Lac Permease of *Escherichia coli*: Histidine-322 and Glutamic Acid-325 May Be Components of a Charge-Relay System

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ABSTRACT: When Glu-325 in the *lac* permease of *Escherichia coli* is replaced with Ala, lactose/H^+ symport is abolished. Thus, the altered permease catalyzes neither uphill lactose accumulation nor efflux. Remarkably, however, permease with Ala-325 catalyzes exchange and counterflow at completely normal rates. Taken together with the results presented in the accompanying paper [Püttner, I. B., Sarkar, H. K., Poonian, M. S., & Kaback, H. R. (1986) *Biochemistry* (preceding paper in this issue)], the findings suggest that the His-322 and Glu-325 may be components of a charge-relay system that plays an important role in the coupled translocation of lactose and H^+.

The preceding paper (Püttner et al., 1986) confirms and extends earlier observations (Padan et al., 1979, 1985; Patel et al., 1982; Garcia et al., 1982) focusing on the importance of His-205 and His-322 in lactose/H^+ symport by the *lac* permease. Evidence was presented indicating that these two residues perform different roles in the mechanism and, more specifically, that His-322 may be directly involved in H^+ translocation.

According to the putative secondary structure model of the permease (Foster et al., 1983), Glu-325 is located in a hydrophobic, transmembrane α-helix (i.e., helix 10). If this is the case, it follows that the functional groups in Glu-325 and His-322 may be in even closer proximity than implied by the residue numbers. Furthermore, structure/function studies on the serine proteases (Blow et al., 1969) indicate that Asp and His may function with a Ser residue as components of a charge-relay system, a mechanism that might easily be adapted in part to H^+ translocation in transport enzymes such as the *lac* permease. For these reasons, Glu-325 was replaced with Ala by using oligonucleotide-directed, site-specific mutagenesis (Zoller & Smith, 1983).

Materials and Methods

All materials utilized were reagent grade and were obtained from commercial sources as described (Sarkar et al., 1985).

Bacterial Strains. All of the bacterial strains used have been described (Püttner et al., 1986; Sarkar et al., 1985).

Site-Directed Mutagenesis. Oligonucleotide-directed, site-specific mutagenesis (Zoller & Smith, 1983) was performed essentially as described (Sarkar et al., 1985), except that the single-stranded DNA template contained several uracil residues in place of thymine (Kunkel, 1985). The codon for Glu-325 in the permease was changed to an Ala codon (GAA → GCA) by using the following synthetic mutagenic primer, which contains a single mismatch (·): 5′-GTACTĠCAAACATATGC-3′. Phage harboring the mutation were identified initially by colony–blot hybridization (Carter et al., 1984), and the presence of the mutation was later verified by dideoxynucleotide sequencing (Sanger et al., 1977; Sanger & Coulson, 1978) with the synthetic sequencing primer used to sequence the His-322 replacements (Pandan et al., 1985). Cloning of the mutated *lac Y* insert into pACYC184 and subsequent transformation of *Escherichia coli* T184 with the recombinant plasmid were performed as described (Sarkar et al., 1985).

Growth of Cells and Preparation of Membrane Vesicles. Cells were grown and induced with isopropyl 1-thio-β-D-galactopyranoside, and right-side-out (RSO)[1] membrane vesicles were prepared as described (Püttner et al., 1986).

Transport Assays. Assays of lactose transport in intact cells were performed with [1-^{14}C]lactose by rapid filtration (Trumble et al., 1984). Assays of efflux, exchange, and counterflow in RSO membrane vesicles were carried out as described (Kaczorowski & Kaback, 1979).

Binding of Monoclonal Antibody. In order to estimate the amount of permease in the membrane, immunoblot analyses were performed with monoclonal antibody 4A10R and ^{125}I-labeled protein A (Carrasco et al., 1985).

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

Results

Although data will not be shown, single-stranded M13mp19 phage DNA containing *lac Y* with the mutation described was isolated and sequenced (Sanger et al., 1977; Sanger & Coulson, 1978) by using a primer complementary to *lac Y* 50–100 bases downstream from the mutation (Padan et al., 1985). The sequence analysis demonstrates that the mutated *lac Y* gene contains an A → C change in codon 325 such that Glu (GAG) is changed to Ala (GCG).

