlikely because both the N and C termini have been shown to be unimportant for activity (37, 38). To introduce mutations into permease dimer, *lacY* genes carrying the appropriate mutation(s) [Cys-less, a functional mutant devoid of cysteine residues (39), mutant E325C (32), N_8C_2, or N_2C_8 (26, 40); the nomenclature of the deletion mutants (N_xC_y) describes the number of putative N-(N_x) and C-terminal (C_y) hydrophobic transmembrane domains before and after the deletion (40)] were restricted from pT7-5/*lacY* and ligated

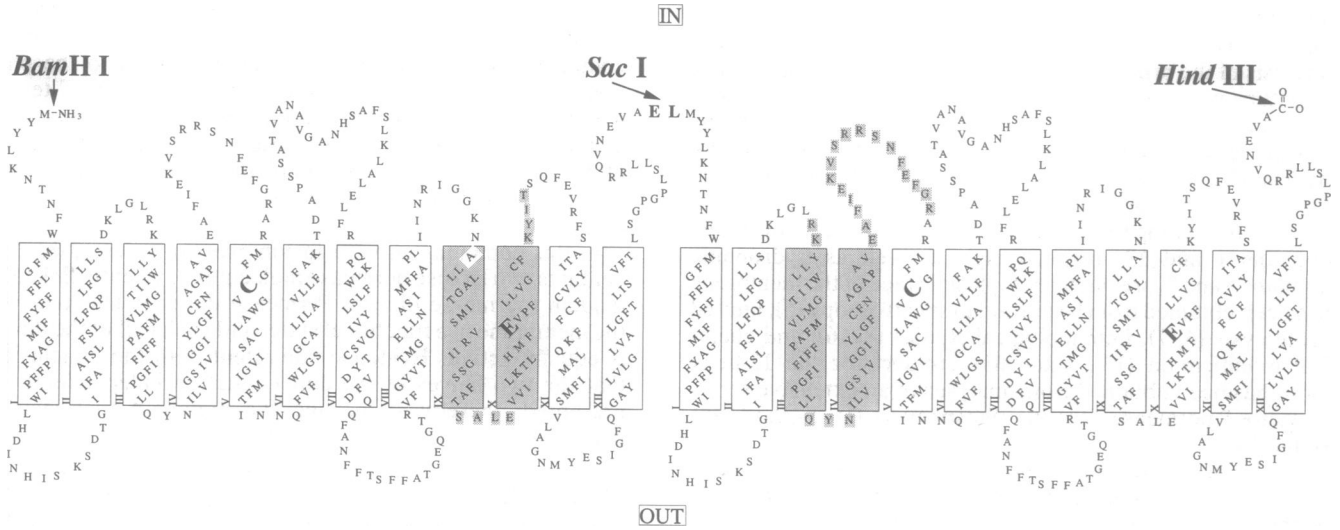

FIG. 1. Secondary-structure model of permease dimer. The single-letter amino acid code is used, and hydrophobic transmembrane helices are shown in boxes. Position of the flanking *Bam*HI and *Hin*dIII and the connecting *Sac* I restriction sites used for construction of permease dimer are indicated. Positions of Glu-325 which was replaced with cysteine and Cys-148, the primary target of NEM, are highlighted in both halves of the dimer. The shaded areas show the regions deleted in permease dimer containing a pair of complementing deletion mutants, N_8C_2/N_2C_8.

into the p3′ or p5′ vectors. The presence of the mutations in p3′ and p5′ was verified by double-stranded DNA sequencing, and mutant dimer permeases were constructed from the precursor plasmids as described above. In addition to permease dimer carrying two wild-type permease molecules (wild-type/wild-type), the following mutant dimers were created: wild-type/E325C; E325C/wild-type, E325C/E325C; wild-type/Cys-less, Cys-less/wild-type, Cys-less/Cys-less, N_8C_2/N_2C_8, and N_2C_8/N_8C_2.

Active Lactose Transport. As a quantitative measure of function, the ability of permease dimer and the mutants to catalyze lactose transport was assayed in *E. coli* T184 (Z^-Y^-). Compared with wild-type permease, permease dimer transports lactose at ≈40–60% of the rate to ≈50% of wild-type steady state (data not shown). Diminished activity may be due to a reduced amount of permease dimer in the membrane relative to wild type (see Fig. 4). Fig. 2 represents time courses of lactose accumulation by permease dimer (wild-type/wild-type) and dimers containing mutation E325C. When the inactivating mutation is introduced into the first (E325C/wild-type) or the second half of the dimer (wild-type/E325C), activity is reduced to ≈50% of permease dimer (wild-type/wild-type) activity in both instances. Permease dimer with the mutation in both halves (E325C/E325C) is completely inactive.

