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The melibiose/Na^+ symporter of *Escherichia coli*: kinetic and molecular properties

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The role of the co-transported cation in the coupling mechanism of the melibiose permease of *Escherichia coli* has been investigated by analysing its sugar-binding activity, facilitated diffusion reactions and energy-dependent transport reactions catalysed by the carrier functioning either as an H^+, Na^+ or Li^+-sugar symporter. The results suggest that the coupling cation not only acts as an activator for sugar-binding on the carrier but also regulates the rate of dissociation of the co-substrates in the cytoplasm by controlling the stability of the ternary complex cation–sugar–carrier facing the cell interior. Furthermore, there is some evidence that the membrane potential enhances the rate of symport activity by increasing the rate of dissociation of the co-substrates from the carrier in the cellular compartment.

Identification of the melibiose permease as a membrane protein of 39 kDa by using a T7 RNA polymerase/promoter expression system is described. Site-directed mutagenesis has been used to replace individual carrier histidine residues by arginine to probe the functional contribution of each of the seven histidine residues to the symport mechanism. Only substitution of arginine for His94 greatly interferes with the carrier function. It is finally shown that mutations affecting the glutamate residue in position 361 inactivate translocation of the co-substrates but not their recognition by the permease.

Introduction

Much of our present knowledge of the mechanism underlying substrate-cation co-transport or symport activity in bacteria has been obtained by extensive analysis of the properties of the lactose permease of *Escherichia coli*, which mediates selective and obligatory coupled flows of H^+ with β-galactosides (Wright *et al.* 1986; Kaback 1986, 1988). Recently, however, growing interest in the mechanism of α-galactoside-symport by the melibiose permease of *Escherichia coli* or *Salmonella typhimurium* arose because this carrier accepts Na^+ or Li^+, in addition to H^+, as coupling cation, depending on the substrate and ionic environment (Wilson & Tsuchyia 1978; Wilson & Wilson 1987; Leblanc *et al.* 1988).

The first aim of the present report is to review recent mechanistic information obtained by analysing the effects of permutation of the three coupling cations on (i) the sugar-binding activity and facilitated diffusion reactions catalysed by the melibiose permease of *E. coli* in deenergized membrane vesicles, and (ii) the activating effect of the transmembrane electrical potential on the carrier activity.

In the second part of this report we consider the general molecular characteristics of the

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transport protein. Moreover, studies of the melibiose-symport coupling mechanism at the molecular level have recently been undertaken by using oligonucleotide-directed, site-specific mutagenesis techniques. The functional consequences of selective substitution of each of the seven histidine residues of the permease will be detailed. In addition, mutations of residue Glu361 of the carrier, which produce selective reduction of the rate of translocation of the substrates but do not modify the binding activity of the carrier, are reported.

1. Mechanistic studies of the melibiose/Na^+ symport

Demonstration that the melibiose carrier can function either as an Na^+, H^+ or Li^+-coupled sugar symporter was obtained by measuring with H^+- or cation-sensitive glass electrodes the inward movements of cations associated with the downhill entry of galactosides in de-energized cells (Tsuchiya & Wilson 1978). Recently, it was demonstrated with the same technique that the cationic selectivity of the carrier changes according to whether the galactoside transported has an α or β configuration (Wilson & Wilson 1987). Moreover, kinetic analysis of the co-transport of the physiological substrate melibiose with either H^+, Na^+ or Li^+ as coupling cation revealed significant differences in the kinetic parameters of transport depending on the chemical identity of the coupling ion (Tanaka *et al.* 1980; Bassilana *et al.* 1985). These observations clearly suggest that analysis of the influence of the chemical identity of the coupling cation on either certain steps of the transport cycle, such as substrate-binding activity and reorientations of the loaded and unloaded carrier, or on the activating effect of the electrical potential ($\Delta\Psi$) on the rate of active transport, should provide mechanistic information on the role of the co-transported cation in the coupling mechanism of the melibiose permease.

Sugar binding activity of the melibiose carrier

The sugar analogue *p*-nitrophenyl-α-D-galactoside (NPG) is a suitable substrate to monitor the sugar-binding activity of the melibiose carrier (Cohn & Kaback 1980). It is co-transported by the permease with either H^+ or Na^+ as coupling cation (Wilson & Wilson 1987) and is competitively displaced from the permease by the physiological substrate melibiose (Damiano *et al.* 1986).

The effects of Na^+ (or Li^+) and H^+ on the NPG binding constants were studied in de-energized membrane vesicles prepared from *E. coli* RA11 and incubated in media containing various concentrations of the co-transported cations, at constant or variable pH. At a given pH (6.6), raising the Na^+ concentration from 25 μM to 10 mM produced a progressive decrease of the apparent K_d value from 15 μM to 0.7 μM with no effect on the maximal number of NPG binding sites. These data indicate that Na^+ ions activate NPG binding by selectively increasing the permease affinity for the co-transported sugar. Very similar dependence of the apparent K_d value on the concentration of Li^+ ions was observed. This suggests that the two monovalent cations have comparable activation strengths. It was next observed that raising the H^+ concentration progressively and selectively inhibits the activation of NPG binding by Na^+ or Li^+. It is of interest that the H^+ inhibitory effect was more marked at low than at high Na^+ concentrations and no longer occurred at Na^+ concentrations above 10 mM. This probably indicates that Na^+ (or Li^+) and H^+ compete for a common cationic binding site.

