Mutations in the lacY gene of Escherichia coli define functional organization of lactose permease
(membrane transport/membrane protein/quaternary structure/negative dominance/active site)

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ABSTRACT Mutations in the lacY gene of Escherichia coli have been used to analyze the functional organization of lactose permease. Deletions suggest that the NH2 terminus of lactose permease is not essential and can be replaced by residues of the cytoplasmic enzyme β-galactosidase. Negative dominant mutations in the lacY gene can be explained by the assumption that membrane-associated lactose permease is active as a dimer or oligomer. The map positions of these mutations and other point mutations that lower or alter the sugar specificity define regions of lactose permease involved in sugar or proton binding and transport.

Lactose permease transports and accumulates galactosides through the inner membrane into the cytoplasm of Escherichia coli (1). Genetic analysis of the lacY gene coding for lactose permease has shown that the permease is made of one polypeptide chain (2). The sequence of the Y gene and its reading frame have been determined (3). The NH2-terminal protein sequences of lactose permease synthesized in vivo and in vitro are in agreement with the DNA sequence and define the start codon (4). According to these analyses, lactose permease is a protein composed of 417 residues.

Lactose permease is well analyzed functionally. Mitchell's chemosmosic theory applies to it. It transports one proton with every molecule of galactoside (5). Vesicles containing it need not be energized to bind galactosides (6). It is symmetrically accessible in the membrane to its substrates (7). The insolubility of lactose permease, however, has rendered its biochemical analysis difficult. It has at least one essential SH group which can be protected by various galactosides (8). This property has been used as assay for its partial purification (8). It has been labeled by a photoaffinity label (9). But so far, nothing is known about the functional organization of the domains a protein of this size is expected to have.

We used genetic methods to elucidate the molecular mode of action of lactose permease. A similar analysis has been performed previously with the lac repressor producing I gene in order to define the functional domains of lac repressor (10, 11). As in the case of the I gene we isolated negative dominant mutants of the Y gene. They indicate a dimeric or oligomeric structure for lactose permease. We isolated mutants of the Y gene with decreased affinity for lactose and increased affinity for maltose (which is normally not transported by lactose permease) to define the sugar-binding site. We also isolated a deletion mutant that replaces the intercistronic DNA in front of the ribosomal binding site of the Y gene and others that replace the extreme NH2 terminus of lactose permease with the NH2 terminus of β-galactosidase. Random deletions (2) and defined deletions with known end points produced in vitro by partial digestion with Hha I and subsequent ligation were used to map the mutations.

MATERIALS AND METHODS

Enzymes and Chemicals. HinfI and Hha I were purchased from New England Biolabs. Hpa II, EcoRI, and the Klenow fragment of DNA polymerase I were from Boehringer Mannheim. Chemicals for electrophoresis were obtained from BDH Biochemicals (Poole, England). [32P]dATP was purchased from Amersham Buchler. Dimethylsulfoxide and hydrazine were kindly provided by E. Vogel (Institut für Organische Chemie, Cologne). 5-Bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) and isopropyl thiogalactoside were obtained from Bachem Fine Chemicals (Torrance, CA). Plates and media were as described (2). Other chemicals were purchased from Sigma (Munich) or Merck (Darmstadt).

Production and Analysis of the Hha I Deletions in the lacY Gene. The 2100-base-pair EcoRI fragment containing the Y gene (3) was inserted into the plasmid pBR32 (12). Recombinant plasmids were selected for growth on melibiose plates at 42°C in a bacterial strain carrying a lacpro deletion. The DNA of one of the recombinant plasmids was partially cleaved by Hha I. Linear DNA molecules not more than 500 base pairs shorter than the original linearized plasmid were purified by electrophoresis and subsequent isolation from a low-melting agarose gel and circularized by ligation. Transformants of a recA lacY strain (ara z [lacpro], thi strA recA rif, F-lacFPZ Y- A" MAAS13 A- pro") were tested for growth on lactose plates. Only those transformants that could not grow on lactose plates contained plasmids with internal deletions in the Y gene, which was shown by the examination of the EcoRI-cleaved plasmids. The deletions were mapped by restriction analysis with Hpa II.

