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THE LACTOSE PERMEASE MEETS FRANKENSTEIN

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Summary

The lactose permease (lac) of *Escherichia coli* is a paradigm for membrane transport proteins. Encoded by the *lacY* gene, the permease has been solubilized, purified to homogeneity, reconstituted into phospholipid vesicles and shown to catalyse the coupled translocation of β-galactosides and H^+ with a stoichiometry of unity. Circular dichroism and other spectroscopic approaches demonstrate that the purified permease is about 80 % helical. Based on hydropathy analysis of the primary amino-acid sequence, a secondary structure has been proposed in which the protein has 12 hydrophobic domains in α-helical conformation that traverse the membrane in zigzag fashion connected by hydrophilic loops. A variety of other approaches are consistent with the model and demonstrate that both the N and C termini are on the inner surface of the membrane, and studies on an extensive series of lac permease/alkaline phosphatase fusion proteins provide exclusive support for the topological predictions of the 12-helix motif. This presentation concentrates on the use of site-directed fluorescence spectroscopy to study structure–function relationships in the permease.

Introduction

An unsolved basic biochemical problem of critical importance is the mechanism of energy transduction in biological membranes. Although the driving force for a variety of seemingly unrelated phenomena (e.g. secondary active transport, oxidative phosphorylation, rotation of the bacterial flagellar motor) is a bulk-phase, transmembrane electrochemical ion gradient, the molecular mechanism(s) by which free energy stored in such gradients is transduced into work or into chemical energy remains enigmatic. Nonetheless, gene sequencing and analyses of deduced amino-acid sequences indicate that many biological machines involved in energy transduction, secondary transport proteins in particular (Henderson, 1990; Marger and Saier, 1993), fall into families encompassing proteins from archaebacteria to the mammalian central nervous system, thereby suggesting that the members may have common basic structural features and mechanisms of action. In addition, certain of these proteins have been implicated in human disease (e.g. glucose/galactose malabsorption, certain forms of drug abuse).

Key words: bioenergetics, transport, site-directed mutagenesis, site-directed spectroscopy, membrane proteins, cys-scanning mutagenesis.

As postulated originally by Peter Mitchell (1963, 1968) and demonstrated conclusively in bacterial membrane vesicles (Kaback, 1986, 1989, 1992), accumulation of a wide variety of solutes against a concentration gradient is driven by a proton electrochemical gradient ($\Delta\bar{\mu}_H$; interior negative and/or alkaline). The work discussed here focuses on one specific secondary transport protein, the lactose or lac permease as a paradigm. β-galactoside accumulation in *Escherichia coli* is catalyzed by lac permease, a hydrophobic polytopic cytoplasmic membrane protein that carries out the coupled stoichiometric translocation of four β-galactosides with H^+ (i.e. β-galactoside/H^+ symport or cotransport). Physiologically, the permease utilizes free energy released from downhill translocation of H^+ to drive accumulation of β-galactosides against a concentration gradient. In the absence of $\Delta\bar{\mu}_H$, lac permease catalyzes the converse reaction, utilizing free energy released from downhill translocation of β-galactosides to drive uphill translocation of H^+ with the generation of a $\Delta\bar{\mu}_H$, the polarity of which depends upon the direction of the substrate concentration gradient.

Lac permease is encoded by the *lacY* gene, the second structural gene in the *lac* operon, which has been cloned into a recombinant plasmid (Teather *et al.* 1978) and sequenced (Buchel *et al.* 1980). By combining overexpression of *lacY* with the use of a highly specific photoaffinity probe (Kaczorowski *et al.* 1980) and reconstitution of transport activity in artificial phospholipid vesicles (i.e. proteoliposomes, Newman and Wilson, 1980), the permease was the first symporter to be solubilized from the membrane, purified to homogeneity (Newman *et al.* 1981; Foster *et al.* 1982; Viitanen *et al.* 1986) and shown to catalyze all the translocation reactions typical of the transport system *in vivo* with similar turnover numbers (Viitanen *et al.* 1984; Matsushita *et al.* 1983). The findings demonstrate that the product of the *lacY* gene is solely responsible for all of the translocation reactions catalyzed by the β-galactoside transport system.

