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What's New with Lactose Permease

H. Ronald Kaback,[1] Kirsten Jung,[1] Heinrich Jung,[1] Jianhua Wu,[1] Gilbert G. Privé,[1] and Kevin Zen[1]

The lactose permease of *Escherichia coli* is a paradigm for polytopic membrane transport proteins that transduce free energy stored in an electrochemical ion gradient into work in the form of a concentration gradient. Although the permease consists of 12 hydrophobic transmembrane domains in probable α-helical conformation that traverse the membrane in zigzag fashion connected by hydrophilic "loops", little information is available regarding the folded tertiary structure of the molecule. In a recent approach site-directed fluorescence labeling is being used to study proximity relationships in lactose permease. The experiments are based upon site-directed pyrene labeling of combinations of paired Cys replacements in a mutant devoid of Cys residues. Since pyrene exhibits excimer fluorescence if two molecules are within about 3.5Å, the proximity between paired labeled residues can be determined. The results demonstrate that putative helices VIII and IX are close to helix X. Taken together with other findings indicating that helix VII is close to helices X and XI, the data lead to a model that describes the packing of helices VII to XI.

KEY WORDS: Lactose permease; site-directed mutagenesis; cysteine modification; pyrene; excimer fluorescence.

INTRODUCTION

Although it is now widely accepted that the driving force for a wide range of seemingly unrelated phenomena (e.g., secondary active transport, oxidative phosphorylation, and 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 other forms of chemical energy like ATP remains a puzzle. On the other hand, gene sequencing and analyses of deduced amino acid sequences indicate that many of the biological machines that catalyze energy transduction, secondary transport proteins in particular (Henderson *et al.*, 1990; Marger and Saier, 1993), fall into large families encompassing proteins from archaebacteria to the mammalian central nervous system, thereby suggesting that the members of each family have common basic structural features and mechanisms of action. In order to gain insight into the general problem of ion-gradient coupled active transport, this laboratory is concentrating on the lactose (lac)[2] permease of *Escherichia coli* as a paradigm.

Accumulation of β-galactosides against a concentration gradient in *E. coli* occurs via lac permease, a hydrophobic polytopic cytoplasmic membrane protein that catalyzes the coupled translocation of a single β-galactoside with a single H^+ (i.e., β-galactoside/H^+ symport or cotransport) (see Kaback, 1983, 1989, 1992 for reviews). Under physiological conditions, the proton electrochemical gradient across the

[1] Howard Hughes Medical Institute, Departments of Physiology and Microbiology and Molecular Genetics, Molecular Biology Institute, University of California Los Angeles, Los Angeles, California 90024-1662; K. Jung, H. Jung and G. G. Privé are Postdoctoral Fellows of the Deutscher Akademischer Austauschdienst, the European Molecular Biology Organization, and the American Cancer Society (California Division), respectively.

[2] Abbreviations: lac, lactose; $\Delta\bar{\mu}_{H^+}$, the proton electrochemical gradient across the membrane; *N*-(1-pyrene)maleimide, pyrene maleimide.

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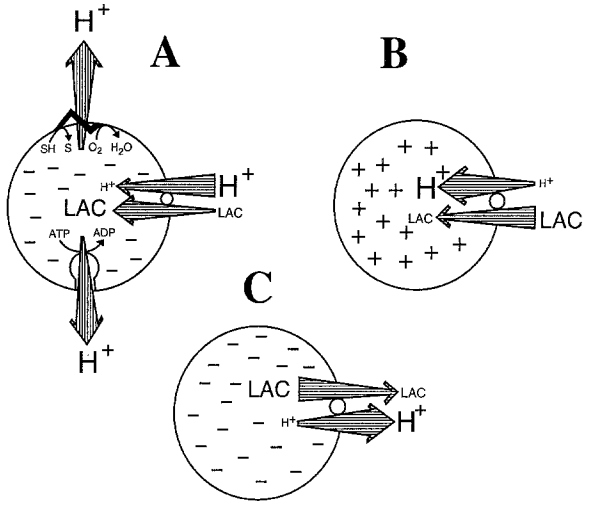

Fig. 1. H^+/lactose symport in *E. coli*. (A) Lactose accumulation in response to $\Delta\bar{\mu}_{H^+}$ (interior negative and alkaline) generated either by respiration or ATP hydrolysis. (B) Uphill H^+ transport in response to an inwardly directed lactose gradient. (C) Uphill H^+ transport in response to an outwardly directed lactose gradient.

