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Review

Molecular biology of the lactose carrier of *Escherichia coli*

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1. Introduction

The biological membrane, which encloses all living cells, is composed of a phospholipid bilayer that hinders the entrance and exit of water-soluble solutes, such as ions, metabolites and antimicrobial agents. However, a specialized class of integral membrane proteins, called transporters or carrier proteins, serve to overcome this membrane barricade by translocating water-soluble solutes across the lipid bilayer [56,89]. Life cannot occur without membrane carrier proteins. Moreover, when carrier proteins are faulty in humans, medical problems can arise. For example, glucose-galactose malabsorption is a serious disease in which a mutation has occurred in the Na^+/glucose carrier of the small intestine and the absorption of glucose and galactose is severely impaired [102]. A defect in a chloride ion channel results in cystic fibrosis [48].

1.1. The chemiosmotic hypothesis

In the early 1960s, Peter Mitchell formulated his now famous chemiosmotic theory, in which an electrochemical gradient of protons, generated by the respiratory chain, was the energetic driving force for the synthesis of ATP [78]. He later predicted that the lactose carrier of *Escherichia coli* accomplished obligatory coupling between proton and sugar movement across the membrane and that the downhill entry of protons resulted in sugar entry and accumulation [79]. This important ion gradient-dependent biological process is known as secondary active transport, distinguished from primary active transport, which directly utilizes chemical, redox or light energy to actively accumulate solutes, and from uniport, which uses neither an ion gradient nor other energy source, but allows transport via the solute gradient itself [80]. Mitchell assigned the term symport to describe solute and ion transport across the membrane in the same direction [79]. One of the most extensively investigated secondary active transporters is the *E. coli* lactose carrier. This review article will focus on the molecular biology, biochemistry and physiology of the lactose carrier.

2. General features of the lactose carrier

2.1. Assays for transport

A qualitative assay for transport is the fermentation of lactose by clones on an indicator plate such as MacConkey agar on which lactose positive clones are red. This is a convenient screening method for *lac*Y mutants (white clones indicate low transport activity). The commonest assay for transport of a sugar is to expose cells lacking β-galactosidase to radioactive lactose. The lactose entering such cells is not metabolized and is accumulated against a lactose concentration gradient (sometimes as high as 200-fold). Since thiomethylgalactoside (TMG) is not metabolized its accumulation may be measured in cells containing β-galactosidase. Another useful assay involves entry of lactose into β-galactosidase-containing cells. In this case the lactose is metabolized and the internal concentration of free lactose remains very low. Thus the entry of lactose is thermodynamically 'downhill'.

The sugar-coupled proton transport is measured in energy-depleted cells deprived of oxygen. Sugar is added and the pH of the external medium is measured. The rise in external pH results from sugar-H + entry into the cells. The stoichiometry of the cotransport may be calculated by correlating the uptake of radioactive sugar with proton entry calculated from the change in pH.

2.2. Discovery and demonstration of lactose-proton co-transport

The lactose carrier has become an important general model for the study of membrane transport systems. It was first described as one of the three structural genes of the celebrated lac operon in 1956 by Monod and co-workers [94]. Lactose-H^+ symport (cotransport) across the membrane was demonstrated from experiments performed by West [109] and West and Mitchell [110] who showed translocation of H^+ into energy-depleted *E. coli* cells immediately after addition of lactose. This lactose-H^+ symport process takes place in a 1:1 stoichiometry for substrate and ion [111]. Biophysical measurements of the size of the carrier (20 ± 2 Å) [22], kinetic studies [24], and fusion protein studies containing two lactose carrier molecules [97] are consistent with the view that the lactose carrier functions as a monomer, although this is still a matter of controversy. Kinetic analyses of the lactose carrier has also provided invaluable information about the order of binding, translocation and release of substrate and ion ([65,66,87,90,116], and reviewed in Ref. [56] and Ref. [89]). However, because it is experimentally difficult to measure kinetic parameters for the lactose carrier under conditions where both lactose and protons are saturated, any mention of K_m throughout this article should be understood to be the apparent K_m rather than the true K_m. Although the lactose carrier is probably the best-understood secondary membrane transport protein, little is known

about the precise mechanism of lactose-H^+ coupling and transport.

2.3. Sequence and structure

The lactose carrier gene (*lac*Y) was cloned by Teather et al. [100] and sequenced by Büchel et al. [16]. *lac*Y was predicted to encode a largely hydrophobic 417 amino acid polypeptide [27]. Alkaline-phosphatase fusion studies have been tremendously useful in the study of the structure of the lactose carrier [17]. The current model of the lactose carrier shows a polytopic protein with 12 membrane-spanning α-helices [17,27,107], where the N- and C-termini of the carrier are present on the cytoplasmic side of the inner membrane of *E. coli* [17,47] (see Fig. 1). With the use of an overproducing *E. coli* strain, Newman et al. [82] purified the carrier to homogeneity in functional form. Electron microscopy analysis revealed the presence of a cleft in the lactose carrier molecule, suggesting a possible site for lactose binding [63]. A high resolution three-dimensional structure of the lactose carrier has not yet been determined nor has one been determined for any symport, antiport or uniport protein to date.

2.4. Expression of two complementary protein fragments of the lactose carrier

Two incomplete fragments of the lactose carrier can insert into the cytoplasmic membrane and show a functional conformation that permits active transport of lactose [3,117]. For example, two non-covalently bound segments of the lactose carrier, amino acids 1–71 (N2) and amino acids 70–417 (C10) placed in a cell showed transport activity. In additional experiments of this type, Bibi and Kaback [3] showed that, when the lacY gene was split into two equal-sized fragments (N6 and C6), the cells expressing both together showed significant transport activity, while cells expressing each half alone showed no activity. Thus two separate portions of the carrier must associate with each other in the membrane in a manner that results in function. When the two fragments were expressed individually little protein was found in the membrane but when expressed together both N-terminal and C-terminal fragments were found in the membrane. Thus the association of the two polypeptides results in a stable and catalytically active complex.

2.5. Deletions in the lactose carrier

The deletion of the C-terminal 16 amino acids has little or no effect on activity. However, deletions of 17, 18, or 19 amino acids results in 40%, 30% and 25% of normal activity respectively [71]. Loss of the C-terminal 20 or 21 amino acids results in complete loss of activity. Truncations of more than 20 amino acids are unstable and are proteolyzed at a rapid rate [95]. At the N-terminal end of the carrier a deletion of the first 23 amino acids does not abolish transport while loss of 38 amino acids results in no activity [5,7,76].

Bibi et al. [4] have shown that internal deletions of the lacY gene which result in the loss of 1 to 6 α-helices fail to accumulate lactose. However, the downhill lactose entry into β-galactosidase-containing cells is still present in such deletions if the C-terminal four α-helices are present. Presumably there is sugar recognition information in the C-terminal 4 helices.