Lac permease contains 12 transmembrane domains in α-helical conformation

Circular dichroic measurements on purified lac permease demonstrate that the protein is 75–80 % helical, an estimate consistent with the hydropathy profile of the permease which suggests that approximately 70 % of its 417 amino acid residues are found in hydrophobic domains with a mean length of 24±4 residues. Based on these findings, it was proposed (Foster *et al.* 1983) that the permease is composed of a hydrophilic N terminus followed by 12 hydrophobic segments in α-helical conformation that traverse the membrane in zigzag fashion connected by hydrophilic domains (loops) with a 17-residue C-terminal hydrophilic tail (Fig. 1). Support for general features of the model and evidence that the N and C termini, as well as the second and third cytoplasmic loops, are exposed to the cytoplasmic face of the membrane were then obtained from laser Raman (Vogel *et al.* 1985) and Fourier transform infrared (P. D. Roepe, K. Rothschild and H. R. Kaback, unpublished information) spectroscopy, immunological studies (Carrasco *et al.* 1982, 1984*a*,*b*; Seckler *et al.* 1983, 1986; Seckler and Wright, 1984; Herzlinger *et al.* 1984, 1985; Danho *et al.* 1985), limited proteolysis (Goldkorn *et al.* 1983; Stochaj *et al.* 1986) and chemical modification (Page and Rosenbusch, 1988). However, none of these

approaches is able to differentiate between the 12-helix motif and other models containing 10 (Vogel *et al.* 1985) or 13 (Bieseler *et al.* 1985) transmembrane domains.

Calamia and Manoil (1990) provided elegant, unequivocal support for the topological predictions of the 12-helix model by analyzing an extensive series of lac permease–alkaline phosphatase *(lacY–phoA)* fusion proteins. In addition, it was shown that the alkaline phosphatase activity of fusion proteins engineered at every third amino-acid residue in putative helices III and V increases abruptly as the fusion junction proceeds from the eighth to the eleventh residue. Thus, approximately half a transmembrane domain is needed to translocate alkaline phosphatase through the membrane to the external surface. When fusions are constructed at each amino-acid residue in putative helices IX and X, the data are in excellent agreement with the model (M. L. Ujwal, E. Bibi, C. Manoil and H. R. Kaback, in preparation).

Lac permease is functional as a monomer

One difficult problem to resolve with hydrophobic membrane proteins is their functional oligomeric state. Notwithstanding strong evidence that lac permease is functional as a monomer (Dornmair *et al.* 1985; Costello *et al.* 1987), Bibi and Kaback (1992) demonstrated 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 occurs at the level of the protein. 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 is probably related to the phenomenon whereby independently expressed N- and C-terminal fragments of the permease interact to form a functional complex (Bibi and Kaback, 1990; see below). In any case, the observation that certain pairs of deletion mutants can interact rekindled concern regarding the oligomerization state of wild-type permease.

Sahin-Tóth *et al.* (1994) recently engineered a fusion protein containing two lac 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 exhibits equal activity. Thus, point mutations in either half of the *lacY* tandem repeat lead to 50% inactivation of transport. Furthermore, the activity of a permease dimer composed of wild-type permease and a mutant devoid of Cys is inactivated by approximately by 60% by *N*-ethylmaleimide (NEM). In order to test the caveat that oligomerization between dimers might occur in the *trans* position, a permease dimer was constructed that contains two different deletion mutants which complement when expressed as untethered molecules. This construct does not catalyze lactose accumulation to any extent whatsoever, suggesting that permease dimers do not undergo oligomerization in the *trans* position. The experiments are clearly consistent with the conclusion that wild-type lac permease is functional as a monomer. It is also noteworthy that this approach can be applied to other membrane transport proteins that have not been solubilized and purified.