As described for the His-322 mutations (Püttner et al., 1986; Padan et al., 1985), when *E. coli* HB101 (Z^+Y^-) is transformed with pA325 and grown on eosin–methylene blue indicator plates at 25 mM lactose, the cells form red colonies similar to those observed in HB101 transformed with pGM21 (not shown). However, T184 cells transformed with the same plasmid (i.e., strain A325) do not catalyze active lactose transport (Figure 1). Similarly, the rate of efflux is almost negligible in RSO membrane vesicles from A325 (Figure 2A; $t_{1/2} \cong 10$ s for T206 vesicles and 540 s for A325 vesicles). In striking contrast, the rate of exchange in A325 vesicles is identical with that observed in T206 vesicles within experi-

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[1] Abbreviation: RSO, right side out.

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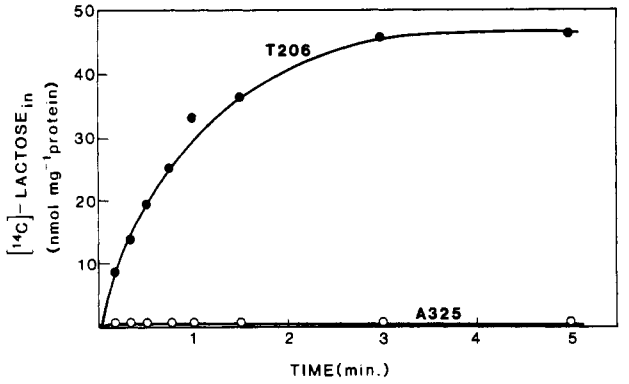

FIGURE 1: Lactose transport in *E. coli* T206 (●) and A325 (○). Transport was measured at given times as described (Trumble et al., 1984) with [1-^{14}C]lactose (17.5 mCi/mmol) at a final concentration of 0.38 mM.

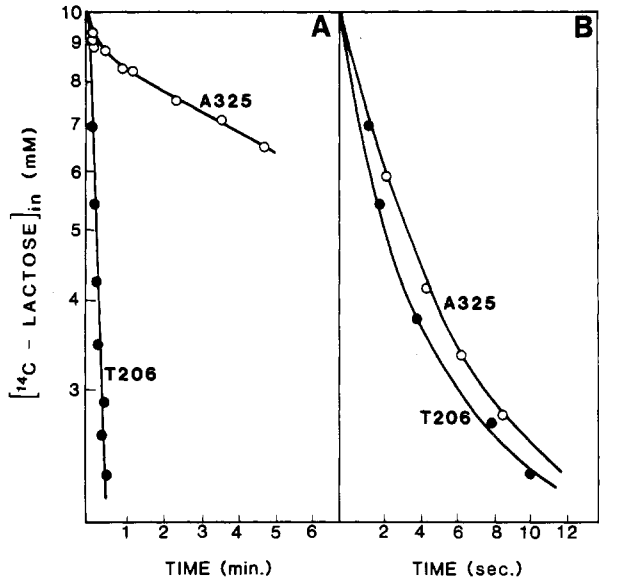

FIGURE 2: Lactose efflux (A) and exchange (B) in RSO membrane vesicles from *E. coli* T206 (●) and A325 (○). The experiments were performed essentially as described (Kaczorowski & Kaback, 1979) except that the stop solution contained 20 mM $HgCl_2$ in addition to 0.1 M KP_i (pH 5.5)/0.1 M LiCl.

mental error (Figure 2B). Moreover, A325 membrane vesicles catalyze counterflow at essentially the same rate and to the same extent as T206 vesicles, but the internal concentration of [1-^{14}C]lactose in A325 vesicles is maintained at high levels for a markedly prolonged period relative to vesicles containing wild-type permease (Figure 3). The effect is consistent with a marked defect in efflux coupled with unimpaired exchange activity (Kaczorowski & Kaback, 1979; Kaczorowski et al., 1979; Viitanen et al., 1983; Carrasco et al., 1984; Herzlinger et al., 1985).

The defect observed in A325 is not due to a difference in the amount of permease in the membrane as demonstrated by immunoblot analysis [not shown; cf. Padan et al. (1985)]. Thus, the intensity of the 33-kDa band corresponding to *lac* permease in A325 membranes is comparable to that in T206 membranes.

Discussion

The provocative implication of the data presented here and in the preceding paper (Püttner et al., 1986) is that important information regarding the mechanism of lactose/H^+ symport can be obtained at the level of individual amino acid residues in lieu of a defined three-dimensional structure.