NEM, a membrane-permeable sulfhydryl reagent, completely inactivates permease dimer (wild-type/wild-type) (Fig. 3). In contrast, cells expressing permease dimers with the first or the second half replaced with Cys-less permease (Cys-less/wild-type or wild-type/Cys-less) retain 40–45% activity after NEM treatment. As expected, permease dimer containing two Cys-less permeases (Cys-less/Cys-less) is practically unaffected by the alkylating reagent.

Expression of Permease Dimer. To demonstrate that lactose transport observed is due to the activity of full-length permease dimer and not to monomeric wild-type permease arising from partial translation or proteolytic degradation of

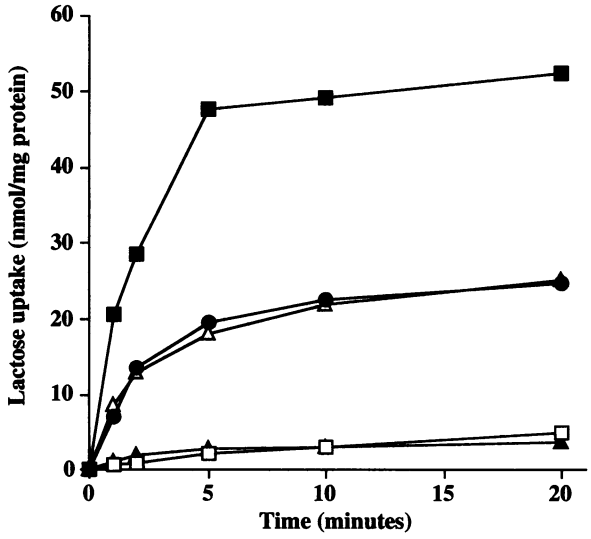

FIG. 2. Lactose transport by *E. coli* T184 expressing permease dimer (■, wild-type/wild-type), no permease (□, pT7-5 with no *lacY* insert), or dimers with mutation E325C in the first half (●, E325C/wild-type), the second half (△, wild-type/E325C), or both halves (▲, E325C/E325C) of the dimer. Cells were grown at 37°C as described. Aliquots of cell suspensions (50 μl containing 35 μg of protein) in 100 mM potassium phosphate, pH 7.5/10 mM magnesium sulfate were assayed at room temperature. Transport was initiated by the addition of [1-^{14}C]lactose (10 mCi/mmol; 1 Ci = 37 GBq) to a final concentration of 0.4 mM. Reactions were quenched by addition of 3.0 ml of 100 mM potassium phosphate, pH 5.5/100 mM lithium chloride and rapidly filtered through Whatman GF/F filters.

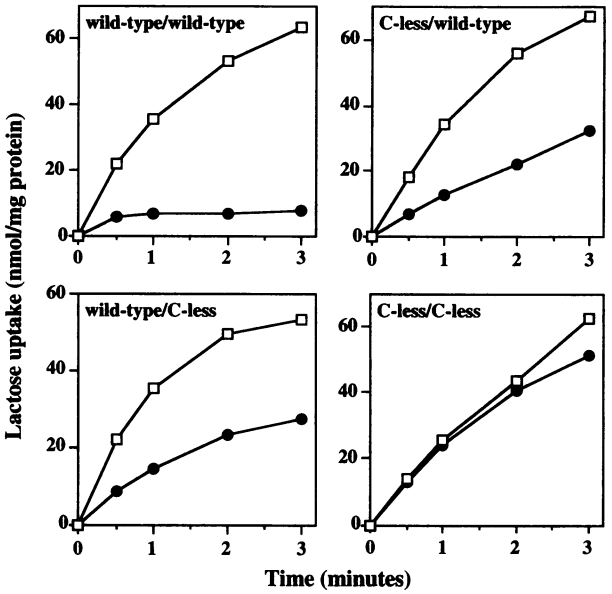

FIG. 3. Effect of NEM on rates of lactose accumulation by permease dimer (wild-type/wild-type) or dimers containing Cys-less (C-less) permease in the first half (Cys-less/wild-type), the second half (wild-type/Cys-less), or in both halves (Cys-less/Cys-less) of the dimer. *E. coli* T184 expressing given permease dimers were incubated in the absence (□) or presence (●) of 1 mM NEM (final concentration) at room temperature for 5 min, the reaction was quenched by addition of 10 mM dithiothreitol (final concentration), and cells were assayed for lactose transport in the presence of 20 mM potassium ascorbate and 0.2 mM phenazine methosulfate (44).