cytoplasmic membrane ($\Delta\bar{\mu}_{H^+}$) is interior negative and/or alkaline, and the permease utilizes free energy released from downhill translocation of H^+ to drive accumulation of β-galactosides against a concentration gradient (Fig. 1). In the absence of $\Delta\bar{\mu}_{H^+}$, the permease catalyzes the converse reaction, utilizing free energy released from downhill translocation of β-galactosides to drive uphill translocation of H^+ with 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 (Büchel *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 solubilized from the membrane, purified to homogeneity (Newman *et al.*, 1981; Foster *et al.*, 1982; Viitanen *et al.*, 1986; see also Wright *et al.*, 1986), and shown to catalyze all the translocation reactions typical of the β-galactoside transport system *in vivo* with comparable turnover numbers (Matsushita *et al.*, 1983; Viitanen *et al.*, 1984). Therefore, the product of *lacY* gene is solely responsible for all of the translocation reactions catalyzed by the β-galactoside transport system.

SECONDARY STRUCTURE

Circular dichroic measurements on purified lac permease reveal that the protein is over 80% helical in conformation, 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 (Foster *et al.*, 1983). Based on these findings, a secondary structure was proposed in which the permease is composed of a hydrophilic N-terminus followed by 12 hydrophobic segments in α-helical conformation that traverse the membrane in zig-zag fashion connected by hydrophilic domains (loops) with a 17-residue C-terminal hydrophilic tail (Fig. 2). Support for the general features of the model and evidence that both the N and C termini of the permease are exposed to the cytoplasmic face of the membrane was obtained subsequently from laser Raman spectroscopy (Vogel *et al.*, 1985), immunological studies (Carrasco *et al.*, 1982, 1984a,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) have 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*) chimeras. Under normal conditions, alkaline phosphatase is synthesized as an inactive precursor in the cytoplasm of *E. coli* with a short signal sequence that directs its secretion into the periplasm where it dimerizes to form active enzyme. If the signal sequence is deleted, however, the enzyme remains in the cytoplasm in an inactive form. When alkaline phosphatase devoid of the signal sequence is fused to the C-termini of fragments of a cytoplasmic membrane protein *in vivo*, enzyme activity reflects the ability of the N-terminal portions of the chimeric polypeptides to translocate the enzyme to the outer surface of the membrane (Manoil and Beckwith,

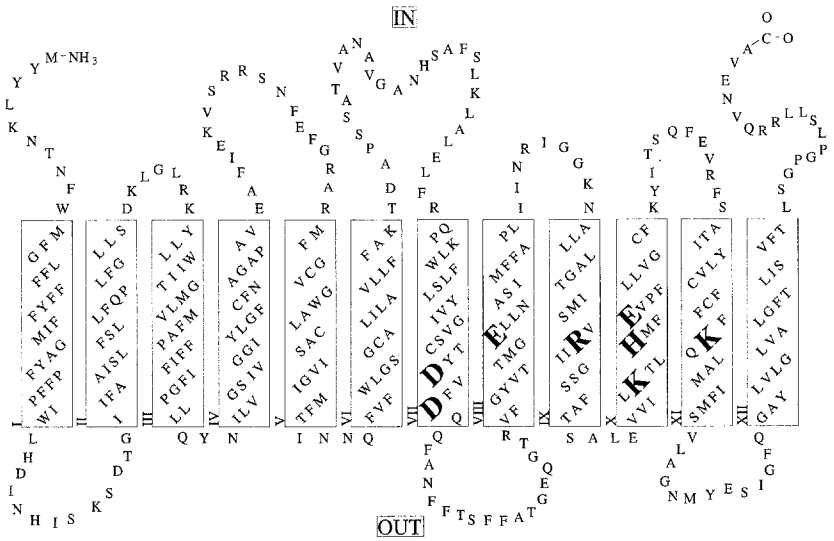

Fig. 2. Secondary structure of lac permease. The model is based primarily on hydropathy analysis (Foster *et al.*, 1983). The single-letter amino acid code is used, and Asp237, Asp240, Glu269, Arg302, Lys319, His322, Glu325, and Lys358 are highlighted. Hydrophobic transmembrane helices are shown in boxes, and the topology of helix VII was modified according to results obtained from a series of *lacY-phoA* fusions in this domain (M. L. Ujwal and H. R. Kaback, unpublished observations).

1986). Alkaline phosphatase activity in cells independently expressing each of 36 *lacY-phoA* fusions exclusively favors the topological predictions of the model with 12 transmembrane domains.