2.6. The role of phospholipids in lactose transport

The lactose carrier was partially purified and transport activity reconstituted in artificial liposomes by Newman and Wilson [83]. This permitted the study of the role of different phospholipids in the artificial liposomes on the transport activity of the carrier. Maximum accumulation of lactose was observed with natural *E. coli* lipids or mixtures of phosphatidylethanolamine (PE) or phosphatidylserine (PS) [20,99]. On the other hand, phosphatidylcholine (PC) or mixtures of PC and phosphatidylglycerol or cardiolipin showed poor accumulation. Page et al. [88] showed that sugar binding was normal in PE-deficient liposomes, although accumulation was defective. Bogdanov and Dowhan [9] studied lactose transport in an *E. coli* mutant which could not synthesize PE and found that accumulation of sugar was reduced 5- to 10-fold relative to cells containing PE. On the other hand lactose downhill entry into β-galactosidase-containing cells (no accumulation required) was normal. Thus carrier-mediated facilitated diffusion was normal while the energy coupling was defective. The K_m for transport was normal in the cells without PE, suggesting that the binding site was normal. The localized positive charge at the surface of the membrane would repel H^+ and attract OH^- making the surface of the membrane more alkaline. This could alter the localized surface environment for charged residues especially histidine. How these changes affect transport is not yet understood.

3. Evolution of the lactose carrier

3.1. A superfamily of transporters

One of the useful techniques in molecular biology is the analysis of proteins that share a common evolutionary origin. In this regard, the lactose carrier has been postulated [68] to be a member of a superfamily of related transporters independently designated as either the transporter superfamily (TSF) [37], major facilitator superfamily (MFS) [68], or most recently, the uniporter, symporter, antiporter superfamily (USA) [34]. This superfamily includes passive and secondary-active carriers, found in organisms ranging from bacteria to humans, which trans-

port diverse substrates such as sugars, amino acids, citric acid cycle intermediates, antibiotics, and antiseptics [35]. Similarities in the structure (12–14 transmembrane domains) and sequences (conserved amino acids) of these functionally-distinct carrier proteins suggest that the members of this superfamily have a common evolutionary origin and thus similar three-dimensional structures, suggesting that they operate by a common mechanism of action [35–37,104,105]. Therefore, findings pertaining to one member of the superfamily may be applicable to all other members of the superfamily.

3.2. A family of homologous carriers within the superfamily

Carriers that are homologous with the lactose-H^+ carrier of *E. coli* include the raffinose-H^+ carrier (RafB) of *E. coli* [1], the sucrose-H^+ carrier (CscB) of *E. coli* [8], the lactose-H^+ carrier (LacY-Kp) of *Klebsiella pneumoniae* [73], and the lactose-H^+ carrier (LacY-Cf) of *Citrobacter freundii* [61]. These carriers are homologous to each other and constitute a family within the superfamily [34,68]. Fig. 2 shows the multiple amino acid sequence alignments of each member of the lactose carrier family.

3.3. The 'GRR' motif

Multiple amino acid sequence alignments have revealed conserved residues, such as the 'G $(X)_3$ D R X G R R' motif [35,36] in the loop between helices 2 and 3 of all members of the superfamily (Figs. 1 and 2). Insertional mutagenesis of several contiguous histidine residues into the loop 2–3, where the motif resides, resulted in significantly reduced lactose transport, indicating the importance of this loop [72]. Recent work has shown that only Asp-68 of the 'G $(X)_3$ D R X G R R' motif is critical for full activity of the lactose carrier [42]. Surprisingly, earlier work on the tetracycline/H + carrier not only showed that Asp-66 (corresponding to Asp-68 of the lactose carrier) is necessary, but that Gly-62, Gly-69, and Arg-70 of the motif were required for function as well [119,120]. Perhaps an analysis of the 'G $(X)_3$ D R X G R R' motif in other members of the superfamily will be necessary to determine if it has a direct functional role in solute transport, and if so, what that functional role might be.

3.4. Other amino acid motifs

The lactose carrier also shares some elements of the 'G $(X)_3$ D R X G R R' motif between helices 8 and 9 as well as the 'R $(X)_3$ G $(X)_3$ G' motif immediately preceding helix 4 [36]. Both motifs are found in almost all members of the superfamily. The significance of these motifs has yet not been evaluated for the lactose carrier. Surprisingly, the lactose carrier does not seem to have either the 'P E S P R' motif (after helix 6) nor the 'P E T K' motif (after helix 12), both of which have primarily been found in symporters and uniporters of the superfamily [36].

4. The history of mutagenesis of the lactose carrier

Extensive mutagenesis of the *lac*Y gene (over 500 mutants) was first reported by Hobson et al. [39]. One interesting set of 179 mutants failed to grow on 5 mM lactose but grew on 100 mM lactose. These 'leaky' or 'K_m' mutants were located primarily in the second half of the *lac*Y gene. Two of these K_m mutants were later cloned and sequenced revealing His-322 → Tyr [49,51] and Asp-237 → Asn [55]. Additional discussion of other mutations of these two amino acids follows later on in this article.

Some of the early studies of the structure–function relationships of the lactose transport protein focused attention on the cysteine residues. Kennedy and co-workers [28] first observed that *N*-ethylmaliemide (NEM), a sulfhydryl-specific reagent, inhibited lactose carrier activity and that the inhibition was greatly reduced by the addition of thiodigalactoside (a high affinity substrate of the carrier). This suggested that one or more Cys residues somehow dictated the substrate-binding site of the lactose carrier. It was later shown by site-directed mutagenesis that Cys-148 was the amino acid protected by substrate against the inhibitory effects of NEM [2,44,118]. However, since the mutant Cys-148 → Ser was active, Cys-148 itself was not critical for full activity of the carrier [81,98]. Eventually, the remaining Cys residues were individually changed to serine and each found to be active [12,74,75,81,98,101,103,106]. When all of the 6 cysteines in membrane spanning helices were changed to serine, the cysteine-less carrier showed 60% normal activity [103]. Starting with the Cys-less carrier, residues of a particular helix (or region) of the carrier were individually replaced by a Cys residue. To date, cysteine-scanning mutagenesis has been performed on helices 1, 3, 5, 7, 9, 10 and 11 of the carrier [47,108]. It was concluded from these studies that only relatively few residues were essential for activity (see below).