The model of co-substrate binding on the permease shown in table 1 (*a*) accounts for all the data. This model assumes firstly that binding of the coupled cation and sugar substrate is

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TABLE 1. MODEL OF CO-SUBSTRATE BINDING ON THE MELIBIOSE PERMEASE. K_d (H^+), K_d (Na^+) AND K_d (Li^+) ARE THE TRUE DISSOCIATION CONSTANTS FOR H^+, Na^+ AND Li^+ RESPECTIVELY; K_d^H (S), K_d^{Na} (S) AND K_d^{Li} (S) ARE THE TRUE DISSOCIATION CONSTANTS FOR NPG (S) IN THE PRESENCE OF H^+, Na^+ OR Li^+ AS COUPLING ION

(*a*)

$$\begin{array}{ccccccc} & S & & H^+ & Na^+ & & S \\ & \downarrow & & \downarrow & \downarrow & & \downarrow \\ CH^+S & \leftrightarrow & CH^+ & \leftrightarrow & C & \leftrightarrow & CNa^+ \leftrightarrow CNa^+S \\ & K_d^H(S) & & K_d(H) & K_d(Na) & & K_d^{Na}(S) \end{array}$$

(*b*)

(S) = NPG		CATIONS	
K_d^H (S)	20 μM	K_d (H)	0.5×10^{-6} M (pK 6.3)
K_d^{Na} (S)	0.7 μM	K_d (Na)	0.3 mM
K_d^{Li} (S)	0.7 μM	K_d (Li)	0.5 mM

sequential and occurs in the order shown, the cation–sugar–carrier stoichiometry being unity, and secondly that Na^+, Li^+ and H^+ compete for a common cationic binding site. The co-substrate binding constants calculated from the mathematical equation derived for this model are listed in table 1 and show several interesting features. Firstly, the calculated dissociation constant value of Na^+ ions (K_d (Na^+) = 0.3 mM) coincides with the Na^+ concentration that produces half-activation of the transport activity of the carrier (Lopilato *et al.* 1978). Secondly, the true NPG dissociation binding constant in the presence of H^+ (K_d^H (NPG)) is twenty-times higher than that in the presence of Na^+ (K_d^{Na} (NPG)); this indicates that H^+ is a less efficient activator of the sugar binding process than Na^+ or Li^+; this is consistent with the higher K_t value recorded for H^+-coupled reaction (Bassilana *et al.* 1985). Thirdly, the existence of a common cationic binding site explains the observed competitive behaviour of Na^+ ions on inward movement of H^+ associated with sugar entry during sugar-pulse experiments (Tsuchiya *et al.* 1985). Finally, the true proton dissociation constant value (K_d (H^+)) suggests that a residue(s) of the cationic binding site with a pK_a around 6.3–6.5 (histidines?) may be involved in the cation recognition process.

Facilitated diffusion reactions

Analysis of the facilitated-diffusion reactions or flows of co-substrates catalysed by the co-transporter in de-energized membrane vesicles or cells (Kaback 1986; Wright 1986; Bassilana *et al.* 1987, 1988) provides complementary information on the basic mechanism of carrier cycling when not complicated by the activating effect of the electrochemical potential gradient of the coupling cation. These reactions – influx or efflux down a sugar concentration gradient and sugar exchange at equilibrium – were first studied in conditions in which the carrier functioned as an Na^+-melibiose symporter. From the values of the maximal rate of sugar flow recorded during each reaction (figure 1*a*, upper columns), two important conclusions can be drawn.

Firstly, the V_{max} of efflux (EFF) is many times greater than the V_{max} for influx (INF). This suggests that the carrier functions asymmetrically. Secondly, the V_{max} of the exchange reaction (EXC) is again much greater than the V_{max} of influx (INF), indicating that the presence of melibiose in the trans (cytoplasmic) compartment strongly stimulates the rate of sugar entry. A similar trans-stimulating effect of intravesicular sugar on the initial rate of sugar inflow is

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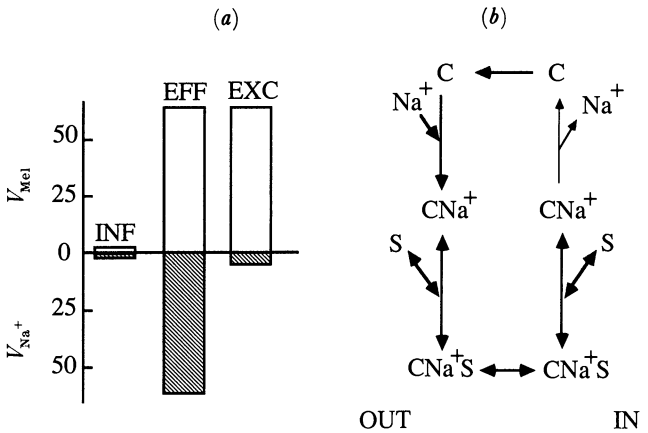