In addition, Calamia and Manoil (1990) demonstrated that approximately half a transmembrane domain is needed to translocate alkaline phosphatase to the external surface of the membrane. Thus, the alkaline phosphatase activity of fusions engineered at every third amino acid residue in putative helices III and V (Fig. 2) increases abruptly as the fusion junction proceeds from the 8th to the 11th residue of each of these transmembrane domains. Furthermore, when fusions are constructed at each amino acid residue in putative helix X of the permease, the data obtained are in good agreement with the model. That is, the alkaline phosphatase activity of the fusions increases sharply as the fusion junction proceeds from Phe320 to His322 (Fig. 2; M. L. Ujwal, E. Bibi, C. Manoil, and H. R. Kaback, unpublished information), indicating that these residues are located in approximately the middle of helix X.

Bibi and Kaback (1990) restricted the *lacY* gene into two approximately equal-size fragments that were subcloned individually or together under separate *lac* operator/promoters. Under these conditions, lac permease is expressed in two portions: (i) the N terminus, the first six putative transmembrane helices and most of putative loop 7; and (ii) the last six putative transmembrane helices and the C terminus. Cells expressing both fragments transport lactose at about 30% the rate of cells expressing intact permease to a comparable steady-state level of accumulation. In contrast, cells expressing either half of the permease independently do not transport lactose. [^{35}S]Methionine labeling and immunoblotting experiments demonstrate that intact permease is completely absent from the membrane of cells expressing *lacY* fragments either individually or together. Thus, transport activity must result from an association between independently synthesized pieces 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 the gene fragments are expressed together, polypeptides identified as the N- and C-terminal moieties of the permease are found in the membrane. The results are consistent with the conclusion that the N- or C-terminal halves of lac permease are proteolyzed when synthesized independently and that association between the two complementing polypeptides leads to a more stable, catalytically active complex. More recent experiments demonstrate that co-expression of independently cloned fragments of the *lacY* gene encoding N_2 and C_{10} (Wrubel *et al.*, 1990), N_1, and C_{11} or N_7 and C_5 (K. Zen, E. McKenna, D. Hardy, E. Bibi, and H. R. Kaback, in preparation) also

form stable molecules in the membrane which interact to form functional permease, while expression of the fragments by themselves yields polypeptides that are relatively unstable and exhibit no transport activity. In contrast, *lacY* gene fragments encoding duplex permeases split in putative transmembrane domains III or VII (Fig. 2) are unable to form functional complexes, implying that the "split permease approach" might be useful for approximating helical boundaries. In addition, the demonstration that polypeptides corresponding to N_I and C_{II} 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.

Purified lac permease reconstituted into proteoliposomes exhibits a notch or cleft (Costello *et al.*, 1984, 1987), an observation also documented by Li and Tooth (1987) using different techniques. The presence of a solvent-filled cleft in the molecule may have important implications with regard to the mechanism of β-galactoside/H^+ symport, as the barrier within the permease may be thinner than the full thickness of the membrane. Therefore, the number of amino acid residues in the protein directly involved in translocation may be fewer than required for lactose and H^+ to traverse the entire thickness of the membrane. A solvent-filled cleft may also represent a caveate with respect to interpretation of spectroscopic experiments designed to test the accessibility of specific regions of the protein to solvent.

The remainder of this discussion deals with very recent experiments utilizing site-directed mutagenesis and protein engineering combined with fluorescence spectroscopy to obtain information regarding proximity relationships in the C-terminal half of lac permease. Although other aspects of permease structure and function will not be covered, a noteworthy and surprising conclusion derived from extensive site-directed mutagenesis studies (see Kaback, 1992) is that very few of the residues in the polypeptide are directly involved in the mechanism of secondary active transport.

FUNCTIONAL INTERACTIONS BETWEEN PUTATIVE INTRAMEMBRANE CHARGED RESIDUES

Recently, King *et al.* (1991) found that lac permease mutants with Thr in place of Lys358 or Asn in place of Asp237 are defective with respect to active transport. Second-site suppressor mutations of K358T[3] yield neutral amino acid substitutions for Asp237 (Asn, Gly, or Tyr), while suppressors of D237N exhibit Gln in place of Lys358. Based on these findings, it was proposed that Asp237 and Lys358 interact via a salt bridge, thereby neutralizing each other. It is presumed that replacement of either charged residue with a neutral residue creates an unpaired charge that causes a functional defect, while neutral substitutions for both residues do not cause inactivation because the unpaired charge is removed. Consequently, the secondary-structure model proposed for the permease was altered to accommodate a putative salt bridge between Asp237 and Lys358 in the low dielectric domain of the membrane by moving Asp237 into the middle of transmembrane helix VII from the hydrophilic domain between helices VII and VIII (King *et al.*, 1991). As discussed below, however, spectroscopic studies and a series of *lacY-phoA* fusions in helix VII suggest that Asp237 and Asp240 may be close to the membrane–water interface. Therefore, these residues are placed near the N terminus of putative helix VII in Fig. 2.