Further, lactose accumulation was severely defective when several residues in helix 8 were replaced by other amino acids [38]. Using amber suppressor analysis, in which as many as 12 amino acids can be placed into the position of a single stop codon, Huang et al. showed that substitutions for Trp-10, Gln-219, Gln-256, and Gln-379 had modest-to-good transport activity [41]. However, Trp-33 changed to Pro drastically reduced lactose carrier function while Lys, Leu, Cys or Glu mutant moderately reduced lactose carrier function [41]. Other replacements for Trp-33 resulted in changes in sugar specificity where, for instance, Ser, Gln, Tyr, Ala, Gly or Phe mutants increased the rate of transport for the lactose analog methyl-β-D-thiogalactopyranoside (TMG). Fig. 1 illustrates the locations

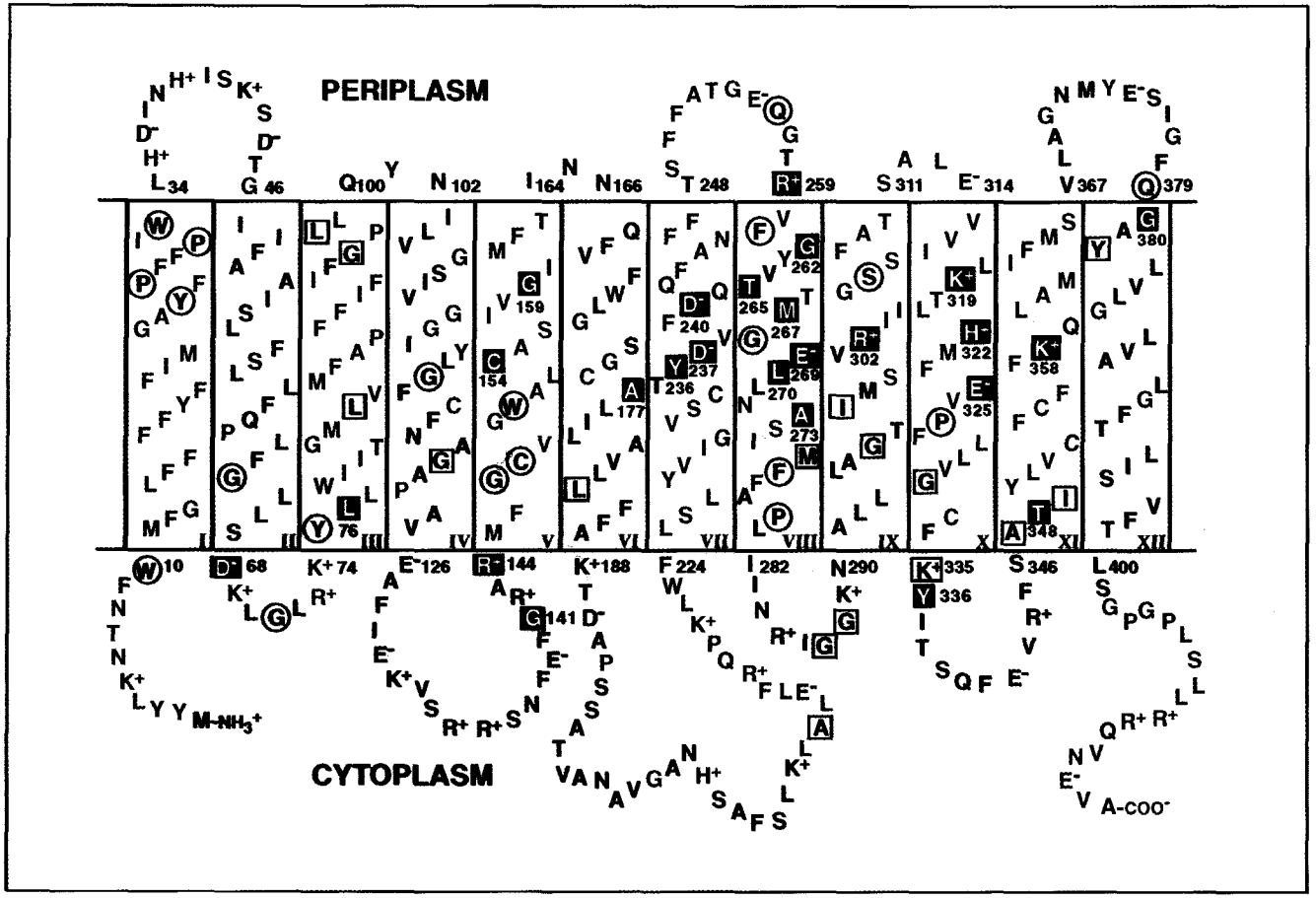

Fig. 1. Mutations in the lactose carrier of *E. coli*. Amino acid residues in dark squares with white lettering indicate that replacements at this position result in less than 25% normal lactose accumulation. Circled residues have been replaced by several other amino acids. One or more substitutions were inactive (less than 25% of normal), while others were more active (between 25–100% of normal). Residues in white squares represent mutations that were moderately active (25–50% of normal). Residues shown in red are those in which mutations resulted in greater than 50% activity. The remaining residues (black) have not been mutated.

and effects of specific mutations on active lactose accumulation in a two-dimensional model of the lactose carrier. Kaback's laboratory has examined all or most of the glycines, serines, prolines, histidines, tryptophans, and tyrosines (last reviewed in Ref. [47]). Taken together with many other mutants, the lactose carrier is probably the