FIGURE 1. Kinetic model and facilitated diffusion properties of melibiose carrier. (*a*) maximal rate of melibiose (V_{Mel}, upper columns) and coupled Na^+ flows (V_{Na}, lower columns) during facilitated reactions (influx, efflux and exchange) catalysed by the carrier in de-energized membrane vesicles. Fluxes are expressed in nmol per mg membrane protein per minute. (*b*) Schematic representation of reactions involved in Na^+ coupled melibiose influx and exchange reactions; (S) melibiose, (C) permease.

observed during counterflow experiments. Analysis of the flows of $^{22}Na^+$ associated with the sugar flux during each of the three reactions provides key information for interpreting the facilitated diffusion data (Bassilana *et al.* 1987). Figure 1*a* (lower columns) indicates that Na^+ entry during the influx reaction is small and comparable in size to the rate of sugar entry. During the efflux reaction, the Na^+ and melibiose fluxes are again tightly coupled and the measured stoichiometry is very near to unity. Surprisingly however, no, or a negligible, flow of Na^+ is associated with sugar movement during the exchange reaction: Na^+ is kinetically occluded during the sugar exchange reaction.

Functional asymmetry of the carrier and kinetic occlusion of the co-transported cation during the sugar exchange reaction can be accounted for by the mirror-type model of the Na^+-melibiose co-transport mechanism depicted in figure 1*b*. Sequential co-substrate binding on the permease facing the outer medium (Na^+ binding first) is proposed on the basis of the NPG binding studies described above. This model also suggests that Na^+ and sugar release from the permease in the intravesicular compartment is again sequential, but in the reverse order, the sugar being released first. Finally it may be assumed that the release of Na^+ ions into the cytoplasm is the slowest partial step. Given the order of co-substrate release and the reduced rate of Na^+ release, sugar exchange proceeds without an associated cation exchange and at the same time the net coupled flows of Na^+ and melibiose in the counter-clockwise direction are hampered.

Comparative analysis of the different facilitated diffusion reactions catalysed by the melibiose carrier functioning as a sugar symporter coupled to either Na^+, Li^+ or H^+ demonstrates that the rates of these reactions are strongly dependent on the chemical identity of the coupling cation (Bassilana *et al.* 1987, 1988). Two results (table 2) support this conclusion. In the first place, the V_{max} of H^+-coupled melibiose influx down a sugar concentration gradient is high, whereas that catalysed in the presence of Na^+ or Li^+ is at the lowest limit of detection. This implies that protons are released at a much greater rate in the cytoplasmic compartment than are Na^+ or Li^+ ions during an influx cycle. Secondly, the Li^+-coupled melibiose exchange activity, unlike the Na^+- or H^+-coupled activities, is unexpectedly

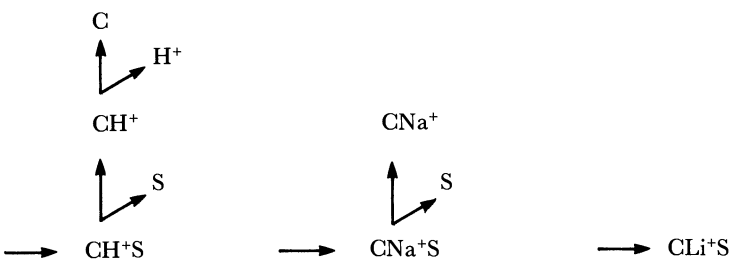
small (Table 2). Thus the rate at which the sugar is released from the carrier into the vesicular compartment in de-energized membranes also varies as a function of the coupling cation.

These observations can be interpreted in terms of the kinetic model of cation-sugar co-transport proposed in figure 1*b* provided one assumes that the rate of release of the co-substrates from the carrier into the inner compartment varies according to the chemical identity of the coupled cation.