As part of an extensive site-directed mutagenesis study with an engineered form of lac permease that is functional but devoid of Cys residues (C-less permease; van Iwaarden *et al.*, 1991), putative intramembrane residues Asp237, Asp240, Glu269, Arg302, Lys319, His322, Glu325, and Lys358 were systematically replaced with Cys (Fig. 2). Individual replacement of any of the residues essentially abolishes active lactose transport (Sahin-Tóth *et al.*, 1992). By using the single-Cys mutants D237C and K358C, a double-Cys mutant was constructed containing Cys replacements for *both* Asp237 and Lys358 in the same molecule. D237C/K358C transports lactose at about half the rate of C-less permease to almost the same steady-state level of accumulation. Remarkably, replacement of Asp237 and Lys358, respectively, with Ala and Cys or Cys and Ala or even interchanging Asp237 with Lys358 causes little change in activity. Subsequently (Sahin-Tóth and Kaback, 1993a), the side chains of Asp237 and/or Lys358 were extended by replacement with Glu and/or Arg

[3] 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 *lac* permease. The sequence is followed by a second letter denoting the amino acid replacement at this position.

or by site-specific derivatization of single-Cys replacement mutants. Iodoacetic acid was used to carboxymethylate Cys, or methanethiosulfonate derivatives (Akabas *et al.*, 1992) were used to attach negatively charged ethylsulfonate or positively charge ethylammonium groups. Replacement of Asp237 with Glu, carboxymethyl-Cys, or sulfonylethylthio-Cys yields active permease with Lys or Arg at position 358. Similarly, the permease tolerates replacement of Lys358 with Arg or ammonium ethylthio-Cys with Asp or Glu at position 237. Moreover, permease with Lys, Arg, or ammonium ethylthio-Cys in place of Asp237 is highly active when Lys358 is replaced with Asp or Glu, confirming the conclusion that the polarity of the charge interaction can be reversed without loss of activity and demonstrating that the distance between positions 237 and 358 can be extended by up to five bond lengths (Sahin-Tóth and Kaback, 1993a). The results demonstrate that neither Asp237, Lys358, nor the interaction between these residues is important for permease activity and provide a strong indication that Asp237 and Lys358 interact in a "flexible" manner to form a salt bridge.

Lac permease mutants in the charge pair Asp237-Lys358 are inserted into the membrane at wild-type levels if the charge pair is maintained with either polarity. On the other hand, disruption of the interacting pair often causes a marked decrease in the amount of protein inserted into the membrane, suggesting a role for the salt bridge in permease folding and/or stability (Dunten *et al.*, 1993a; Sahin-Tóth and Kaback, 1993a). Interestingly, [^{35}S]methionine pulse-chase experiments (Dunten *et al.*, 1993a) indicate that these permease mutants, as opposed to certain C-terminal truncation mutants (Roepe *et al.*, 1989; McKenna *et al.*, 1991, 1992), may be degraded prior to or during insertion into the membrane, as the mutant proteins are inserted into the membrane in a stable form if they are overproduced at a high rate from the T_7 promoter. In any case, the observations raise the possibility that Asp237 and Lys358 may interact in a folding intermediate, but not in the mature molecule. As discussed above, however, inactive single mutants with Cys in place of Asp237 or Lys358 regain full activity upon carboxymethylation or treatment with methanethiosulfonate ethylammonium, respectively, which restores a negative charge at position 237 or a positive charge at position 358, and similar results are obtained when the charges are reversed. Therefore, it seems very likely that although neither residue nor the putative charge pair is important for activity, the interaction between Asp237 and Lys358 plays a role in folding/stability, and the residues maintain proximity in the mature permease.

To test the possibility that other charged residues in transmembrane helices are neutralized by charge-pairing ("charge-pair neutralization"), 13 additional double mutants were constructed in which all possible interhelical combinations of negative and positively charged residues were replaced pairwise with Cys (Sahin-Tóth *et al.*, 1992). Out of all the combinations of double-Cys mutants, only D240C/K319C exhibits significant transport activity. However, the functional interaction between Asp240 and Lys319 is different phenomenologically from Asp237–Lys358. Thus, D240C/K319C catalyzes lactose transport as about half the rate of C-less to a steady-state level of accumulation that is only about 25–30% of the control. Moreover, although significant activity is observed with the double-Ala mutant or with the two possible Ala-Cys combinations, interchanging Asp240 and Lys319 completely abolishes active transport. In addition, replacement of Asp240 with Glu abolishes lactose transport, and permease with carboxymethyl-Cys at position 240 is inactive when paired with Lys319, but exhibits significant activity with Arg319. Sulfonylethylthio-Cys substitution for Asp240 also results in significant transport activity. Permease with Arg or ammonium ethylthio-Cys in place of Lys319 exhibits high activity with Asp240 as the negative counterion, but no lactose transport is observed when either of these modifications is paired with Glu240. Finally, mutations in Asp240-Lys319 do not affect insertion of the permease into the membrane. Therefore, although neither Asp240 nor Lys319 *per se* is mandatory for active transport, the interaction between this pair of charged residues exhibits much more stringent properties than Asp237-Lys358, and the polarity of the interaction appears to be important for activity.