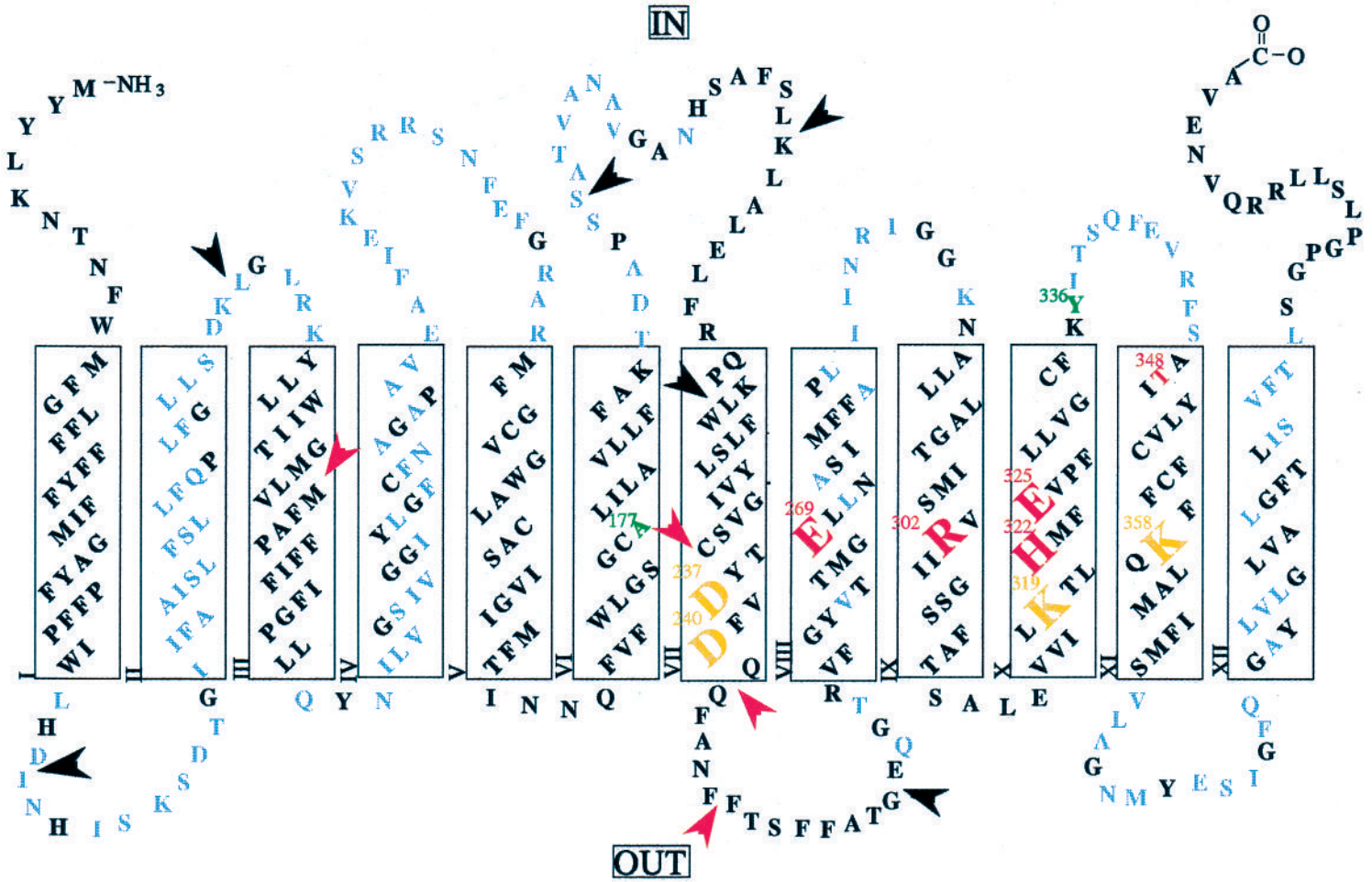
Site-directed mutagenesis reveals that few amino-acid residues are essential for activity