TABLE 2. FACILITATED DIFFUSION REACTIONS MEDIATED BY THE MELIBIOSE CARRIER. (*a*) MAXIMAL RATE OF INFLUX, EFFLUX AND EXCHANGE IN DE-ENERGIZED MEMBRANE VESICLES WITH H^+, Na^+ OR Li^+ AS COUPLING CATION; (*b*) DEPENDENCE OF THE PATTERN OF CO-SUBSTRATES RELEASE FROM THE CARRIER INTO THE CYTOPLASM AS A FUNCTION OF THE COUPLING ION

	MEL-H^+	MEL-Na^+	MEL-Li^+
(*a*)	V_{max}	(nmol per mg protein per min)	
influx	42	2	1
efflux	200	57	37
exchange	60	55	1

(*b*)



The proposed mechanism of co-substrate release from the permease into the cytoplasmic compartment prevailing in the presence of each coupling cation is shown schematically in table 2*b*. At one extreme (H^+-melibiose symport), release of both co-substrates occurs. It is interesting that the similarity between the V_{max} values for H^+-coupled melibiose influx and exchange reactions (table 2) suggests that the rate of sugar release is slower than that of H^+ and is rate limiting for carrier turnover in de-energized membranes. At the other extreme (Li^+-melibiose symport), neither co-substrate is released or at best is released at a very reduced rate. Incidentally, the failure of the melibiose carrier to catalyse Li^+-coupled melibiose exchange or influx is apparently not due to an impaired capacity of the Li^+-containing ternary complex to re-orientate as substantial Li^+-coupled exchange activity is observed when the sugar substrate is methyl α D-galactopyranoside or methyl-1-thio-β, D-galactopyranoside (TMG) (Bassilana *et al.* 1988).

Such diversity in the pattern of co-substrate release in the presence of the three different coupled cations can be explained by assuming that the rate of co-substrate release from the carrier into the inner compartment in de-energized conditions is inversely proportional to the stability of the ternary complex cation–sugar–carrier facing the intravesicular medium. It is probable that the stability of the ternary complex is governed by interactions both of the sugar and the coupling cation with the transporter. Furthermore, the strengths of the interactions of the different coupling cations, i.e. Na^+, Li^+ or H^+ (or hydronium, see Boyer (1988)) with the carrier are likely to differ for each coupling cation. This would lead to differences in the stabilities of the ternary complex conformations and to unequal patterns and rates of

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co-substrate release. Our data are consistent with ternary complex stabilities decreasing in the order Li^+, > Na^+, > H^+.

The profile of sugar transport activation by $\Delta\Psi$ varies according to the coupling cation

Kinetic studies of sugar/Na^+ co-transport by the melibiose permease in cells or right-side out membrane vesicles have established that the electrical membrane potential generated during substrate oxidation enhances the initial rate of sugar influx (Lopilato *et al.* 1978; Cohn & Kaback 1980; Bassilana *et al.* 1985). Detailed examination of the relation between initial rate of melibiose influx and magnitude of the electrical potential in membrane vesicles (Bassilana *et al.* 1985; Leblanc *et al.* 1988) indicates that the profile of sugar influx activation by $\Delta\Psi$ varies according to the coupling cation. Indeed, it was initially observed that when the carrier functions according to a Na^+ (or Li^+) symport mode, the maximal rate of influx increases exponentially as a function of $\Delta\Psi$ whereas the apparent affinity transport constants, K_t, for both Na^+ and Li^+ coupled reactions remain constant at values around 0.1–0.2 mM. Strikingly, at all imposed $\Delta\Psi$ values, the stimulating effect of $\Delta\Psi$ is much less marked with Li^+ than with Na^+ as the coupling cation. In contrast to these findings the V_{max} of the H^+-coupled melibiose influx is already high in de-energized membranes and is only slightly enhanced by $\Delta\Psi$; the major effect of the electrical potential on transport activity is indeed to decrease specifically the K_t value for the H^+-coupled sugar transport reaction from 10 mM in the absence of energy down to 1 mM in the presence of a $\Delta\Psi$ of 160 mV. These studies also demonstrate that, at any given $\Delta\Psi$ value, the rate of melibiose influx coupled to Li^+ is systematically lower than that coupled to Na^+ and even lower than the H^+ coupled sugar influx.

Bearing in mind the suggestion that the ternary complex stability decreases as a function of the coupled cation in the order Li^+, Na^+, H^+, we suggest that the extent of the activating effect of $\Delta\Psi$ on the symporter correlates with the reciprocal of the stability of the cation/sugar/carrier ternary complex. In other words, one of the major effects of $\Delta\Psi$ on the melibiose carrier activity is to decrease the stability of the ternary complex facing the cytoplasmic compartment with a resulting increase of the rate of dissociation of the co-substrates in the inner compartment. It is very tempting to mention the possible resemblance between the mechanism of activation of the co-transport reaction by $\Delta\Psi$ proposed above and the mechanism of ATP synthesis by the adenosine triphosphatase from beef-heart mitochondria suggested by the work of Penefsky (1985). This author provided evidence to support the proposal that the energy conserved during the oxidation of substrates by the mitochondrial respiratory chain can be utilized to reduce the very tight binding of product ATP in catalytic sites and to promote dissociation of the nucleotide.