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                    ========Helix 1==========
LacY-Cf       MYYLK NTNFWMFGFF FFFYFFIMGA YFPFFPIWLH EVNHISKGDT
LacY-Ec       MYYLK NTNFWMFGLF FFFYFFIMGA YFPFFPIWLH DINHISKSDT 45
LacY-Kp  MKLSELAPRE RHNFIYFMLF FFFYYFIMSA YFPFFPVWLA EVNHLTKTET
   RafB    MNSASTHK NTDFWIFGLF FFLYFFIMAT CFPFLPVWLS DVVGLSKTDT
   CscB    MALNIPFR NAYYRFASSY SFLFFISWSL WWSLYAIWLK GHLGLTGTEL
Consensus  ---------- ---------- -F-------- -------WL- ----------
            ========Helix 2========       ==========Helix 3=====
LacY-Cf  GIIFACISLF SLLFQPIFGL LSDKLGLRKH LLWVITGMLV MFAPFFIYVF
LacY-Ec  GIIFAAISLF SLLFQPLFGL LSDKLGLRKY LLWIITGMLV MFAPFFIFIF 95
LacY-Kp  GIVFSCISLF AIIFQPVFGL ISDKLGLRKH LLWTITILLI LFAPFFIFVF
   RafB  GIVFSCLSLF AISFQPLLGV ISDRLGLKKN LIWSISLLLV FFAPFFLYVF
   CscB  GTLYSVNQFT SILFMMFYGI VQDKLGLKKP LIWCMSFILV LTGPFMIYVY
Consensus G---------  ---F----G- --D-LGL-K- L-W-----L- ---PF-----
          ====   ==========Helix 4========                    ==
LacY-Cf  GPLLQVNILL GSIVGGIYLG FIYNAGAPAI EAYIEKASRR SNFEFGRARM
LacY-Ec  GPLLQYNILV GSIVGGIYLG FCFNAGAPAV EAFIEKVSRR SNFEFGRARM 145
LacY-Kp  SPLLQMNIMA GALVGGVYLG IVFSSRSGAV EAYIERVSRA NRFEYGKVRV
   RafB  APLLHLNIWA GALTGGVFIG FVFSAGAGAI EAYIERVSRS SGFEYGKARM
   CscB  EPLLQSNFSV GLILGALFFG LGYLAGCGLL DSFTEKMARN FHFEYGTARA
Consensus -PLL--N--- G---G----G ---------- ----E---R- --FE-G--R-
          =====Helix 5=======    ========Helix 6========
LacY-Cf  FGCVGWALCA SIAGIMFTIN NQFVFWLGSG CAVILALLLL FSKTDVPSSA
LacY-Ec  FGCVGWALCA SIVGIMFTIN NQFVFWLGSG CALILAVLLF FAKTDAPSSA 195
LacY-Kp  SGCVGWALCA SITGILFSID PNITFWIASG FALILGVLLW VSKPESSNSA
   RafB  FGCLGWALCA TMAGILFNVD PSLVFWMGSG GALLLLLLLY LARPSTSQTA
   CscB  WGSFGYAIGA FFAGIFFSIS PHINFWLVSL FGAVF.MMIN MRFKDKDHQC
Consensus -G--G-A--A ---GI-F--- ----FW--S- ---------- ----------
                                        ==========Helix 7======
LacY-Cf  KVADAVGANN SAFSLKLALE LFKQPKLWLI SLYVVGVSCT YDVFDQQ.FA
LacY-Ec  TVANAVGANH SAFSLKLALE LFRQPKLWFL SLYVIGVSCT YDVFDQQ.FA 244
LacY-Kp  EVIDALGANR QAFSMRTAAE LFRMPRFWGF IIYVVGVASV YDVFDQQ.FA
   RafB  MVMNALGANS SLISTRMVFS LFRMRQMWMF VLYTIGVACV YDVFDQQ.FA
   CscB  IAADAGGVKK EDF.....IA VFKDRNFWVF VIFIVGTWSF YNIFDQQLFP
Consensus ----A-G--- ---------- -F-----W-- -----G---- Y--FDQQ-F-
          ===            ========Helix 8=========         ====
LacY-Cf  NFFTSFFATG EQGTRVFGYV TTMGELLNAS IMFFAPLIVN RIGGKNALLL
LacY-Ec  NFFTSFFATG EQGTRVFGYV TTMGELLNAS IMFFAPLIIN RIGGKNALLL 294
LacY-Kp  NFFKGFFSSP QRGTEVFGFV TTGGELLNAL IMFCAPAIIN RIGAKNALLI
   RafB  IFFRSFFDTP QAGIKAFGFA TTAGEICNAI IMFCTPWIIN RIGAKNTLLV
   CscB  VFYAGLFESH DVGTRLYGYL NSFQVVLEAL CMAIIPFFVN RVGPKNALLI
Consensus -F----F--- --G----G-- --------A- -M---P---N R-G-KN-LL-
          ====Helix 9======     =======Helix 10======
LacY-Cf  AGTIMSVRII GSHSHT.ALE VVILKTLHMF EIPFLIVGCF KYITSQFEVR
LacY-Ec  AGTIMSVRII GSSFATSALE VVILKTLHMF EVPFLLVGCF KYITSQFEVR 344
LacY-Kp  AGLIMSVRIL GSSFATSAVE VIILKMLHMF EIPFLLVGTF KYISSAFKGK
   RafB  AGGIMTIRIT GSAFATTMTE VVILKMLHAL EVPFLLVGAF KYITGVFDTR
   CscB  GVVIMALRIL SCALFVNPWI ISLVKLLHAI EVPLCVISVF KYSVANFDKR
Consensus ---IM--RI- ---------- ----K-LH-- E-P------F KY----F---
            =======Helix 11=======              =====Helix 12===
LacY-Cf  FSATIYLVCF CFFKQLAMIF MSVLAGKMYE SIGFQGAYLV LGIIRVSFTL
LacY-Ec  FSATIYLVCF CFFKQLAMIF MSVLAGNMYE SIGFQGAYLV LGLVALGFTL 394
LacY-Kp  LSATLFLIGF NLSKQLSSVV LSAWVGRMYD TVGFHQAYLI LGCITLSFTV
   RafB  LSATVYLIGF QFSKQLAAIL LSTFAGHLYD RMGFQNTYFV LGMIVLTVTV
   CscB  LSSTIFLIGF QIASSLGIVL LSTPTGILFD HAGYQTVFFA ISGIVCLMLL
Consensus -S-T--L--F -----L---- -S---G---- --G------- ----------
          =====
LacY-Cf  ISVFTLSGPG PFSLLRRRES VAL
LacY-Ec  ISVFTLSGPG PLSLLRRQVN EVA                              417
LacY-Kp  ISLFTLKGSK TLLPATA
   RafB  ISAFTLSSSP GIVHPSVEKA PVAHSEIN
   CscB  FGIFFLSKKR EQIVMETPVP SAI
Consensus ---F-L---- ---------- --------
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Fig. 2. Multiple amino acid sequence alignment. This alignment was generated by the Genetics Computer Group PILEUP computer program according to the parameters used in Refs. [35] and [104]. The locations of transmembrane domains are denoted as a double-line above the alignments. Amino acids that are conserved in all members of this family are shown in the consensus line. LacY-Cf, the lactose-H^+ carrier of *Citrobacter freundii* [61]; LacY-Ec, the lactose-H^+ carrier of *E. coli* [16]; LacY-Kp, the lactose-H^+ carrier of *Klebsiella pneumoniae* [73]; RafB, the raffinose-H^+ carrier of *E. coli* [1]; CscB, the sucrose-H^+ carrier of *E. coli* [8]. The numbers refer to the residues of the LacY-Ec sequence. This figure was provided courtesy of Dr. Jeffrey K. Griffith of the University of New Mexico, School of Medicine.

most mutagenized transport protein ever examined. Surprisingly, only about a dozen or so of the amino acids in the lactose carrier were found to be absolutely essential for lactose carrier function, while about the same number were shown to be moderately important. Of these critical residues many of them reside within transmembrane domains (Fig. 1) and about half of the critical residues are conserved in members of the family that includes the lactose carrier (Fig. 2 [34]).

5. The role of His-322

Substitution of His-322 by Arg, Asn, Gln or Lys resulted in a carrier with defective lactose accumulation and proton translocation, but normal downhill lactose transport [86,91]. Additional work indicated the importance of Glu-325 and Arg-302 for the activity of the lactose carrier (see Kaback et al. [47] for a review). On the basis of these and other experiments, Kaback [46] proposed that H^+ is translocated through the carrier by protonation and deprotonation of residues Arg-302, His-322 and Glu-325 in a manner similar to the charge-relay mechanism found in chymotrypsin [6].

Cloning and sequencing of a mutant with poor apparent affinity for lactose revealed that His-322 was changed to Tyr [49,54]. Site-directed mutagenesis of His-322 to Phe or Tyr showed poor downhill entry at low sugar concentration but good entry at 30 mM concentrations (red on MacConkey plates) [50]. No accumulation of lactose and melibiose was observed, whereas proton uptake was detected in both Phe-322 and Tyr-322 mutants. Although sugar accumulation and downhill transport were significantly reduced for the His-322 → Asn mutant, it showed a normal lactose:H^+ stoichiometry [30]. One possible explanation consistent with these data is that the mutant carrier has a very much reduced affinity for lactose. Brooker thus concluded that an ionizable histidine residue at 322 is not absolutely essential for proton translocation. Although these studies are consistent with the notion that His-322 may not be required for proton uptake, it is clear that His-322 is essential for substrate selection, binding or transport (see below).