By using site-directed mutagenesis with wild-type permease or a functional mutant devoid of Cys residues (C-less permease, van Iwaarden *et al.* 1991*b*), individual amino-acid residues in the permease that are important mechanistically have been identified (Fig. 1). About 300 of the 417 residues in C-less permease have been replaced with Cys and, remarkably, over 95 % of the mutants retain activity (Sahin-Tóth *et al.* 1994; Dunten *et al.* 1993*a*) [in addition to helices I, IX, X and XI, Cys-scanning mutagenesis has been carried out with helices III (M. Sahin-Tóth, S. Frillingos, E. Bibi, A. Gonzalez and H. R. Kaback, in preparation), V (C. Weizmann, M. Sahin-Tóth and H. R. Kaback, unpublished information) and VII (S. Frillingos, M. Sahin-Tóth and H. R. Kaback, in preparation) (see Fig. 1)]. Of the few mutants that do not catalyze active transport, most retain the ability to catalyze partial reactions or bind ligand. More specifically, none of the eight Cys (van Iwaarden *et al.* 1991*a*,*b*; Trumble *et al.* 1984; Menick *et al.* 1985, 1987*b*; Viitanen *et al.* 1985; Neuhaus *et al.* 1985; Sarkar *et al.* 1986; Brooker and Wilson, 1986), six Trp (Menezes *et al.* 1990) or twelve Pro (Lolkema *et al.* 1988; Consler *et al.* 1991) residues in the permease is obligatory for activity. Only one out of four His residues (Padan *et al.* 1985; Püttner *et al.* 1986, 1989; Püttner and Kaback, 1988), one of the 14 Tyr residues (Roepe and Kaback, 1989) (although Tyr→Phe replacements indicate that three Tyr residues are important for activity, cys-scanning mutagenesis has revealed that Tyr-26 and Tyr-236 can be replaced with Cys with retention of significant activity) and one out of 36 Gly residues (K. Jung, H. Jung, P. Colachurchio and H. R. Kaback, in preparation) are important for active transport. However, Glu-269 (helix VIII) (Ujwal *et al.* 1994), Arg-302 (helix IX) (Menick *et al.* 1987*a*), His-322 (helix X) (Padan *et al.* 1985; Püttner *et al.* 1986, 1989; Püttner and Kaback, 1988) and Glu-325 (helix X) (Carrasco *et al.* 1986, 1989) are essential for substrate accumulation and/or binding. Moreover, differences in the properties of the mutants suggest that Arg-302, His-322 and Glu-325 may function in an H^+ translocation pathway, although it is possible that the residues also form part of a coordination site for H_3O^+ (see Kaback, 1992, for a discussion). In any event, very few residues are mandatory for binding or transport, and it is unlikely that individual amino-acid replacements cause global conformational changes.

Site-directed fluorescent labeling and mutagenesis yield helix packing in the C-terminal half of the permease

The observations described above highlight a need for static and dynamic information at high resolution in order to define the transport mechanism, and recent studies with

Fig. 1. Secondary structure model of lac permease based on the hydropathy profile of the protein. The single amino-acid code is used, and hydrophobic segments are shown in boxes as transmembrane (α-helical domains connected by hydrophilic loops). Residues labeled black yield active permease when replaced with Cys; residues labeled red yield inactive permease when replaced with a number of different residues; residues labeled yellow are charge-paired (Asp237–Lys358 and Asp240–Lys3l9); residues labeled light blue have not been mutagenized. Permease with A177C (green) is inactive, but other replacements have not been made; permease with Y336F (green) is inactive, but other replacements have not been made. Black arrowheads indicate active split permease constructs; red arrowheads signify inactive constructs.