2. Molecular properties of the melibiose carrier

General molecular characteristics of the transport protein

The melibiose carrier is coded by the *melB* gene in the melibiose operon located at 93 min on the genetic map of *Escherichia coli*. The *melB* gene is intercalated between the *melA* gene, which codes for the α-galactosidase responsible for the hydrolysis of the transported sugars, and a putative third gene, *melC*, the function of which is unknown (Hanatani *et al.* 1984). Determination of the nucleotide sequence of a DNA fragment carrying *melABC* genes suggests that the *melB* gene is composed of 1407 nucleotides (Yazyu *et al.* 1984). The amino acid

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sequence inferred from these data indicates that the transport protein consists of 469 amino acids and has a theoretical molecular weight of 52029 Da. As with the lactose permease, the melibiose carrier lacks a typical NH_2 terminal signal sequence. Examination of the amino acid composition of the carrier further suggests that the transport protein has a low polarity (30% polar, 70% non-polar) and is basic. Surprisingly, very little homology between the primary structures of the melibiose and lactose transport proteins of *E. coli* is observed although both carriers share several sugars as substrates (melibiose, NPG, TMG). In contrast, very significant homologies between the NH_2 terminal end of the lactose carrier of *Streptococcus thermophilus* (*lacS* carrier) and the melibiose carrier of *E. coli* have been reported (Poolman *et al.* 1989).

Calculation of the hydropathy (hydrophilicity and hydrophobicity) along the amino acid sequence of the melibiose carrier according to the method of Kyte & Doolittle (1982) suggests that the melibiose carrier is organized into a succession of relatively long hydrophobic segments punctuated by shorter hydrophilic segments in a manner similar to the Lac, AraE and XylE proteins (Henderson 1988). By using Chou & Fasman's procedure for predictions of the presence of α-helix, β-pleated sheet and reverse turn in proteins (Chou & Fasman 1974), a tentative model of the secondary structure of the melibiose carrier protein has been established (figure 2). The transport protein in postulated to consist of 12 α-helical segments (average length 23 residues) that span the membrane in a zig-zag manner. The NH_2- and COOH protein termini are both assumed to face the cytoplasmic compartment.

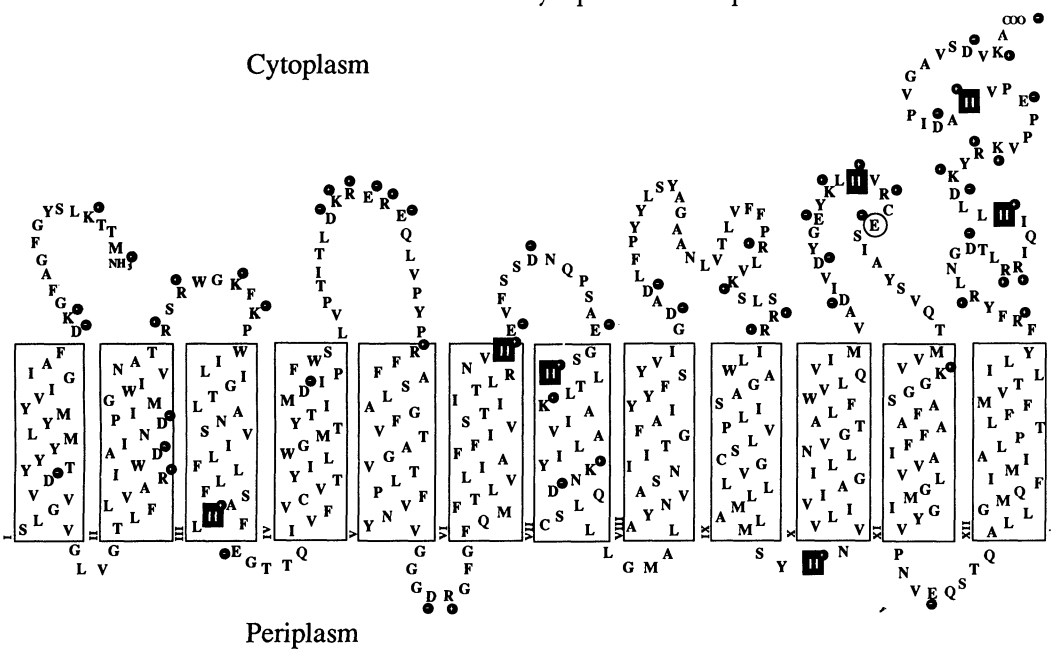

FIGURE 2. Secondary structure model of melibiose permease. α-Helical transmembrane domains are shown in boxes; NH_2 and COOH termini are both located on the cytoplasmic surface of the membrane. Black squares and open circles indicate the respective locations of the seven histidine residues and of glutamate in position 361, the functional importance of which is analysed (see text).