Isolation and Mapping of the Y- Mutations. A logarithmic-phase culture of the heterogenote lac- LB recAR/F-lacZ- M330b Y' pro' growing in rich medium was treated with the mutagen N-methyl-N'-nitro-N-nitrosoguanidine for 3 min at a final concentration of 40 μg of mutagen per ml. The recA mutation did not abolish mutagenesis as control experiments showed. To stop further mutagen activity the culture was diluted 1:200 in liquid rich medium and grown overnight at 30°C. Cells were plated approximately 100 cells per plate on minimal glucose plates containing streptomycin and incubated for 48 hr at 30°C. Colonies were then replica plated on minimal lactose and minimal glucose plates. Colonies that grew on minimal glucose plates but not on minimal lactose plates after incubation at 30°C for 48 hr were purified and the epispore was crossed into a nalidixic acid-resistant strain carrying a [lacpro] deletion. Colonies that were unable to grow in this nalidixic acid-resistant background on minimal melibiose plates at 42°C and which therefore had a mutation in the lacY gene of the F-lac Z- M330b Y- pro' epispore were further analyzed. The F-lac Z- M330b Y- pro' epispores were crossed back again into a lac" LB recA strain and
the negative dominant character of the epissomal lacY<sup>+</sup> mutation was verified by the failure of the lac<sup>+</sup>L8 recA/F'lacZ<sup>−</sup> ΔM330 Y<sup>−</sup>-pro<sup>−</sup> heterogenote to grow on minimal lactose plates. For mapping, the episodes were crossed into a set of chromosomal lac<sup>+</sup> deletions. The lac<sup>−</sup>/F' lacZ M 330 Y<sup>−</sup>-pro<sup>−</sup> heterogenotes were grown overnight in liquid rich medium, and drops of these overnight cultures were spotted on minimal melibiose plates; recombination was analyzed after 48-hr incubation at 42°C.

RESULTS

Deletion of the Intercistronic Region Between lacY and lacZ. The intercistronic region between the lac Z and lac Y genes (3) includes a typical Shine–Dalgarno (13) sequence, T-A-A-G-G-A, which precedes the ATG start codon for the enzyme by eight base pairs. A stem and loop structure can be constructed from the intercistronic region beginning with the three ochre codons at the end of the Z gene and the region coding for the NH<sub>2</sub> terminus of lactose permease (3). This structure has no apparent function because all of the intercistronic DNA preceding the Shine–Dalgarno sequence can be deleted without decreasing the expression of lactose permease. We conclude this from the DNA sequence of the internal Z deletion M330b which lacks the DNA from codon 38 of the Z gene to the first base pair of the Shine–Dalgarno sequence (Fig. 1). M330b was isolated as a revertant of the strongly polar ochre mutation U118 in codon 18 of the Z gene on melibiose at 42°C (17). The activity of lactose permease is the same in mutant and wild type. Thus, the Shine–Dalgarno sequence is sufficient to initiate normal translation of the lactose permease.

Fusions of the lacY Gene. We wondered whether the whole intercistronic region between the Z and Y genes and maybe even the first codons of the Y gene could be replaced by other suitable DNA sequences without impairing the activity of lactose permease. We used Z<sup>−</sup>Y<sup>+</sup> revertants of the Y<sup>+</sup> mutant MAB16 (2) to study this. MAB16 is a nonsuppressible (2), non-polar Y<sup>+</sup> mutant. It maps very close to the Z gene. Some of its Z<sup>−</sup>Y<sup>+</sup> revertants are temperature sensitive, suggesting that MAB16 lies in the structural part (i.e., in one of the first codons of the Y gene).

In order to isolate Z<sup>del</sup><sup>−</sup> revertants from Z<sup>−</sup>Y<sup>+</sup>MAB16 we plated 0.1 ml of 1000 independent overnight cultures on melibiase plates containing the β-galactosidase indicator 5-bromo-4-chloro-3-indolyl β-D-galactoside (18). The plates were incubated for 2 days at 42°C and screened for white revertant colonies. We found six Z<sup>−</sup>Y<sup>+</sup> revertants. Fig. 2 shows the map positions of these mutations. One mutant arose by a double event. The five others carried deletions that extended from the Y proximal end of the Z gene into or beyond the Z gene. One deletion ended in the middle of the Z gene and presumably fused a Z restart to lactose permease. Two others ended close to the lac operator in the Z gene. One of them (MAB16RZ-36) still contained codon 23 of the Z gene (i.e., it recombined with Z<sup>+</sup> (B. Gronenborn, personal communication; ref. 19)). The other deletion (R26) lacked all known Z markers including U118 but left the I gene functionally intact. Three deletions (R2, R22, R23) destroyed I gene activity. They presumably ended in the I gene because similar I<sup>−</sup>–Z<sup>−</sup> fusions have not been found to extend beyond the start codon of the I gene (20).