IN
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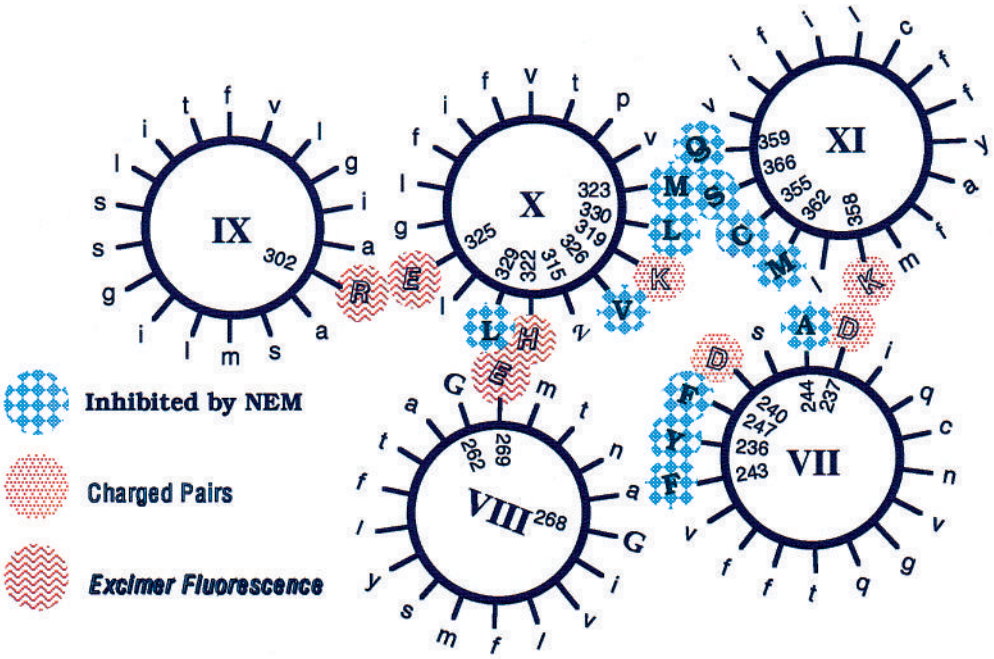

Fig. 2. Helical wheel model of putative helices VII–XI in lac permease viewed from the periplasmic surface. In addition to the symbols given on the figure, G268 and G262 (helix VIII) which are sites of second-site suppressors for D240 and E325, respectively, are emboldened, and V315 is highlighted (see Jung *et al.* 1993).

permease molecules engineered specifically for spectroscopic approaches (van Iwaarden *et al.* 1991*b*; Menezes *et al.* 1990) are providing exciting new insights. Since none of the individual Cys residues in the permease is required for activity, all eight Cys residues have been replaced simultaneously with Ser or Val to yield a Cys-less permease that retains at least 50 % of wild-type activity (van Iwaarden *et al.* 1991*b*). Molecules containing one or two Cys replacements at any desired position(s) can then be tagged with thiol-specific biophysical probes after solubilization and purification. By using this approach, Jung *et al.* (1993) established proximity relationships between transmembrane helices in the C-terminal half of the permease. Pairs of charged amino-acid residues in transmembrane domains were replaced with Cys in a C-less construct containing a biotin-acceptor domain in the middle cytoplasmic loop to facilitate purification by monovalent avidin affinity chromatography (Consler *et al.* 1993). As a fluorophore, *N*-(1-pyrenyl)maleimide (PM) was selected, since it can form an excited state dimer (excimer) that exhibits a unique emission maximum if two conjugated ring systems are within about 0.35 nm and correctly oriented (Kinnunen *et al.* 1993). The findings obtained indicate: (i) that transmembrane domain X is in α-helical conformation; (ii) helices IX (Arg-302) and X (Glu-325) are in close proximity; and (iii) helix VIII (Glu-269) is in close proximity to helix X (His-322) (Fig. 2).