6. Sugar recognition mutants of the lactose carrier

In studies designed to delineate amino acid residues that are important for sugar selection or recognition, in 1985 Brooker and Wilson found lactose carrier mutants with an increased capacity to transport the disaccharide maltose by incubating *E. coli* cells containing a plasmid encoding a normal lactose carrier on minimal plates with maltose and selecting for mutants that grew better than normal [10]. DNA sequencing of these maltose-specific mutants revealed mutations at two sites for the lactose carrier: Ala-177 changed to Val or Thr, and Tyr-236 changed to Phe, Asn, Ser or His (see Table 1). All mutants showed higher maltose transport rates than normal [10], and a normal β-galactoside:H^+ stoichiometry [11]. When cells were selected for growth on sucrose a cell was isolated with the Ala-177 → Val mutation [52]. The Val-177 mutation confers an enhanced transport for sucrose as well as the maltose analog 4-nitrophenyl-α-maltoside [52]. Therefore, the Val-177 mutation was more closely studied [53]. *E. coli* containing this particular mutant achieved downhill transport, but not accumulation, of sucrose. A kinetic analysis of Val-177 showed that the V_{max} values for both melibiose and TMG were decreased [53]. The K_m for melibiose was increased, whereas the K_m for TMG was decreased. Interestingly, the K_m value for the lactose analog 2-nitrophenyl-β-D-galactopyranoside (β-ONPG) was unchanged, whereas the V_{max} was increased several-fold. Various sugars [maltose, cellobiose (glucose β-1,4-glucoside) and palatinose (glucose α-1,6-fructofuranoside)] were able to block β-ONPG transport in the Val-177 mutant, but not in the wild-type. Markgraf et al. showed that Thr-266 → Ile of the lactose carrier substantially reduced lactose transport, but enhanced maltose transport [69]. Goswitz and Brooker later observed that mutants which grew well on minimal plates containing arabinose also turned out to be Ala-177 changed to Val [33]. Mutants in which Ala-177 was changed to Ile, Leu, Phe or Pro, by site-directed mutagenesis recognized arabinose. Taken together, these various data (Table 1) clearly demonstrate that Ala-177, Tyr-236 and Thr-266 mediate sugar selection

Table 1
Sugar-recognition mutants of the lactose carrier

Sugar(s) used for selection	Cell used for selection	Mutants	Ref.
Sucrose	normal	A177V	[52]
Arabinose	normal	A177V, I, L, F or P	[33]
Maltose	normal	A177V or T	[10,69]
	normal	Y236F, N, S, or H	
	normal	T266I	
Maltose plus	normal	Y236F	[21]
Cellobiose	normal	S306T	
	normal	A386P	
	V177	A177V/Y236F or N	
	V177	A177V/S306T or L	
	V177	A177V/K319N	
	V177	A177V/H322Y, N or Q	
Maltose	normal	Y236H, F, or N	[26,29]
plus TDG	V177	A177V/Y236H	
	V177	A177V/I303F	
	V177	A177V/H322N	
	T306	S306T/Y236F or H	
	V177/T306	A177V/S306T/Y236N	
Maltotriose	V177	A177V/F236H or N	[84]
	V177	A177V/S306T	
	V177	A177V/H322N	
	H236	Y236H/S306T	
	V177/N236	A177V/Y236N/S306T	

properties of the lactose carrier, possibly through modulation of either the V_{max} or k_{cat}/K_m ratio of the carrier.

Another type of sugar recognition mutant was isolated by selection on maltose minimal plates containing cellobiose, which inhibits maltose transport [21]. Sequencing of the plasmid DNA derived from *E. coli* cells selected by the above procedure showed three types of mutants: Tyr-236 → Phe, Ser-306 → Thr, or Ala-386 → Pro. Sequencing of DNA derived from the Val-177 mutants, which grew on maltose-cellobiose minimal plates, revealed the original Val-177 mutation plus the following second-site mutants (Table 1): Tyr-236 → Phe or Asn, Ser-306 → Thr or Leu, Lys-319 → Asn or His-322 → Tyr, Asn or Gln. The Val-177/Asn-319 double mutant was able transport various sugars without protons as well as mediate a proton leak under conditions of an electrochemical gradient of protons [14]. Third-site revertants of the double mutant were isolated in which cells were able to recognize maltose and were resistant to TDG inhibition of growth [26]. In another experiment, normal cells and maltose-positive mutants were plated on maltose plus TDG (a potent inhibitor of the lactose carrier) [29]. Sequencing of the mutants that grew well under these conditions revealed changes occurring at Tyr-236, Ile-303 or His-322 (see Table 1). Two types of revertants were found that retained the original double mutations plus a new mutation at a third site: Ile-303 changed to Phe and Tyr-236 changed to Asn or His. These triple mutants were similar in that each showed some lactose downhill transport, but very little lactose accumulation. Olsen et al. showed that two or three mutations within the same lactose carrier molecule conferred the ability to recognize the trisaccharide maltotriose [84]. The mutations occurred at Ala-177, Tyr-236, Ser-306 or His-322. In brief, these results, summarized in Table 1, suggested that all or some of these amino acids (Ala-177, Tyr-236, Ile-303, Ser-306, Lys-319, His-322, and Ala-386) either line the aqueous-facing binding site [77] (see Fig. 3), or that these amino acids form supporting structures for the actual sugar-binding site.

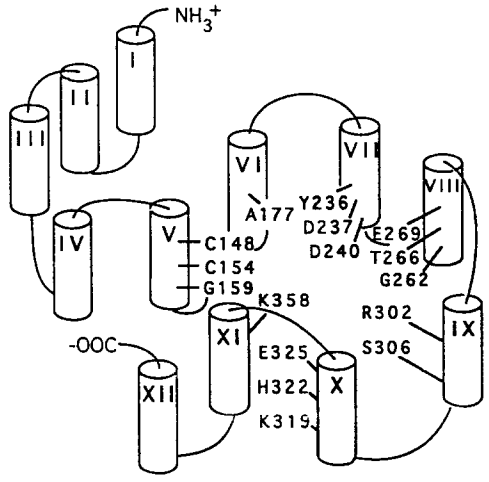

Fig. 3. Residues involved in sugar recognition. Hypothetical three-dimensional model of the lactose carrier indicating the position of residues involved in sugar recognition (from Matos et al. [70] and adapted from Collins et al. [21]).

7. Energy coupling and uncoupling in the lactose carrier

7.1. Mutants that transport sugars without protons

The coupling of proton transport to lactose transport is essential for the process of sugar accumulation. Several mutants have been isolated in which this coupling is defective and various types of energy uncoupled phenotypes have been observed. In 1970, Wong et al. [115] isolated an *E. coli* mutant which failed to accumulate thiomethylgalactoside (TMG) or the lactose analog o-nitrophenyl-β-galactoside (ONPG) but showed normal downhill lactose entry and downhill ONPG entry 400% of normal [115]. West and Wilson showed that this mutant was unable to translocate protons across the membrane after the addition of TMG [112]. This was the first demonstration of a mutant with a defect in the coupling between cation entry and sugar accumulation in a carrier protein. In essence, the lactose carrier was converted from a symporter into a uniporter. The cloning and sequencing of this uncoupled mutant revealed that Gly-159 was changed to Cys [70]. The mutant showed an increased sensitivity to several sulfhydryl reagents, including NEM, which is consistent with the view that Gly-159 is in or near the sugar recognition site (see Fig. 3) and the energy coupling region of the carrier. It is not yet known whether substrate protects the Cys-159 mutant from the effects of NEM.