Revertant MAB16RZ-36 containing codon 23 of the Z gene was used to test whether the NH<sub>2</sub> terminus of β-galactosidase may be fused to functional lactose permease. We crossed the ochre mutation Z<sup>−</sup>U118 which lies in codon 18 of the Z gene (21) into the revertant and thereby destroyed the lactose permease activity. For this purpose we plated the heterogenote Z<sup>−</sup>U118/F' lacproMAB16RZ-36 on lactose plates and isolated revertants. In some of the revertants the F' lacproMAB16RZ-36 had become permease negative by reciprocal recombination as shown by crossing into a (lacpro<sup>del</sup>) background. The simultaneous presence of the Z deletion MAB16RZ-36 and the U118 mutation on the F' lacpro episome was verified by recombination analysis with Z<sup>−</sup> point mutations (Fig. 2).

Negative Dominant Mutations in the lacY Gene. The existence of negative dominant constitutive mutations (I<sup>+</sup>) in the lacI (repressor) gene could be interpreted by assuming lac repressor to be an oligomer (22). In this interpretation, one inactive subunit is sufficient to alter or even abolish the function of the tetramer. Mapping (23) and sequence determination (24) of the I<sup>−</sup> mutants suggested that only the NH<sub>2</sub> terminus of lac repressor was involved in operator binding and recognition. This was fully confirmed later by in vitro experiments using the NH<sub>2</sub>-terminal headpiece of lac repressor in lac operator shielding experiments (25).

The symmetrical behavior (7) of lactose permease in kinetic experiments led us to suspect lactose permease to be dimeric or oligomeric. Before induction, only a few molecules of lactose permease are present in the membrane of each cell. Thus, under suitable conditions, negative dominant I<sup>−</sup> mutants may be isolated. By definition, a Y<sup>−</sup> mutant permease would have lost the capacity to bind or transport galactosides or protons without losing the capacity to enter the membrane and to aggregate. The map positions of such mutants therefore outline the regions or domains of sugar and proton binding and transport.

In order to decrease the concentration of active lactose permease as much as possible, we used the lac promoter mutant L8 (26) as the Y<sup>−</sup> wild type allele in the construction of the heterogenote. The promoter mutation L8 reduces the expression of the lac operon to 8% without abolishing growth on lactose (26). We isolated four Y<sup>−</sup> mutants on an F' lacproMAB16RZ-36 episome. They inhibit, in a recA background, growth of lac<sup>+</sup>L8 on lactose and even on lactose plus isopropyl thiogalactoside (1 mM). We also constructed controls carrying other Y<sup>−</sup> mutations of the F' lacproMAB16RZ-36 episome. They were not dominant, indicating that dominance could not be produced with just any Y<sup>−</sup> mutation.

One of the mutants (Y<sup>−</sup>-4) mapped in deletion group V (Fig. 3). The nonsense mutation NE4 had been shown to have an exchange in codon 33 (5) and to map in deletion group IV (2). Thus Y<sup>−</sup>-4 has a defect somewhere between codons 33 and 43, assuming a statistical distribution of the deletions. The DNA between codons 33 and 45 of lactose permease codes for a small hydrophilic region between two long lipophilic sequences which presumably anchor lactose permease in the membrane (Fig. 3).

One other Y<sup>−</sup> mutation maps in deletion group XXI which corresponds to the region between codons 152 and 191 (Fig. 3). This is not far from the cysteine which is shielded by thiogalactoside against alkylolation with N-ethylmaleimide (K. Beyruth, personal communication). The last two Y<sup>−</sup> mutations map in deletion group XXVIII somewhere between codons 191 and 279. If we assume a random distribution of the deletions, the exchanges are to be found somewhere between residues 250 and 279.

Mutations in the lacY Gene Which Change Affinity and Specificity of Lactose Permease. Another way to look for mutations that make lactose permease specifically defective in sugar or proton binding and transport is to look for mutations that decrease or alter the specificity of lactose permease. We screened our collection of "leaky" (i.e., easily reverting) Y<sup>−</sup> mutants (2) for mutants that grew normally on a high concentration of lactose. Usual lactose plates contain 5 mM lactose. For our screening we used plates containing 0.1 M lactose. We
found 18 mutants that grew on these plates but not on the usual lactose plates. We called these mutants \( Y^{-K} \) to indicate that they might be altered in their binding constants (\( K \)) for galactosides or protons.

One of the \( Y^{-K} \) mutations (MUB7) mapped in deletion group III. This implies that the mutation lies somewhere before codon 33 (Fig. 3). The other \( Y^{-K} \) mutations mapped in the region coding for the COOH-terminal part of lactose permease—i.e., in deletion groups XXV to XXXV (Fig. 3). Thus, all \( Y^{-K} \) mutations but one are due to exchanges between codons 191 and 360 of lactose permease.