Second-site suppressor analysis (King *et al.* 1991) and site-directed mutagenesis combined with chemical modification (Sahin-Tóth *et al.* 1992; Dunten *et al.* 1993*b*; Sahin-Tóth and Kaback, 1993*b*) demonstrate that Asp-237 (helix VII) interacts with Lys-358 (helix XI), probably forming a salt bridge. Individual replacement of Asp-237 or Lys-358 in C-less permease with Cys or Ala abolishes active lactose transport, whereas simultaneous replacement of both charged residues with Cys and/or Ala or *reversal* of the residues leads to fully active permease. Remarkably, mutant D237C is restored to full activity by carboxylmethylation, which recreates a negative charge at position 237, and mutant K358C is restored to full activity by treatment with ethylammonium methanethiosulfonate, which recreates a positive charge at position 358. It has also been shown by site-directed mutagenesis (Sahin-Tóth *et al.* 1992; Sahin-Tóth and Kaback, 1993*b*) and second-site suppressor analysis (Lee *et al.* 1992) that Asp-240 (helix VIII) interacts with Lys-3l9 (helix X). Individual replacement of either Asp-240 or Lys-3l9 in C-less permease with neutral amino acid residues inactivates the permease, but double neutral mutants retain significant activity. In contrast to Asp-237/Lys-358, however, the polarity of the interaction between Asp-240 and Lys-3l9 is important, as reversal of the residues inactivates the permease. In any case, the findings indicate that helix VII (Asp-237 and Asp-240) is in close proximity to helices X (Lys-3l9) and XI (Lys-358). It is also apparent that none of these residues plays a direct role in the mechanism. Taken together with the conclusions derived from site-directed pyrene labeling, the observations describe the packing of helices VII–XI (Fig. 2) provide the first tertiary structure information regarding this class of membrane proteins (Jung *et al.* 1993; Kaback *et al.* 1993).

Ligand binding induces widespread conformational changes

Jung *et al.* (1994*b*) have demonstrated that excimer fluorescence can also be used to study dynamic aspects of permease folding. Excimer fluorescence formed *between*

transmembrane domains is markedly diminished by denaturants, whereas the excimer fluorescence observed within helix X is unaffected, indicating that tertiary interactions are disrupted with little effect on secondary structure. Consistently, interacting helices do not exhibit excimer fluorescence in detergent, but only after reconstitution into membranes. One of the excimers described also exhibits ligand-induced alterations. Excimer fluorescence due to the interaction between helices VII (E269C) and X (H322C) is quenched by Tl^+, and the effect is markedly and specifically attenuated by permease substrates. The reactivity of single Cys residues placed in many transmembrane domains is also dramatically altered in the presence of ligand, implying that transport involves widespread changes in tertiary structure. In one Cys replacement mutant (V315C) tested thus far, the effect of ligand is mimicked by imposition of $\Delta\bar{\mu}_H$, providing an exciting preliminary suggestion that either ligand binding or $\Delta\bar{\mu}_H$ may cause the permease to assume the same conformation (Sahin-Tóth and Kaback, 1993*a*,*b*). The initial observations made with right-side-out membrane vesicles have been confirmed recently with purified V315C permease (Jung *et al.* 1994*a*).

Cys-148 is in a substrate-binding site

Fox and Kennedy (1965) demonstrated that lac permease is irreversibly inactivated by *N*-ethylmaleimide (NEM) and that protection is afforded by certain substrates. On the basis of these findings, it was postulated that a Cys residue is at or near the substrate-binding site in the permease, and it was shown later by Bayreuther *et al.* (1981) that the substrate-protectable Cys is at position 148. Since Cys-l48 can be replaced with Gly (Trumble *et al.* 1984; Viitanen *et al.* 1985) or Ser (Neuhaus *et al.* 1985; Sarkar *et al.* 1986) with little or no effect on activity, it is apparent that this residue is not essential. However, the observations also raise the possibility that substrate protection may be due to a long-range conformational effect. Recently (H. Jung, K. Jung and H. R. Kaback, in preparation), Cys-148 was replaced with hydrophobic (Ala, Val, Ile, Phe), hydrophilic (Ser, Thr) or charged (Asp, Lys) residues, and the properties of the replacement mutants were analyzed. Although Cys-l48 is not essential for transport, the size and polarity of the side chain at this position modify transport activity and substrate specificity. Thus, small hydrophobic side-chains (Ala, Val) generally increase the apparent affinity of the permease for substrate, whereas hydrophilic side-chains (Ser, Thr, Asp) decrease the apparent affinity and bulky or positively charged side-chains (Phe, Lys) virtually abolish activity. In addition, hydrophilic substitutions (Ser, Thr, Asp) alter the activity of the permease towards monosaccharides relative to disaccharides.