Another energy-uncoupled mutant was isolated in 1973, which showed defective thiomethylgalactoside accumulation, but maintained an ability to mediate thiogalactoside downhill transport [57,114]. The ONPG downhill transport rate of the mutant was twice that of the parent. Cloning and sequencing of the lacY in this mutant found that Gly-262 was replaced by Cys [13]. Under conditions where both the mutant and normal carriers transport lactose downhill at comparable rates, no sugar-induced proton translocation was detected in the mutant. Furthermore, the mutant carrier was more sensitive to the sulfhydryl reagents than the wild-type carrier and was protected by substrate against their inhibitory effects. This indicated that, like Gly-159, Gly-262 also played a role in energy-coupling and affected the substrate-binding site. Changing many charged residues to neutral amino acids results in the loss of lactose accumulation while downhill entry of lactose remains intact. For example, Püttner et al. demonstrated that His-322 changed to Asn or Gln showed complete loss of lactose accumulation, but were positive on indicator plates [93].

7.2. Mutants that transport protons without sugars

A second type of uncoupled mutant transports protons in the absence of sugar [53]. When expression of the carrier was induced, proton transport (or 'leak') caused a severe inhibition of cell growth by reducing the protonmotive force generated by the respiratory chain. These proton-leaky mutants had been isolated as maltose-positive [10] and sucrose-positive [52] mutants where Ala-177 was changed to Val and Tyr-236 was changed to His, Asn, Phe or Ser. The Val-177 mutant showed a lower than normal protonmotive force and a higher than normal rate of proton entry into the cells [53]. Brooker showed that the double mutant Val-177/Asn-319 was able to transport protons without sugar [14,15].

7.3. Mutants that 'leak' protons in the presence of sugar

Brooker [15] discovered an unusual proton leak mechanism which he designated as the leak B pathway (the leak A pathway was proton leak in the absence of sugar). He found that sugars inhibited the growth of A177V/K319N on rich plates (complete inhibition with 0.5 mM TDG and partial inhibition with 2.5 mM TMG or 2.5 mM lactose). In the presence of the sugar there is an abnormally rapid leak of protons which reduces the protonmotive force and

Table 2
Properties of mutants of charged residues

Mutant	Accumulation (% normal)			Downhill (% normal)	Fermentation (MacConkey)	Expression (% normal)	Ref.
	lactose	TMG	melibiose	lactose	melibiose		
D237N	0	0	0		white		[55]
K358T	0	3	0		white		[55]
K358T/D237Y	5	25	45		red		[55]
K358T/D237N	30	80	85		red		[55]
K358T/D237G	60	95	84		red		[55]
D237N/K358Q	50	42	83		red		[55]
D237C	5					25	[25]
D237A	0					40	[25]
D237K	0					15	[25]
K358C	0					5	[25]
K358A	0					5	[25]
K358D	0					40	[25]
D237C/K358C	75					15	[25]
D237C/K358A	75					15	[25]
D237A/K358C	70					15	[25]
D237K/K358D	60					50	[25]
D240A	5	0	15	30	white		[59]
D240A/K319Q	20	10	35	30	red		[59]
K319N	0	0	0	25	white	94	[60]
K319N/D240V	40	30	25	100	red	83	[60]
K319N/D240G	25	20	15	80	red	83	[60]
K319L	0	0	0	10	white	40	[60]
K319L/D240A	30	0	0	75	red	48	[60]
K319L/D240Y	30	0	25	45	red	43	[60]
E269Q	0	0	0	0	white	74	[60]
E269Q/K319N	0	0	0	0	red	84	[60]
D240C	0					100	[96]
D240A	0					100	[96]
K319C	0						[96]
D240C/K319C	25					100	[96]
D240C/K319A	33					100	[96]
D240A/KK319C	33					100	[96]
D240A/K319A	35					100	[96]
D240K	0						[96]
K319D	0						[96]
D240K/K319D	0					100	[96]
E325S	0	0	0	5	white	47	[62]
H322D	2	2	1	1	white	101	[62]
E325S/H322N	0	0	3	0	red	36	[62]
E325S/H322D	0	0	2	2	red	119	[62]
H322N	0	0	3	25	red	95	[62]
E325N	0	0	0	1	white	68	[62]
H322N/E325N	0	0	0	38	red	70	[62]

inhibits growth. The explanation for the phenomenon appears to be influx of sugar together with protons and subsequent efflux of sugar without protons [26,59]. A kinetic model has been proposed to explain the behavior of various uncoupled mutants of the lactose carrier in which two leak pathways involving a reorientation of either the proton binding site (a proton leak) or the substrate binding site (a substrate leak) [67]. This model is consistent with earlier work suggesting that in the normal carrier, two conformations exist: one with the binding site open to the cytoplasm of the cell while closed to the periplasm and the opposite situation in which the binding site is open to the periplasm but closed to the cytoplasm [23,85].

8. Charged amino acids and salt-bridges

It is striking that some of the charged amino acids in the lactose carrier are highly conserved throughout evolution, are important for substrate selectivity and play a role in energy transduction. The presence of charged amino acid residues within a transmembrane region of a carrier is thermodynamically unfavorable unless they face the aqueous environment or are neutralized by another residue with the opposite charge.

8.1. The Asp-237–Lys-358 salt bridge

In 1991, King and Wilson studied two mutants in which a charged amino acid was changed to a neutral residue (Lys-358 → Thr and Asp-237 → Asn) [55]. Each mutant showed a severe defect in the accumulation of lactose, melibiose and TMG (Table 2). The Lys-358 → Thr mutant displayed no lactose or melibiose downhill entry as judged by white colonies on melibiose MacConkey plates and by the sugar transport assay [55]. After several days incubation at 37°C on melibiose MacConkey plates, red revertants of Thr-358 were isolated. Revertants from the Asn-237 were isolated by growth on melibiose minimal medium. All revertants of Lys-358 → Thr contained the original Thr-358 mutation plus a second mutation at Asp-237 which was changed to a neutral amino acid (Asn, Gly or Tyr). Revertants from the Asn-237 mutant were isolated in the same manner. These revertants retained the original Asn-237 mutation plus Lys-358 changed to Gln. All of the second-site revertants showed sugar transport better than the starting single mutation but less than normal (Table 2). Because both types of revertants resulted in the neutralization of an amino acid of opposite charge, it was reasonable to conclude that Asp-237 and Lys-358 were in locations close enough in the three-dimensional space of the carrier that a salt-bridge could form between both residues (see Fig. 4.) [55]. Dunten et al. showed that replacement of either residue with Cys showed severe defects in transport, whereas the double mutant Cys-237/Cys-358 showed 75% of normal transport [25]. When a mutant was constructed