Lee *et al.* (1992) have also provided evidence that there is an interaction between Asp240 and Lys319 by using a different experimental approach. These workers replaced Asp240 with Ala by site-directed mutagenesis and found little or no active sugar transport. Two second-site revertants were then isolated, one with Gln in place of Lys319 and the other with Val in place of Gly268. The double mutants exhibit little or no accumulation of sugar, but manifest significant rates of lactose entry down a concentration gradient. Although suppression of D240A by G268V

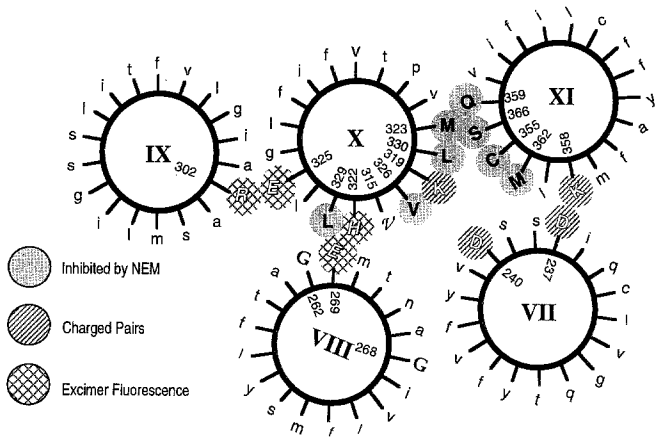

Fig. 3. Helical wheel model of putative helices Vii-IX in lac permease viewed from the periplasmic surface. In addition to the paired residues described in the text (i.e., R302 and E325, E325 and H322, H322 and E269, D240 and K319, D237 and K358), also shown are G268 and G262 which are second-site suppressors for D240 and E325, respectively, and V315. Residues marked with gray exhibit strong inhibition by *N*-ethylmaleimide when they are replaced with Cys.

was not explained (see below), the properties of the double mutant D240A/K319Q are consistent with the observations of Sahin-Tóth *et al.* (1992) and Sahin-Tóth and Kaback (1993a). However, the suggestion that Asp240-Lys319 may play a direct role in H^+ translocation (Lee *et al.*, 1992) is inconsistent with the observation that various combinations of Cys-Ala replacements at these positions yield permease that is able to concentrate lactose against a concentration gradient (Sahin-Tóth *et al.*, 1992).

It should be emphasized that the charge-pair neutralization approach is dependent upon permease activity and will not reveal charge-paired residues if they are essential for activity. In this regard, it is noteworthy that double-Cys mutants involving residues suggested to be H-bonded and directly involved in lactose-coupled H^+ translocation and/or substrate recognition [i.e., Arg302, His322, and Glu325 (see Kaback, 1989, 1992), as well as Glu269 which has also been shown to be an important residue (Hinkle *et al.*, 1990; Ujwal *et al.*, 1993)], are defective with respect to active lactose transport. On the other hand, as revealed below, it is likely that certain pairs of these residues are in close proximity in the tertiary structure of the permease and probably interact.

Despite the indication that Asp237-Lys358 and Asp240-Lys319 may participate in salt bridges, the evidence for the interactions is indirect. Moreover, other approaches are required to determine the location of the residues relative to the plane of the membrane and to demonstrate directly that the pairs are in close proximity. C-less permease mutants containing double-Cys or paired Cys-Ala replacements will be particularly useful in this respect. Preliminary efforts to estimate the accessibility of Cys residues at positions 237 and 358 with water- or lipid-soluble sulfhydryl reagents suggest that Cys residues at the two positions are accessible to both types of reagents, although the lipid-soluble reagents are relatively more effective (Dunten *et al.*, 1993a). Other preliminary experiments with permease mutants specifically labeled at positions 237 or 358 with paramagnetic or fluorescent probes (Jung *et al.*, 1993a), as well as a series of alkaline phosphatase fusions in helix VII (M. L. Ujwal and H. R. Kaback, unpublished observations), are consistent with placement of both residues near the interface at the external surface of the membrane rather than in the middle of helix VII (Fig. 2). Efforts to demonstrate disulfide bond formation directly by oxidation of appropriate double-Cys mutants are also in progress. In any event, based on the evidence currently available, it is clear that Asp237-Lys358 and Asp240-Lys319 interact functionally, and it is reasonable to suggest that both pairs of residues may be in close proximity. It follows that putative helix VII (Asp237 and Asp240) may neighbor helices X (Lys319) and XI