A mutant lactose permease that could transport maltose has been described (24). Because the mutant has not lost the capacity to transport lactose and melibiose it could not be
mapped. We looked for other such mutants. A deletion of the maltose transport system (malB 107) (28) was crossed in a (lac-pro)Δ48 strain into which an F′lacpro′Z′Y′ episome was introduced. In this background, a strain having the genotype F′I′Z′Y′ can grow on 0.1 M maltose plus 1 mM isopropyl thiogalactoside but not on 5 mM maltose plus inducer. Thus, we plated 0.1 ml of 50 independent overnight cultures on plates containing 5 mM maltose and 1 mM isopropyl thiogalactoside.

From each plate one revertant was picked and purified. All revertants could still grow on lactose and melibiose at 42°C and be transferred with the episome the capacity to make a (lac-pro)Δ48(malB 107) strain grow on plates containing 5 mM maltose plus inducer.

If growth on 5 mM maltose can be acquired relatively easily through mutation in the lac operon, one may ask whether some of the Y−X mutants have acquired the capacity to grow on plates...
containing 5 mM maltose and isopropyl thiogalactoside. We checked all the Y-κ mutants for growth on such plates. Wild type and all but one (AJ33) of the Y-κ mutants did not grow. AJ33 mapped in deletion group XXVIII, corresponding to a location of the mutation close to codon 265. Five of the Y-κ mutants (MUB7, AV38, AJ36, MA36, and AN14) mapping in deletion groups XXXI to XXXII (Fig. 3) had kept the capacity to grow on 0.1 M maltose plus inducer, thus showing a possible differential destruction of lactose versus maltose transport capacity.

**DISCUSSION**

Study of the revertants of the Y- mutation MAB16 allows the conclusion that the NH₂ terminus of β-galactosidase can replace the most NH₂ terminal residues of lactose permease. Thus, there are no specialized ribosomes for starting translation of the integral membrane protein lactose permease. The NH₂ terminus of this mutant permease is long enough to be probed with antibodies against β-galactosidase in order to decide whether it is present on both sides of the membrane.

The existence of negative dominant mutations suggests that lactose permease is a dimer or oligomer in its active state in the membrane. All other explanations for the negative dominance seem less plausible to us. Why, when normally active as a monomer in the membrane, should lactose permease be inactivated by the Y-κ products? It does not make sense either to assume a limited number of sites in the membrane which the Y-d product would occupy and thereby prohibit the proper positioning of native lactose permease in the membrane. The membranes can support a 10-fold excess of active lactose permease over the wild-type level (14). The simplest explanation for negative dominance seems to be that normally occurring dimers or oligomers of lactose permease that may be formed in the cytoplasm or, less likely, in the membrane (see below) are inactive when one of the subunits is damaged, as in the Y-d products.

A dimeric or oligomeric structure would provide the basis of symmetry of the sugar-binding sites on both sides of the membranes (7). A symmetrical structure, however, creates the problem of how lactose permease enters the membrane. Either one of the subunits rotates in the membrane at least once before aggregation, or lactose permease aggregates first in the cytoplasm and enters the membrane as dimer or oligomer. Neither possibility is excluded. However, we tend to prefer aggregation in the cytoplasm.

The sequence of lactose permease and the existence of the Y-κ mutations favor two models in our opinion. In the first model, the symmetrically dimeric (or possibly tetrameric) lactose permease forms a channel with some of its helical lipophilic sequences. Three helices (the first three lipophilic regions?; see Fig. 3) from each subunit would be sufficient to form a channel of the necessary diameter for sugar transport. This would place the machinery of the sugar and proton recognition sites including the allosteric mechanisms of concerted proton/sugar transport on the outside of the membrane. Specificity would be gained by keeping all unwanted substrates from entering the channel. Compatible with such a model is the ease by which specificity can be altered. The other model would place all the sugar and proton recognition sites into the interior of a large channel formed by at least a dozen lipophilic helices of the dimer or tetramer. This interior structure would then recognize sugar and proton and move them in a concerted fashion from one side of the membrane to the other.

The map positions of the Y-d, Y-κ, and Y₅₉₉ mutations define regions of lactose permease involved in sugar or proton binding and transport. The Y-d mutations and the Y-κ mutations which have not gained the capacity to bind and transport maltose could be defective in the capacity to bind and transport galactosides or protons. We think that the Y-d and Y-κ mutations, which map in deletion groups V and III, respectively, are possibly damaged in proton binding and transport because the hydrophilic sequences involved seem to be too short to play a role in sugar recognition. The Y-d and Y-κ mutations mapping in regions downstream from codon 150 could be damaged in sugar binding. The Y-κ mutant which has gained the capacity to transport maltose is certainly altered in its sugar binding site. Another explanation may be that the Y-d and Y-κ mutations affect substrate binding indirectly rather than directly by causing conformational changes of the carrier.

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