permease dimer, immunoblots were carried out on membrane preparations from T184 expressing the tandem fusion protein (Fig. 4). As expected, permease dimer migrates at the same position on SDS/polyacrylamide gels as the routinely observed dimeric aggregates of wild-type permease. However, membrane levels of the fusion protein are somewhat reduced relative to wild-type permease for unknown reasons. Importantly, no other immunoreactive species is observed on the immunoblots, particularly around 33 kDa, where the preponderance of monomeric lactose permease migrates. Although overloading the gels and/or overexposing the immunoblots results in the appearance of background bands at 33 kDa and elsewhere, the intensity at 33 kDa is always <1% of the intensity of the immunoreactive band corresponding to permease dimer.

Permease Dimer Containing Pairs of Complementing Deletions. The results described argue strongly that full-length

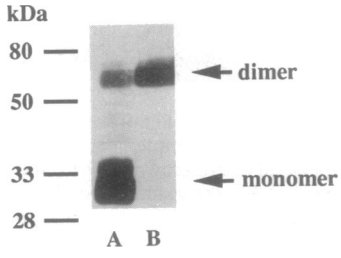

FIG. 4. Immunoblot of membranes containing wild-type permease or permease dimer (wild-type/wild-type). Membrane preparations containing ≈50 μg (wild-type permease; lane A) and 100 μg (permease dimer; lane B) of protein per sample were subjected to SDS/PAGE and electroblotted; the nitrocellulose paper was incubated with anti-C-terminal antibody. After incubation with horseradish peroxidase-conjugated protein A, followed by a short incubation with fluorescent substrate (Amersham), the nitrocellulose paper was exposed to film for 1 min.

permease dimer is in the membrane and that both halves of the dimer are equally active. Furthermore, each half of the dimer appears to function independently. However, it is conceivable that the transport activity observed results from interactions between dimers. To test this possibility, permease dimers were constructed containing different deletion mutants that complement functionally when expressed as individual molecules in the same cell (26). As shown in Fig. 5*A*, N_8C_2/N_2C_8 dimer is completely inactive with respect to lactose transport, although the fusion protein is present in the membrane, although at a reduced level compared with wild type (Fig. 5*B*). Although N_2C_8/N_8C_2 dimer also exhibits no transport activity (Fig. 5*A*), it is hardly detectable on immunoblots (data not shown).

Unstable expression of N_8C_2/N_2C_8 and N_2C_8/N_8C_2 dimers raises the possibility that lack of transport may be due to diminished concentrations of the mutant proteins in the membrane. Therefore, the level of permease dimer (wild-type/wild-type) was varied by inducing cells for different periods of time and then assaying transport activity and estimating the concentration of permease in the membrane by immunoblot analysis. As shown in Fig. 5*A*, wild-type/wild-type dimer catalyzes significant lactose accumulation even without induction, and levels of accumulation are maximal 10 min after induction. Importantly, although N_8C_2/N_2C_8 dimer exhibits no significant transport activity, its concentration in the membrane falls between that observed in cells expressing wild-type/wild-type dimer with no induction and after 10-min induction (Fig. 5*B*). Thus, the complete lack of activity observed with N_8C_2/N_2C_8 dimer cannot be due to decreased expression. The observations demonstrate that the deletion mutants do not complement functionally when they are expressed in a tethered form.