(Lys358) in the tertiary structure of the permease (Fig. 3).

USE OF SITE-DIRECTED FLUORESCENCE LABELING TO STUDY PROXIMITY RELATIONSHIPS

Because of the difficulty inherent in crystallizing hydrophobic membrane proteins (Deisenhofer and Michel, 1989), a high-resolution structure of lac permease is not available, and development of alternative methods for obtaining structural information is essential. Therefore, pairs of charged residues in putative transmembrane helices of C-less permease were replaced with Cys in order to provide specific sites for labeling with *N*-(1-pyrene)maleimide (pyrene maleimide) (Jung, *et al.*, 1993). Pyrene maleimide was chosen as a fluorophore because two pyrene moieties can form an excited-state dimer (excimer) that exhibits a unique emission maximum at approximately 470 nm if the conjugated ring systems are within about 3.5 Å of each other and in the correct orientation (Kinnunen *et al.*, 1993) and because this fluorophore has been used previously to study proximity relationships between Cys residues (e.g., Betcher-Lange and Lehrer, 1978; Ishii and Lehrer, 1987; Lüdi and Hasselbach, 1988; Sen and Chakrabarti, 1990).

Arg320 (putative helix IX), His322 (helix X), and Glu325 (helix X) were studied initially because these residues are important for activity and are thought to interact (Püttner *et al.*, 1986; Carrasco *et al.*, 1986; Püttner and Kaback, 1988; Püttner *et al.*, 1989, Carrasco *et al.*, 1989; Lee *et al.*, 1989). Furthermore, Cys-scanning mutagenesis (Sahin-Tóth and Kaback, 1993b) of putative transmembrane helices IX and X demonstrates that Arg302, His322, and Glu325 are the only three residues in this region of the permease that are crucial for activity. To test the proximity of the residues, the double-Cys mutants H322C/E325C, R302C/H322C, and R302C/E325C and the corresponding single-Cys mutants were constructed with the biotinylation domain from a *Klebsiella pneumoniae* oxalacetate decarboxylase (Cronan, 1990) in the middle cytoplasmic loop, purified by avidin-affinity chromatography (Consler *et al.*, 1993), labeled with pyrene maleimide, reconstituted into proteoliposomes, and studied by fluorescence emission spectroscopy (Jung *et al.*, 1993b).

With the double mutant H322C/E325C, a typical pyrene excimer fluorescence emission band at about 470 nm is observed. The observation is consistent with the postulate that His322 and Glu325 are in a portion of the permease that is α-helical, since the residues would be predicted to lie on the same face of an α-helix. Mutant R302C/E325C labeled with pyrene maleimide also exhibits excimer fluorescence; however, insignificant fluorescence is observed around 470 nm with mutant R302C/H322C. As a whole, the data indicate that helix IX is in close proximity to helix X, but Arg302 appears to be close to Glu325 rather than His322 (see Menick *et al.*, 1987).

Since hydrophobic proteins like lac permease have a strong tendency to aggregate, it is important to determine whether the excimer fluorescence observed results from an intramolecular interaction within single molecules or from an intermolecular interaction between two permease molecules. The following experiments were performed to test these alternatives: (i) Each single-Cys mutants was analyzed, and no excimer band is observed at 470 nm. (ii) Single-Cys mutants R302C and E325C were purified separately, labeled, mixed, and reconstituted into proteoliposomes. No excimer fluorescence at 470 nm is observed, and the fluorescence emission spectrum is identical to those obtained from the unmixed single-Cys mutants. (iii) If excimer fluorescence results from an intermolecular interaction, the intensity of the 470 nm band should be inversely related to the lipid : proton ratio. Therefore, pyrene maleimide-labeled R302C/H322C permease was reconstituted at lipid : protein ratios of 128 : 1, 385 : 1 and 1000 : 1 (w/w). All three samples exhibit essentially no excimer fluorescence. Based on these three control experiments, it seems very likely that the excimer fluorescence observed with E325C/H322C and R302C/E325C permeases results from intramolecular interactions between pyrene molecules attached to Cys residues within single molecules.