Site-directed fluorescence spectroscopy was also used to study Cys-148 and other residues in the vicinity (J. Wu and H. R. Kaback, in preparation). In the absence of ligand, permease with a single Cys residue at position 148 reacts rapidly with 2-(4′-maleimidylanilino)-naphthalene-6-sulfonic acid (MIANS), a fluorophore whose quantum yield increases dramatically upon reaction with a thiol, indicating that this residue is readily accessible to the probe. Various ligands of the permease block the reaction, and the concentration-dependence is commensurate with the affinity of each ligand for the permease (i.e. β-D-galactopyranosyl 1-thio-β-D-galactopyranoside$\ll$lactose$<$galactose), but neither sucrose nor glucose has any effect whatsoever. Interestingly, labeling of Cys-

145, which is presumed to be one helical turn removed from Cys-l48, displays properties similar to those observed with Cys-l48 permease, but the effects of ligand are far less dramatic. In contrast, permease with a single Cys residue at position 146 or 147 behaves in a completely different manner. Studies with iodide show that MIANS at positions 145 or 148 is accessible to the collisional quencher, indicating that this face of helix V is solvent-exposed, whereas MIANS at positions 146 or 147 is not quenched by iodide in the presence or absence of ligand. Finally, iodide-quenching of MIANS at position 145 is clearly diminished in the presence of ligand. Taken together with the findings discussed above, the results indicate that Cys-l48 is a likely component of a substrate-binding site that interacts hydrophobically with the galactosyl portion of the substrate, but does not play an essential role in transport. In addition, the observations indicate that residue 145 is in close proximity to Cys-148.

A novel approach to crystallization of hydrophobic membrane proteins

A major thrust of this laboratory is aimed at two-dimensional and three-dimensional crystallization. Since hydrophobic membrane proteins such as lac permease are particularly difficult to crystallize in three-dimensional form, Privé *et al.* (1994) have devised a novel approach in which a fusion is constructed between the permease and a 'carrier' protein. The carrier is a soluble, stable protein with its C and N termini close together in space at the surface of the protein, so that it can be introduced into an internal position of the permease without distorting either molecule. McKenna *et al.* (1992*b*) demonstrated that all but three of the hydrophilic domains in the permease can be disrupted by the insertion of two or six contiguous His residues without abolishing activity. The carrier is chosen with convenient spectral properties, making the fusion protein easier to characterize than the native molecule. A chimeric protein with *E. coli* cytochrome b_{562} fused into the middle cytoplasmic loop of lac permease and six His residues attached to the C terminus has been constructed, expressed and highly purified by nickel chelate chromatography. The chimera exhibits transport activity similar to that of wild-type lac permease and has a visible absorption spectrum and a redox potential that are identical to those of cytochrome b_{562}. The chimera has a higher proportional polar surface area than wild-type permease and should have better possibilities of forming the strong, directional intermolecular contacts required of a crystal lattice.

Insertion of permease into the membrane

Expression of lactose permease in fragments as a probe for membrane-spanning domains

Lac permease also provides a useful system for studying insertion of polytopic proteins into the membrane. Surprisingly, as shown by Bibi and Kaback (1990), when the *lacY* gene is split into two approximately equal-sized fragments (N6 and C6) (Fig. 1), cells expressing both fragments catalyze significant lactose accumulation, whereas cells expressing either half of the permease individually are devoid of activity. Intact permease is completely absent from the membrane of cells expressing fragments either individually

or together. Thus, transport activity must result from association between independently synthesized portions of lac permease. When the gene fragments are expressed individually, the N-terminal portion of the permease is observed sporadically and the C-terminal portion is not observed; when expressed together, the N- and C-terminal moieties of the permease are found in the membrane. Clearly, the N- or C-terminal halves are proteolyzed when synthesized independently, and association between the two complementing polypeptides leads to a more stable, catalytically active complex.