It is interesting to note that, in agreement with observations on other bacterial cytoplasmic membrane proteins (Von Heijne 1986), the predicted cytoplasmic loops connecting the transmembrane helical segments of the melibiose transport protein contain a high density of positively charged arginine and lysine residues. Recently, Botfield & Wilson (1988) proposed a rather different picture of the secondary structure of the melibiose carrier. Indeed, they

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suggested that there are only 11 transmembrane α-helical segments, exhibiting a mean length of 30 amino acids. Because of this uneven number of transmembrane segments, the NH_2 and COOH termini face the cytoplasm and periplasmic space, respectively. The gene fusions strategy developed by Beckwith and collaborators (Manoil *et al.* (1988)) for analysing membrane protein topology should provide means of testing the validity of these predictions. A program of construction of melibiose carrier-alkaline phosphatase hybrid proteins by using the Tn*Pho*A method, is being carried out in our laboratory to distinguish periplasmic and cytoplasmic domains of the transport protein.

Recently, unambiguous *in vivo* identification of the melibiose carrier has been carried out by using the T7 RNA polymerase/promoter expression system (Pourcher *et al.* 1989). In cells poisoned with rifampicin, this expression system allows preferential expression of cloned genes and selective labelling of the encoded polypeptides (Tabor & Richardson 1985). The selectivity of labelling of the *melB* gene product was improved firstly by cloning a DNA fragment restricted to the sole *melB* gene and secondly, by introducing a strong, synthetic ribosome binding site sequence (Crowl *et al.* 1985) upstream from *melB* in the cloning vector. Fluorographic analysis of the (^{35}S) methionine labelled proteins shows that most (60 %) of the radioactivity incorporated in the cells is concentrated in a broad and diffuse band corresponding to a protein with an apparent molecular mass of about 39 kDa (36–42 kDa). This highly-labelled polypeptide is exclusively associated with the inner-membrane fraction. No equivalent labelled polypeptide is expressed and labelled in the absence of inserted DNA. In addition, insertion of the *melA* gene, which codes for the α-galactosidase, leads to specific labelling of a different polypeptide, essentially associated with the cytoplasmic fraction and migrating with a molecular mass of 52 kDa. These data indicate that the protein with a molecular mass of 39 kDa and associated with the membrane fraction is the melibiose transport protein. However, it should be noted that the apparent molecular mass of the identified carrier (39 kDa) is significantly lower than the value of 52 kDa that would be predicted from the nucleotide sequence of *melB* gene. It is likely that the very hydrophobic melibiose carrier, as in other bacterial permeases (Ehring *et al.* 1980, Henderson 1988 and this symposium), binds large amounts of SDS and migrates faster than a soluble protein of comparable molecular mass. The high degree of selectivity and also intensity of labelling of the melibiose carrier protein obtained by using the T7 RNA polymerase/promoter expression system is now being used to monitor purification of the carrier protein.

Contribution of histidines to the melibiose carrier function

Over the past five years, application of oligonucleotide-directed, site-specific mutagenesis techniques to the study of the symport mechanism catalysed by the *lac* permease has proved to be a successful method for investigating the molecular basis of the symport activity. In particular, one histidine (His322) out of four of the lactose permease has been shown to be crucial for the carrier function. Moreover, several additional observations indicate that this histidine is associated with a glutamate residue in position 325 and an arginine in position 302 to form a triad that could function as a charge relay system (for details see Kaback (1988) and this symposium). We have used the same strategy to analyse the role of the histidine residues in the melibiose symport mechanism and also to define the homologies and differences between the molecular mechanisms of the Na^+- (or H^+- or Li^+-) coupled melibiose transport system and the H^+-lactose coupled symporter.

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Two observations suggest that histidines may indeed contribute to the sugar transport mechanism mediated by the melibiose carrier. Firstly, chemical modification of the carrier with the histidyl reagent diethyl-pyrocarbonate (DEPC) inactivates the energy-dependent transport activity of the carrier as well as the facilitated diffusion reactions (downhill sugar influx or efflux and sugar exchange at equilibrium) in de-energized membrane vesicles (Cohn & Kaback 1980; Leblanc *et al.* 1988). Secondly, analysis of the sugar binding properties of the carrier suggests the presence of a residue with a pK_a of 6.3–6.5 in the cationic binding site, i.e. possibly a histidyl residue.

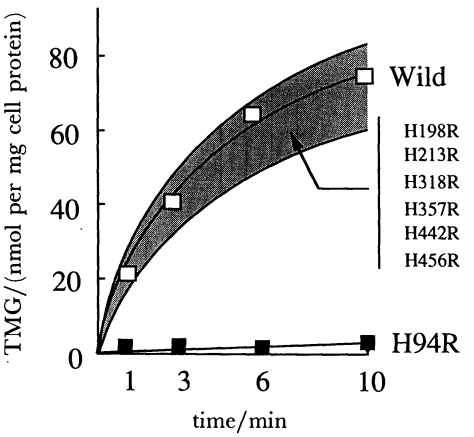

FIGURE 3. Effects of replacing each histidine one at a time by arginine on Na^+-dependent TMG transport in cells; □, wild type; ■, mutant H94R. Curves fitting cell TMG accumulation for the other six mutants (H198R, H213R–H318R–H357R–H442R–H456R) fall within the dotted area and are omitted for clarity.