As discussed above, studies on second-site suppressor mutations (King *et al.*, 1991; Lee *et al.*, 1992) and site-directed mutagenesis studies on C-less permease (Sahin-Tóth *et al.*, 1992; Dunten *et al.*, 1993; Sahin-Tóth and Kaback, 1993a) indicate that helix VII (Asp237 and Asp240) is in close proximity to helices X (Lys319) and XI (Lys358). Combining the results obtained from pyrene fluorescence with the previous studies leads to the model shown in Fig. 3 where helices VII, IX, X, and XI are shown to interact via ion pairs between R302 and E325, K319 and D240, and K358 and D237. Although not shown, a side-view projection of the helices reveals that these

residues are at approximately the same level with respect to depth in the membrane (Jung *et al.*, 1993b).

In addition to neutral substitutions for Lys319, as mentioned above, a second-site suppressor mutant for Asp240 has been described (Lee *et al.*, 1992) with Val in place of Gly268. Also, recent experiments (J. Wu and H. R. Kaback, unpublished information) demonstrate that the phenotype of E325C is suppressed by substitution of Ser for Gly262. These observations suggest that putative helix VIII may interact with helices VII and X in such a manner as to bring Glu269 into proximity with His322 (Fig. 3).

Regarding Glu269, by carrying out cassette mutagenesis of *lacY* DNA encoding putative helix VIII, Hinkle *et al.* (1990) identified mutants that retain the ability to catalyze lactose accumulation. A stripe of residues, largely on one side of helix VIII opposite Glu269, was shown to tolerate mutations with relatively little effect on activity, suggesting that this mutable strip of low information content is probably in contact with the membrane phospholipids. No active mutants in Glu269 were identified, however, and this residue was subjected to site-directed mutagenesis (Ujwal, *et al.*, 1993). Permease with Cys or Gln in place of Glu269 is completely inactive, while Asp269 permease is completely defective with respect to lactose transport, but transports β,D-galactopyranosyl 1-thio-β-D-galactopyranoside (TDG) reasonably well. In addition, it should be noted that paired double mutants containing E269C and Cys replacements for each of the other charged residues in transmembrane domains are inactive (Sahin-Tóth *et al.*, 1992). In brief, therefore, Glu269 plays an important role in transport, but the charge-pair neutralization approach gives no indication as to whether Glu269 interacts with another residue.

To test the notion that Glu269 interacts with His322, the double-Cys mutant E269C/H322C was constructed (Jung *et al.*, 1993b). The mutant and the corresponding single-Cys mutants containing biotinylation domains in the middle cytoplasmic loop were purified, labeled with pyrene maleimide, and reconstituted into proteoliposomes. Remarkably, pyrene maleimide-labeled E269C/H322C permease exhibits a distinct excimer emission band, and importantly, no excimer fluorescence is observed with pyrene maleimide-labeled E269C or H322C nor when the single-Cys mutants are labeled and mixed prior to reconstitution. The results provide a strong indication that Glu269 is close to His322 and imply that helix VIII is in close proximity to helix X (Fig. 3).

Further evidence for the model comes from Cys-scanning mutagenesis of helix XI in C-less permease (Dunten *et al.*, 1993b). When each amino acid residue in helix XI (from Ala347 to Ser366) is replaced with Cys, the great majority of the mutants exhibit highly significant activity. Each active Cys-replacement mutant was then tested for inactivation by *N*-ethylmaleimide, and the single-Cys mutants that exhibit strong inhibition fall on the same face of helix XI (Fig. 3). Similarly, Cys-scanning mutagenesis of helix X (Sahin-Tóth and Kaback, 1993b) reveals that with the exception of Val331, the *N*-ethylmaleimide-inhibitable Cys-replacement mutants are present on the same face of helix X as Val315, Lys319, His322, and Glu325 (Fig. 3).

Other experiments (K. Jung, H. Jung, and H. R. Kaback, in preparation) indicate that excimer fluorescence between pyrene maleimide-labeled Cys residues in transmembrane domains can be used to study certain dynamic aspects of permease folding. The excimer observed with reconstituted pyrene maleimide-labeled R302C/E325C or E269C/H322C permease is markedly diminished by increasing concentrations of sodium dodecylsulfate (up to 0.6%), while the excimer band with the H322C/E325C mutant is unaffected. Apparently, the detergent disrupts tertiary interactions within the permease with little effect on secondary structure. Consistently, the double mutants E269C/H322C and R302C/E325C do not exhibit excimer fluorescence after labeling with pyrene maleimide in octyl-β-D-glucopyranoside, but do so after reconstitution into proteoliposomes.