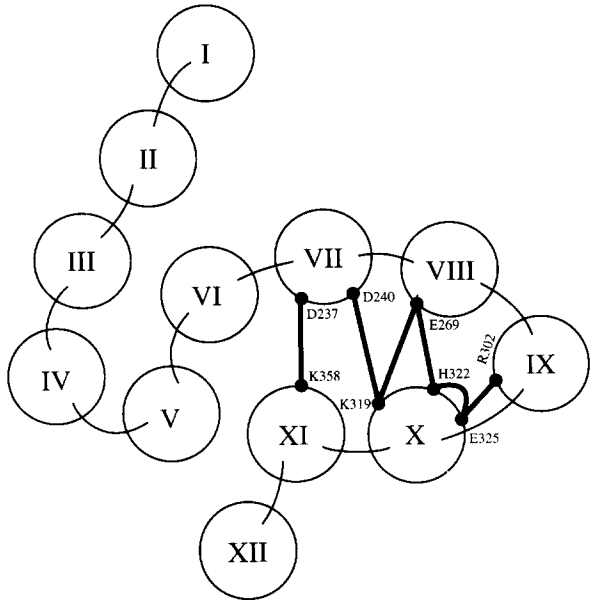

Fig. 4. Postulated salt-bridges within the lactose carrier of *E. coli*.

where both charges were reversed (Lys-237/Asp-358), good transport activity was observed by Sahin-Toth et al. [96]. Consequently, although each amino acid of the salt-bridge is required for full activity, neither the salt-bridge per se nor the orientation of the charges are essential for lactose transport [25]. Sahin-Toth et al. [96] have suggested that the salt-bridge formed by Asp-237 and Lys-358 is necessary for stability or insertion of the lactose carrier in the membrane.

8.2. The Asp-240–Lys-319 salt bridge

Replacement of Asp-240 with an Ala residue in the lactose carrier diminished its ability to accumulate melibiose, lactose and TMG [59]. A fast-growing revertant of the Ala-240 mutant was isolated on melibiose minimal plates. The primary mutation was present plus Lys-319 was changed to Gln. The revertant showed better accumulation than either Ala-240 or Gln-319 (Table 2). The Ala-240/Gln-319 revertant strongly suggested that Asp-240 and Lys-319 interact and probably form a salt-bridge (Fig. 4). When the charges of Asp-240 and Lys-319 were reversed transport activity was poor [96], which is consistent with the notion that the positions of the residues in this salt-bridge are functionally important.

8.3. The Lys-319–Asp-240 and Lys-319–Glu-269 salt bridges

Converting Lys-319 to Asn resulted in the inability of the host cell to ferment melibiose on MacConkey plates [60]. Second-site suppressor analysis of this mutant revealed the original mutation (Asn-319) plus mutations at

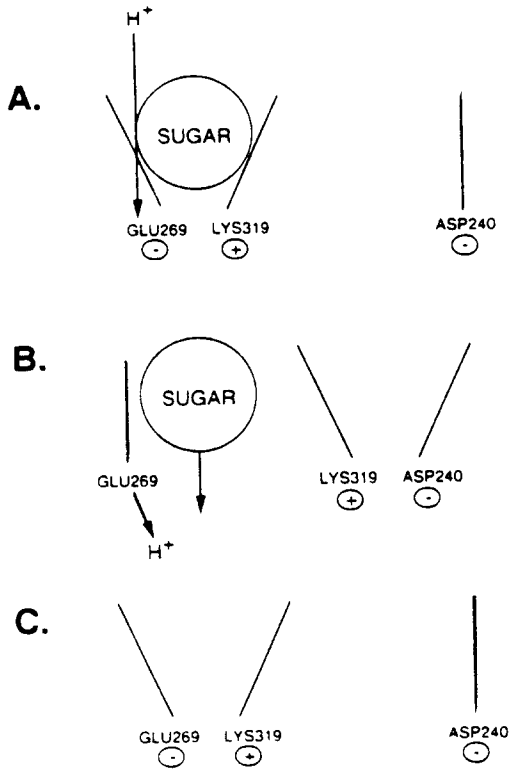

Fig. 5. Proposed mechanism for sugar-proton symport. (A) The sugar in the binding pocket of the carrier with the salt-bridge (between Glu-269 and Lys-319) preventing sugar transport. Protons are drawn into the pocket by an inwardly directed proton motive force. (B) The protonation of Glu-269 with opening of the gate. Asp-240 facilitates this process by attracting Lys-319 and forming a salt-bridge. The sugar then passes through the membrane into the cytoplasm. (C) The loss of the proton from Glu-269 and re-establishment of the original salt-bridge with Lys-319. Adapted from Lee et al. [60].

one of two separate sites: Asp-240 changed to Val or Gly and Glu-269 changed to Asn. Likewise, conversion of Lys-319 to Leu failed to ferment melibiose on MacConkey plates. Revertants of this primary mutant proved to have second-site mutations at Asp-240 (Ala or Tyr). These two revertants showed greater transport than the original Leu-319 mutant. In a second series of experiments, revertants were isolated from the primary mutant Glu-269 → Asn, which grew poorly on melibiose minimal plates. The melibiose-positive revertants included the double mutant Gln-269/Asn-319. Together, these data strongly suggested that Lys-319 forms a salt-bridge with both Asp-240 and Glu-269 (Fig. 4). Since all the revertants isolated from mutants for Glu-269 were severely defective with respect to their ability to accumulate sugars, the salt-bridge between Glu-269 and Lys-319 could be more critical for the function of the carrier than the salt-bridge between Asp-240 and Lys-319. This finding led to the formulation of a hypothetical mechanism, illustrated in Fig. 5, in which binding of the proton to Glu-269 neutralizes it, thus setting free the Lys-319 which then binds to Asp-240. This opens the channel and allows sugar to pass across the membrane.

8.4. The His-322–Glu-325 salt bridge

Substantial evidence has accumulated demonstrating the functional importance of His-322 and Glu-325 in sugar transport [18,19,30,31,49–51,58,86,91–93]. Carisco et al. suggested that Glu-325 and His-322 were ion-paired because both amino acids are only three residues apart in the primary sequence and are believed to reside in an α-helical region of the carrier [18]. Jung et al. [43] converted both charged residues to cysteines and added a fluorescent reagent (pyrene-maleimide). This exhibited excimer fluorescence, indicating that the two residues were within about 3.5 Å of each other. This result would be expected if the two residues were in an α-helical region. A physiological approach to this question was carried out with the initial mutant Glu-325 → Ser, which failed to ferment melibiose (white clones on MacConkey indicator plates) [31,62]. A red revertant was isolated which showed a second site mutation at His-322 (Glu-325 → Ser/His-322 → Asn) [62]. This suggested that His-322 and Glu-325 interact to form a salt-bridge (Fig. 4). In an additional experiment the double mutant His-322 → Asn/Glu-325 → Asn was constructed by site-directed mutagenesis. This double mutant showed a greater V_{max} for lactose transport than either of the single mutants His-322 → Asn or Glu-325 → Asn (Table 2). These results also provided physiological evidence to support the view that His-322 interacts with Glu-325.