Oligonucleotide-directed, site-specific mutagenesis (Sarkar *et al.* 1986) has been used to convert the seven histidine residues of the permease individually into arginine residues. The effect of each His to Arg mutation on the carrier activity was first analysed by measuring the rate of TMG transport in the presence of Na^+ ions as coupling cations in DW2 cells ($MelB^-$) bearing the mutated gene inserted into plasmid pKK223–3. Figure 3 shows that one histidine substitution (His 94) out of seven drastically interferes with the transport activity of the melibiose permease. Indeed, all mutated carriers except the permease with arginine in place of His 94 accumulate TMG at a rate and to an extent not less than 60% of that of the unconverted permease. In contrast, the intracellular level of TMG found after 10 min (or even 90 min) in cells bearing the mutated *H94R* gene corresponds to that expected after simple equilibration of the sugar analogue.

Analysis of the sugar binding activity of the different mutated carriers (table 3) further confirms that only the His to Arg substitution of the H94 histidine residue modifies the carrier function. The apparent NPG binding constant ($K_{d_{app}}$) in the presence of 10 mM Na^+ ions and at pH 6.6, the Na^+ activation constant $K_a(Na^+)$ and the inhibitory constant of the physiological sugar ($K_{i_{Mel}}$) on NPG binding of permeases H198R, H213R, H318R, H357R, H442R and H456R are all close to the values measured for the unmodified carrier. This indicates that these six histidine residues are not implicated in either the process of co-substrate recognition by the carrier or in the mechanism of Na^+ activation of sugar binding on the transport protein. The second important piece of information given in this table is that permeases with an arginine in place of His 94 do not bind NPG. Because a total absence of NPG

Table 3. Effects of replacing each histidine one at a time by arginine on sugar binding properties of the melibiose carrier

	wild type	H94R	other mutants H- > R
V_{max} (nmol per milligram protein)	0.35	not	0.1–0.2
K_d app (micromoles)	0.9	measurable	0.5–1.1
K_a (Na) (millimoles)	0.5	—	0.2–0.5
K_i(mel) (millimoles)	0.9	—	0.4–1.0

binding and TMG transport activities of permease H94R could result from a low membrane carrier content in cells carrying the H94R mutated gene, the level of expression of this mutated gene was compared with that of the wild-type *melB* gene by using the T7 RNA polymerase/promoter expression system described above. The data show no difference in the extent of (^{35}S) methionine incorporated by the polypeptide (molecular mass about 39 kDa) associated with the inner-membrane fraction and previously identified as the *melB* gene product (not shown). It was also verified that the conversion of the CAT codon for histidine in position 94 into a CGT codon for arginine is the sole difference between the native *melB* gene and the mutated gene H94R. Finally, it should be mentioned that the mutated permease H94R is unable to catalyse either H^+, Na^+ or Li^+-coupled facilitated diffusion reactions (efflux, exchange) in de-energized cells or membrane vesicles.

All the data reported above therefore indicate that only the histidine residue in position 94 is crucial for the melibiose carrier function. The absence of binding and thus transport activity of the mutated permease H94R makes difficult a precise suggestion of the contribution of this histidyl residue to the mechanism of sugar symport. One can expect that substitutions of different amino-acids for His94 (glutamine, asparagine, alanine, etc.) should provide more information on the role of this histidyl residue. The possibility that His94 is ion-paired with neighbouring acidic residues will also be investigated. It is relevant here to recall that the lactose permease also contains only one histidine (His 322) the substitution of which inactivates the *lac* carrier (Kaback 1988). It is also interesting to mention that both His 94 of the melibiose carrier (figure 2) and His322 of the lactose permease (Bieseler *et al.* 1985) are located in α-helical segments; apparently however, His94 lies in the proximal portion of the melibiose carrier (helix 3) whereas His322 is located in the distal part of the lactose transport protein (helix 10). This may mean that the topologies of the functional domains of each transport protein are different.

*Mutation of residue Glu*361 *affects reorientation of the loaded carrier*

Looking for a potential implication of His–Glu(Asp) ion pairs in the mechanism of action of the melibiose permease, Sarkar & Kaback (in Kaback (1988)) reported that substitution of Glu-361 with glycine or aspartate (mutants E361G, E361D) inactivates Na^+-TMG or H^+-melibiose transport by the carrier. They tentatively concluded that the His357–Glu361 may form an ion pair important for the carrier function. The results of mutagenesis of the histidine residues of the melibiose carrier reported above do not, however, support this suggestion as His357 is not essential for carrier activity. To define the partial step(s) of the transport cycle modified by the Glu361 substitutions, the properties of the mutated permeases were studied in greater detail. Recombinant pKK223–3 plasmids carrying the mutated genes were constructed to increase

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expression of the mutated proteins and thus to raise their residual transport activities to experimentally detectable levels. *E. coli* DW2 (Mel A^+B^-) transformed with the two recombinant plasmids accumulate TMG at a ten-times slower rate than cells expressing the unmodified carrier.