Although individual replacement of each of the charged residues in transmembrane domains inactivates active lactose transport (Padan *et al.*, 1985; Püttner, *et al.*, 1986, 1989; Carrasco *et al.*, 1986, 1989; Püttner and Kaback, 1988; Lee *et al.*, 1989; King and Wilson, 1989a,b, 1990; Sahin-Tóth *et al.*, 1992; Ujwal, *et al.*, 1993), preliminary experiments (K. Jung, H. Jung, and H. R. Kaback, in preparation) indicate that some of the constructs exhibit ligand-induced conformational alterations. Excimer fluorescence in proteoliposomes containing pyrene maleimide-labeled E269C/H322C permease is quenched by Tl^+, and the effect is markedly attenuated by 5 mM TDG (i.e., Stern–Volmer plots reveal a decrease in the quenching constant for Tl^+ from 27 to 10 M^{-1} in the presence of 5 mM TDG). In contrast, Tl^+ quenching of the excimer band observed with pyrene maleimide-labeled H322C/E325C permease is unaffected by TDG. Interestingly, it has also been demonstrated

recently (Sahin-Tóth and Kaback, 1993b) that C-less permease with a single Cys residue in place of Val315 [presumably the N-terminal residue in helix X (Fig. 2) which is on the same face as His322 and E325 (Fig. 3)] is inactivated by *N*-ethylmaleimide much more rapidly in the presence of TDG or $\Delta\bar{\mu}_{H^+}$. Most recently (H. Jung, M. Sahin-Tóth, and H. R. Kaback, in preparation), V315C permease containing the biotin acceptor domain in the middle cytoplasmic loop (Consler *et al.*, 1993) has been purified, and the kinetics of pyrene maleimide labeling has been studied in the presence and absence of TDG. The studies confirm the observations described with right-side-out membrane vesicles and imply that ligand binding or $\Delta\bar{\mu}_{H^+}$ induces a change in tertiary structure without affecting secondary structure.

SUMMARY AND CONCLUDING REMARKS

The lac permease of *E. coli* is being used as a paradigm for secondary active transport systems that transduce the free energy stored in electrochemical ion gradients into work in the form of a concentration gradient. This hydrophobic, polytopic, cytoplasmic membrane protein catalyzes the coupled, stoichiometric translocation of β-galactosides and H^+, and it has been solubilized, purified, reconstituted into artificial phospholipid vesicles, and shown to be solely responsible for β-galactoside transport. The *lacY* gene which encodes the permease has been cloned and sequenced, and based on spectroscopic analyses of the purified protein and hydropathy profiling of the amino acid sequence, a secondary structure has been proposed in which the protein has 12 transmembrane domains in α-helical configuration that traverse the membrane in zig-zag fashion connected by hydrophilic loops with the N and C termini on the cytoplasmic face of the membrane. Unequivocal support for the topological predictions of the 12-helix model has been obtained by analyzing a large number of lac permease–alkaline phosphatase (*lacY-PhoA*) fusions. Second-site suppressor analysis and application of site-directed mutagenesis and chemical modification to a functional permease devoid of Cys residues (C-less permease) have provided a strong indication that helix VII is probably close to helices X and XI in the tertiary structure of the permease. Very recent experiments in which paired Cys replacements in C-less permease were labeled with pyrene, a fluorophore that exhibits excimer fluorescence when two of the unconjugated ring systems are in close approximation, demonstrate that His322 and Glu325 are probably located in a α-helical region of the permease and that helix IX is probably close to helix X. Other results suggest that, helix VIII (Glu269) is close to helix X (His322), and site-directed pyrene-maleimide labeling experiments are consistent with the prediction. Preliminary results indicating that conformational changes in the permease can be detected as a result of either ligand binding or imposition of $\Delta\bar{\mu}_{H^+}$ are also discussed.

With current developments in molecular biology, it seems probable that the basis structures and mechanisms of action of secondary transport proteins have been conserved throughout evolution. Therefore, studies on bacterial transport systems which are considerably more easy to manipulate than their eukaryotic counterparts have important relevance to secondary transporters in higher-order systems, particularly with respect to the development of new approaches to structure–function relationships. Although it is now possible to manipulate membrane proteins to an extent that was unimaginable only a few years ago, it is unlikely that transport mechanisms can be defined on a molecular level without high-resolution structural information. In addition to structure, however, dynamic information is required at high resolution, and as suggested by some of the experiments discussed here, a combination of site-directed mutagenesis and fluorescence spectroscopy may be particularly useful in this respect.

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