The postulated role of Glu-325 in proton transport [18,31] is supported by the fact that a second-site revertant of Ser-325 (Ser-325/Asp-322), which contains a negative charge substituted for His-322, is capable of sugar-proton cotransport [62]. Although sugar accumulation does not take place, melibiose-stimulated proton uptake is present (30% of normal). Remarkably, the Ser-325/Asp-322 revertant shows very high TDG-stimulated proton uptake. Hence, this double mutant demonstrates that a carboxyl group substituted for His-322 can compensate for the loss of the carboxyl group at the 325 position in proton cotransport. It is interesting to note that a negative charge in this region is more important than an intact His-322. These experiments emphasize the importance of Glu-325 in the physiology of sugar transport, probably in proton binding and transport.

Site-directed pyrene excimer fluorescence studies [43] and EPR spectroscopy [45] suggest that His-322 is close to Glu-269. Thus, His-322 may salt bridge with Glu-269. A second example of a positive residue interacting with two different negative residues is the case of Lys-319 interacting with both Glu-269 and Asp-240 [60]. We postulate that at one phase of the transport cycle Lys-319 interacts with Glu-269 [60] and His-322 interacts with Glu-325 [62]; at another phase of the cycle His-322 interacts with Glu-269,

Glu-325 interacts with Arg-302 [43,45], and Lys-319 interacts with Asp-240 [59,60]. Such alterations in salt bridges may be involved in the conformational changes that occur during the transport cycle.

If His-322 (or other important residue) were one of the elements of the sugar binding site, one would expect that mutation of this amino acid would significantly reduce transport but not necessarily abolish function. The lactose binding site of S-lac Lectin has been studied by analysis of X-ray crystal structure and nine specific interactions (mostly hydrogen bonds) are known to occur between the sugar and specific amino acid residues in the protein structure [64]. One might predict that altering one of the amino acids in the binding site would reduce the affinity of the protein for the sugar but the remaining eight interactions would permit partial binding affinity. Similarly loss of one amino acid residue in the lactose binding site of the lactose carrier would be expected to reduce but not abolish binding of the sugar.

9. Nature of the lactose carrier sugar- and cation-binding site(s)

9.1. A binding site for lactose

The exact three-dimensional structure of the lactose binding site in the lactose carrier is unknown although several amino acid residues indicated above are presumed to be in or near this binding site. Recently X-ray crystallographic data for the lactose binding site of the human S-lac lectin was published [64]. Residues that form hydrogen bonds with the lactose include a lysine, histidine, glutamic acid, two arginines and two asparagines. We speculate that the binding site of the lactose carrier may be similar to that found in this lectin. Fig. 6 shows the proposed hydrogen bonds between the amino acid residues in the binding site of the lactose carrier and the various hydroxyls of the lactose molecule. Shown for comparison is the structure found in the lectin. The charged residues of the lactose carrier given in the figure are among those known to be important for sugar recognition from physiological studies discussed above. His-322, for example, when changed to Phe, Tyr or Asn, results in altered sugar recognition [51,30]. Likewise, mutants of Lys-319 show altered sugar recognition [60]. It is interesting to note that two key residues in the lectin binding site include a histidine (His-45) and an arginine (Arg-49) which are 4 residues apart and in the proposed structure in the lactose carrier binding-site the histidine (His-322) is three residues away from the lysine (Lys-319). We propose (Fig. 4) that in the absence of sugar the charged residues are salt-bridged [His-322 to Glu-325 (and Glu-269), Lys-319 to Asp-240 (and Glu-269) and Arg-302 to Glu-235]. Thus the charged residues essential for sugar binding can be stabilized in the hydrophobic environment of the empty carrier by salt-bridging. X-ray crystallographic structure of the lac repressor (lacR) bound to isopropylthiogalactoside (IPTG) has recently been published [32]. Hydrogen bonding interactions are made with IPTG by two arginines (Arg-101 and Arg-192), two aspartic acids (Asp-149 and Asp-274), an asparagine (Asp-264) and a serine (Ser-69). It will be of interest to see the interactions of this protein with a disaccharide when this protein is crystallized in the presence of lactose (or allolactose, the true inducer).

9.2. A binding site for protons

The residues in the protein responsible for binding of H^+ (or hydronium ion) have not been considered. Of the amino acid residues in the carrier, Glu-325 and Glu-269 are particularly good candidates for possible coordination with the cation. The change of either of these two residues to most neutral amino acids leads to a complete loss of

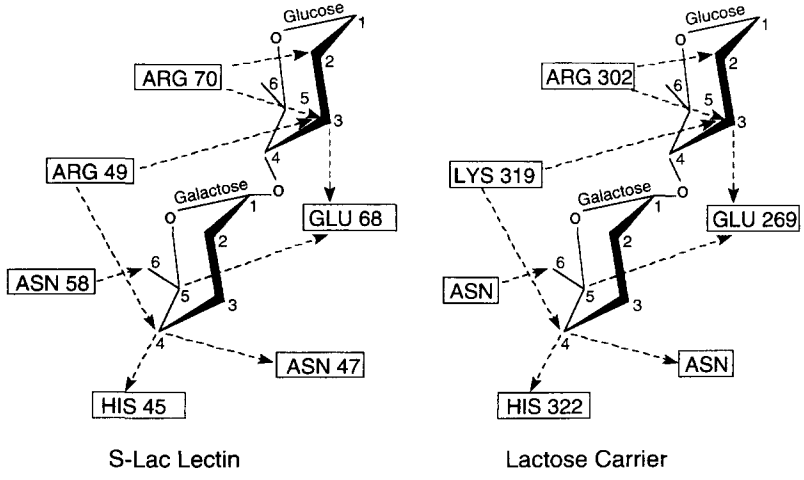

Fig. 6. Hypothetical binding site in the lactose carrier. On the left is the lactose binding site in the S-lac lectin as determined by X-ray crystallography [64]. On the right is a hypothetical binding site in the lactose carrier based on analyses of mutants (see text).

proton transport. Thus an important role in proton transport is indicated. We propose that Glu-325 (and perhaps Glu-269) are involved in hydronium ion binding. One cation binding protein (human molecular chaperone Hsc 70) which has been studied by X-ray crystallography [113] shows coordination sites to two aspartic acids (Asp-206 and Asp-199) plus two additional residues to the sodium ion while another protein (dialkylglycine decarboxylase) shows a coordination site for one aspartic acid (Asp-307) plus three additional residues for sodium ion [40].

We suggest that the hydronium ion interacts with Glu-325 (and perhaps Glu-269) plus several other residues. The possibility that the hydronium ion itself provides a coordination site for sugar binding might be considered. This binding of cation causes an allosteric change which facilitates the binding of the sugar. The presence of both cation and sugar results in an additional allosteric change which opens the exit channel for sugar and cation. This results in the transfer of one sugar molecule and one cation across the membrane into the cell. Further work is required to provide evidence for this hypothesis.

Acknowledgements

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