Measurement of the kinetic-transport parameters of the mutated permeases in RSO membrane vesicles (table 4) shows that the reduction of transport activity in both strains results from a selective decrease of the maximal rate of the energy-dependent transport reaction, the affinity-transport constant K_t displayed by each mutant being similar to that of the non-mutated carrier. This table also shows that the variations of carrier content of the different membrane vesicle preparations is insufficient to account for the drop in V_{max} values. In fact calculation of carrier turnover of the mutated transport proteins indicates that the rate of cycling of the E361G and E361D carriers during active transport is at least ten-times slower than the rate of cycling of the unmodified carrier.

TABLE 4. EFFECTS OF GLY, ASP AND ALA SUBSTITUTIONS FOR GLU 361 ON SUGAR TRANSPORT AND NPG BINDING ACTIVITIES MEDIATED BY THE MELIBIOSE CARRIER IN MEMBRANE VESICLES

		wild type	E361G	E361D	E361A
TMG transport	V_{max} (nmol per milligram protein)	90	5	2	9
	K_t (millimoles)	0.17	0.08	0.14	0.06
NPG binding	ν_{max} (nmol per milligram protein)	0.35	0.19	0.21	0.22
	K_d app (micromoles)	0.9	0.5	0.6	0.2
carrier turn-over	k_{cat} (per second)	4.3	0.36	0.14	0.7
facilitated diffusion	EFF ($t_{\frac{1}{2}}$, per second)	66	> 800	—	> 800
	EXC ($t_{\frac{1}{2}}$, per second)	66	> 800	—	> 800

Analysis of the sugar-binding characteristics of the mutated permeases further establishes that substitution of Glu361 by glycine or aspartate does not interfere with the recognition steps of the co-substrates by the carrier (table 4). Thus, in conditions similar to those used for the TMG transport studies (10 mM Na^+, pH 6.6), the apparent NPG dissociation constants ($K_{d_{app}}$) of the modified and native permeases are identical. Also, melibiose displaces equally well the NPG bound both on mutated and on native permeases, indicating that the molecular interactions of the physiological sugar with the two modified transporters are similar to those of the unmodified carrier. Moreover, Na^+ (or Li^+) ions activate NPG binding on the mutated and non-mutated carriers in a very similar fashion (not shown). Finally, table 4 shows that the permeases E361G and E361D no longer catalyse Na^+-coupled efflux and exchange reactions in de-energized membrane vesicles. According to the kinetic mechanism of Na^+/melibiose symport presented in figure 1, these data strongly suggest that re-orientation of the loaded complex (cation–sugar–carrier) is severely impaired by substituting glycine or aspartate for glutamate in position 361.

Studies of the properties of a mutant in which the glutamate residue in position 361 was replaced by alanine (H361A mutant) were found helpful in interpreting the effect of the Glu361 substitutions. Indeed, the rate of TMG transport in E361A mutant whole cells is about 50% that of the wild strain, as compared with 10% in the E361G and E361D mutants. The rate of TMG transport in membrane vesicles from mutant E361A, however, is unexpectedly

low. This is surprising as there is usually a good correlation between the rates of TMG transport in cells and in derived membrane vesicles. As mechanical fragility of this mutated carrier may reflect folding instability of the carrier, or of a particular region of the carrier, one would expect the thermal stabilities of the mutated E361A carriers and of the native ones to be different. Analysis of the temperature dependency of the rate of TMG active transport in cells carrying the mutated E361A carrier and the native carrier shows that a temperature increase from 25 °C to 37 °C during transport measurement leads to a five-fold *decrease* of the rate of TMG transport by mutant E361A whereas a similar rise in temperature produces a fourfold *increase* in the transport rate of the unmodified permease. It is noteworthy that the residual activities of mutants E361G and E361D, although low, are also reduced by a temperature increase.

Considered together, the properties of the three mutants E361G, E361D and E361A, suggest that the major effect of mutating the aspartic residue in position 361 can be accounted for by a structural modification of the carrier. Nevertheless, the change in protein structure is probably local, as only the step involving reorientation of the loaded carrier is modified. Moreover, the relatively high rate of sugar transport by the alanine mutant suggests that the presence of the negative charge of the glutamate residue is required for stability of the local carrier structure rather than for the conformation in itself. As a corollary, the inactivation of the transport function observed by moving apart the negative charge by about 1.5 Å† when aspartate is substituted for glutamate in position 361 suggests that proper location of the negative charge is required for optimal stability of the local structure of the carrier. Finally, considering the proposed location of Glu361 in a cytoplasmic loop connecting helices 10 and 11 in our model (see figure 2), no conclusion can be drawn as to whether the local conformation of this loop is critical *per se* or whether it is important for interactions between this loop and other region(s) of the protein. In any case these data show that recognition and translocation of the co-substrates by the melibiose carrier can be dissociated.

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† Å = 10^{-10} m = 10^{-1} nm.

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