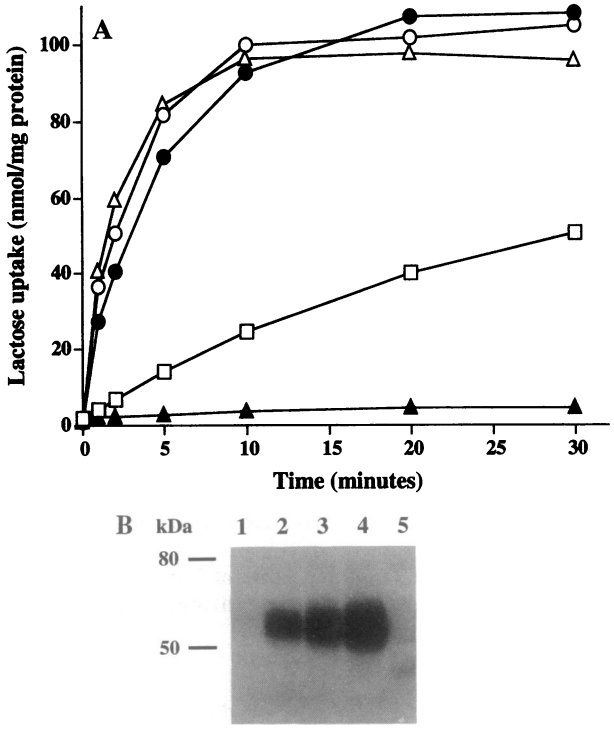

FIG. 5. Lactose transport and expression of wild-type/wild-type dimer and dimers containing complementing deletions. *E. coli* T184 harboring given plasmids were grown at 37°C, and cells were induced with 0.5 mM isopropyl β-D-thiogalactoside (final concentration). (*A*) Lactose transport by cells expressing wild-type/wild-type dimer [before induction (□), after 10-min induction (●), 20-min induction (○), or 40-min induction (△)], no permease (pT7-5; vector with no *lacY* gene) or dimers containing N_8C_2/N_2C_8 or N_2C_8/N_8C_2 (▲). Cells expressing pT7-5 or dimers with deletions were induced for 2 hr. Other experimental details are given in Fig. 2. (*B*) Immunoblot of membranes from cells expressing wild-type/wild-type dimer (lanes 1–4) or N_8C_2/N_2C_8 dimer (lane 5). Lanes: 1, before induction; 2, after 10-min induction; 3, after 20-min induction; 4, after 40-min induction; 5, cells expressing N_8C_2/N_2C_8 dimer after 2-hr induction. Electrophoresis and immunoblotting of membrane preparations were done as described in Fig. 4.

DISCUSSION

Coexpression of wild-type and mutant transport proteins within the same cell is a powerful method to study potential interactions between polypeptides. Suppression of transport activity by an inactive mutant coexpressed with the wild type (negative dominance) or restoration of transport activity by coexpression of two inactive mutants (functional complementation) may provide evidence for oligomeric organization. With the tetracycline (Tet) transporter, a H^+-coupled antiporter with 12 transmembrane domains that is in the same superfamily of transport proteins as lactose permease (42), functional complementation by inactive missense mutations or by inactive interdomain gene hybrids between *TetB* and *TetC* has been demonstrated (41, 43). In contrast, with lactose permease, complementation is observed only with pairs of permease molecules containing large deletions and not with missense mutations or point deletions (26). In this respect, complementation between deletion mutants of lactose permease appears to be a phenomenon that may not reflect the functional oligomeric state of the wild-type protein (for review, see ref. 26).

We demonstrate here that an engineered fusion protein containing two lactose permease molecules is inserted into the membrane and exhibits high transport activity. Permease dimer provides a unique model system that permits the "coupled" coexpression of two permease molecules with the following advantages: (*i*) the two permease molecules are fused, maintaining spatial proximity that is presumably favorable for interaction; and (*ii*) the two halves of the dimer are expressed at identical levels.

Two approaches are used to test negative dominance. In one case, a dimer permease was constructed containing wild-type permease and mutant E325C; in another, the dimer contained wild-type permease and Cys-less permease, and the effect of NEM on transport activity was measured. In both cases, the results show that inactivation of either half of the dimer results in ≈50% decrease in activity, indicating that both halves of the dimer are equally active and that inactivation of half of the dimer does not interfere with the function of the other half. In other words, the two halves of the dimer appear to function independently.

To examine the possibility that trans-complementation between dimers might occur, covalent dimers containing deletion mutants that complement functionally when expressed in an untethered form were constructed and tested for activity. No activity whatsoever is observed for either of the constructs studied, although one of the constructs (N_8C_2/N_2C_8) is detected in the membrane at a level where comparable amounts of wild-type/wild-type dimer catalyze highly significant accumulation. However, there is one caveat to this control experiment. Because complementation between deletion mutants clearly occurs when the mutants are *untethered*, the inability to complement when tethered suggests that some restriction related to linkage prevents cis complementation. This restriction may, in turn, prevent the deletion dimers from complementing in trans. Thus, the type of trans-interaction between deletion dimers might conceivably differ from the interaction between intact molecules. Therefore, the observation that deletion dimers do not complement when tethered does not prove absolutely that the other constructs cannot do so. Nonetheless, taken together with previous observations (22–25), the results provide indepen-

dent support for the contention that lactose permease is functional as a monomer.

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