Co-expression of independently cloned fragments of *lacY* encoding N_2 and C_{10} (Wrubel *et al.* 1990), N_1 and C_{11} or N_7 and C_5 (Zen *et al.* 1994) also yields stable molecules in the membrane which interact to form functional permease (Fig. 1). Thus, *lacY* gene fragments encoding contiguous, non-overlapping peptides with discontinuities in either cytoplasmic or periplasmic loops are able to complement functionally. In striking contrast, Zen *et al.* (1994) have demonstrated that peptide fragments with discontinuities in transmembrane domains are unable to form functional complexes, implying that the 'split permease approach' may be useful for approximating helical boundaries. Based on this notion, a series of contiguous, non-overlapping permease fragments with discontinuities at various positions in loop 6 (cytoplasmic), putative helix VII and loop 7 (periplasmic) were co-expressed in order to approximate the boundaries of transmembrane domain VII (Zen *et al.* 1994). Contiguous fragments with a discontinuity between Leu-222 and Trp-223 (loop 6) or Gly-254 and Glu-255 (loop 7) are functional, but fragments with discontinuities between Cys-234 and Thr-235, Gln-241 and Gln-242 or Phe-247 and Met-248 are inactive (Fig. 1). Therefore, it is likely that Leu-222 and Gly-254 are located in hydrophilic loops 6 and 7, respectively, whereas Cys-234, Gln-241 and Phe-247 are probably within transmembrane domain VII. These and other results are consistent with a secondary structure model of lactose permease in which Asp-237 and Asp-240 are contained within domain VII rather than loop 7, as predicted by hydropathy profiling (King *et al.* 1991; Sahin-Tóth *et al.* 1992; Dunten *et al.* 1993*b*; Sahin-Tóth and Kaback, 1993*b*; Zen *et al.* 1994).

Regarding the contention that contiguous permease fragments with discontinuities in transmembrane domains do not exhibit functional complementation, Roepe *et al.* (1989) and McKenna *et al.* (1991, 1992*a*) have presented evidence that the last turn of putative helix XII must be intact for the permease to insert into the membrane in a stable form. Thus, truncation of the 17-residue C-terminal tail of the permease at position 401 has little or no effect on the activity or stability of the protein, whereas truncation of the permease at position 396 leads to complete loss of activity and rapid degradation (Fig. 1). Truncations at positions 397–400 exhibit progressively increasing activity and are progressively more stable in the membrane. Moreover, replacement of residues 397–400 with Leu-Leu-Leu-Leu yields fully functional permease that is completely stable, while replacement with Gly-Pro-Gly-Pro yields unstable permease with minimal activity. It seems likely, therefore, that incomplete transmembrane domains are not inserted into the membrane in a stable form. However, it should be emphasized that proteolytic degradation cannot explain the lack of transport activity observed with permease duplexes containing discontinuities in transmembrane domains. Thus, an immunoreactive C-terminal fragment is observed with $N_{2.5}/C_{9.5}$. Moreover, permease deleted of the first 22 amino-acid residues (the N terminus and half of the first transmembrane domain) is stable and functional when

expressed at a high rate (Bibi *et al.* 1992). Therefore, it seems reasonable to suggest that the lack of transport activity observed with duplexes split in transmembrane domains may be due to an alteration in the transfer of conformational information from one side of a transmembrane domain to the other.

Membrane insertion may involve multiple mechanisms

The demonstration that polypeptides corresponding to N_1 and C_{11} form a relatively stable, functional complex argues against the notion that the N terminus of the permease inserts into the membrane as a helical hairpin. However, H. K. Zen, T. G. Consler, D. Hardy and H. R. Kaback (in preparation) (see Consler *et al.* 1993) have shown that insertion of the biotin-acceptor domain into the second or the fourth periplasmic loops of the permease (between helices III and IV or helices VII and VIII, respectively; Fig. 1) blocks insertion of transmembrane helices III and IV or VII and VIII without altering the insertion of the remainder of the protein, suggesting that these portions of the permease may be inserted as a helical hairpin. It is also important that Dunten *et al.* (1993*b*) showed that disruption of the salt bridge between Asp-237 and Lys-358 causes the permease to be inserted into the membrane much less efficiently, which raises the possibility that the C-terminal half of the polypeptide may be inserted post-translationally. Finally, the first 22 amino-acid residues in the permease, which represent the N-terminal hydrophilic domain and the first half of putative helix I, are not important for activity, but enhance the efficiency of insertion into the membrane (Bibi *et al.* 1992).

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