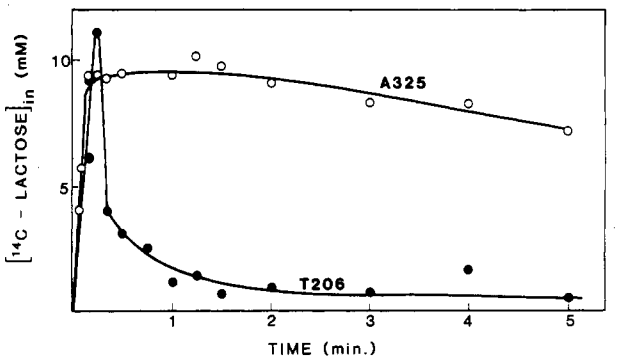

FIGURE 3: Lactose counterflow in RSO vesicles from *E. coli* T206 (●) and A325 (○). The experiments were performed essentially as described (Kaczorowski & Kaback, 1979) with internal lactose at 10 mM and external [1-^{14}C]lactose (10 mCi/mmol) at a final concentration of 1.6 mM.

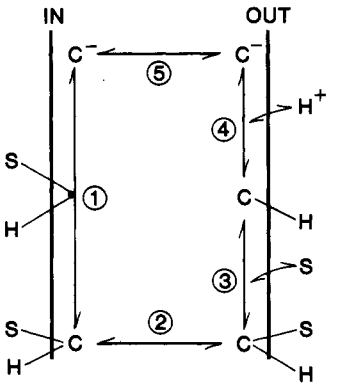

FIGURE 4: Schematic representation of reactions involved in efflux, exchange, and counterflow. C represents the *lac* permease; S, the substrate (lactose). The order of substrate and H^+ binding at the inner surface of the membrane is not implied [from Kaczorowski & Kaback (1979)].

Although the permease is yet to be crystallized, on the basis of circular dichroic and laser Raman spectroscopy in conjunction with various algorithms for secondary structure, models have been proposed for the permease in which the protein contains 12–14 transmembrane α-helical domains that traverse the membrane in a zigzag fashion (Foster et al., 1983; Bieseler et al., 1985; Wright et al., 1985; Vogel et al., 1985). Although it is clear that the structure cannot be resolved until the permease is crystallized, in each of the models proposed, His-322 and Glu-325 are located in a transmembrane α-helix. Furthermore, Bieseler et al. (1985) have provided more direct evidence that this portion of the protein is in α-helical conformation.

When the amino acid sequence of putative helix 10 (Foster et al., 1983) is subjected to three-dimensional computer modeling (V. S. Madison, unpublished information), the carboxylate group in Glu-325 is about 1.5 Å from the imidazole ring in His-322. On these grounds, it seems a likely possibility that the two residues are ion paired. In order to test the hypothesis, Glu-325 is being changed to Asp, which is shorter by one methylene carbon. In addition, Val-326 will be interchanged with Glu-325 in order to move the carboxylate still further from His-322.

Mechanistically, the results are easily rationalized by the kinetic scheme shown in Figure 4. Accordingly, efflux down a concentration gradient consists of a minimum of five steps: (1) binding of substrate and H^+ to the permease on the inner surface (order unspecified); (2) translocation of the ternary complex to the outer surface; (3) release of substrate; (4) release of H^+; (5) return of the unloaded permease to the inner

surface. Alternatively, exchange and counterflow with external lactose at saturating concentrations involve steps 1 to 3 only (Kaczorowski & Kaback, 1979; Kaczorowski et al., 1979; Viitanen et al., 1983; Carrasco et al., 1984; Herzlinger et al., 1985). Since all steps in the mechanism that involve protonation or deprotonation appear to be blocked in the His-322 mutants, it seems reasonable to suggest that protonation of His-322 is intimately involved in step 1. In contrast, replacement of Glu-325 with Ala results in a permease that is defective in all steps involving net H^+ translocation, but catalyzes exchange and counterflow normally. Clearly, therefore, permease with Ala at position 325 is probably blocked in step 4 of the mechanism (i.e., it is unable to lose H^+).

If these considerations are correct, they provide the basis for a strategy to delineate other residues involved in H^+ translocation both before and after His-322 and Glu-325. By functional analyses of a population of uncoupled mutants, it should be straightforward to determine which mutants do and which do not catalyze exchange and counterflow. Alterations in residues in the pathway before His-322 should not catalyze either exchange or counterflow, while alterations in residues after Glu-325 should catalyze both reactions, and DNA sequence analyses of *lac Y* in the two subclasses of uncoupled mutants should reveal the residues involved.

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Registry No. L-Glu, 56-86-0; L-His, 71-00-1; *lac* permease, 9068-45-5.

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