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Biochimica et Biophysica Acta 1505 (2001) 121–130

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Review

Sodium-substrate cotransport in bacteria

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Received 28 July 2000; received in revised form 24 October 2000; accepted 16 November 2000

Abstract

A variety of sodium-substrate cotransport systems are known in bacteria. Sodium enters the cell down an electrochemical concentration gradient. There is obligatory coupling between the entry of the ion and the entry of substrate with a stoichiometry (in the cases studied) of 1:1. Thus, the downhill movement of sodium ion into the cell leads to the accumulation of substrate within the cell. The melibiose carrier of *Escherichia coli* is perhaps the most carefully studied of the sodium cotransport systems in bacteria. This carrier is of special interest because it can also use protons or lithium ions for cotransport. Other sodium cotransport carriers that have been studied recently are for proline, glutamate, serine-threonine, citrate and branched chain amino acids.

Keywords: Cotransport; Melibiose; Proline; Glutamate; Serine-threonine; Citrate; Branched chain amino acid

1. Introduction

Sodium-substrate cotransport is an extremely common method of substrate uptake in all living cells [1,2]. Sodium and substrate enter the cell together on the membrane carrier usually with a stoichiometry of 1:1 [3–5]. A model (Fig. 1) is given by Bassilana et al. [4]. There is obligatory coupling between entry of substrate and entry of cation. Sodium ion enters the cell down its electrochemical gradient, this ion gradient providing the energy for entry of the substrate against a concentration gradient. For example, in the presence of 10 mM sodium ion the entry of melibiose into an α-galactosidase-negative cell occurs until the internal concentration is about 200 times that outside the cell. The Na^+ which enters the cell normally exchanges with external protons via the Na^+/H^+ exchange carrier. The proton gradient is maintained by the proton ATPase (Fig. 2). In some organisms sodium ion is extruded from the cell by a Na^+/ATPase [3].

2. Melibiose

The first demonstration of sodium-dependent sugar transport in bacteria was reported by Stock and Roseman [6]. They showed that cells of *Salmonella typhimurium*, induced for the melibiose carrier, were able to accumulate thiomethyl galactoside (TMG) against a concentration gradient. Addition of 5 mM NaCl stimulated TMG uptake by 100-fold. The addition of TMG (20 mM) stimulated uptake of sodium ion. They concluded that there was cotransport of sugar and sodium ion by the membrane transport

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0005-2728/01/$ – see front matter
PII: S0005-2728(00)00282-6

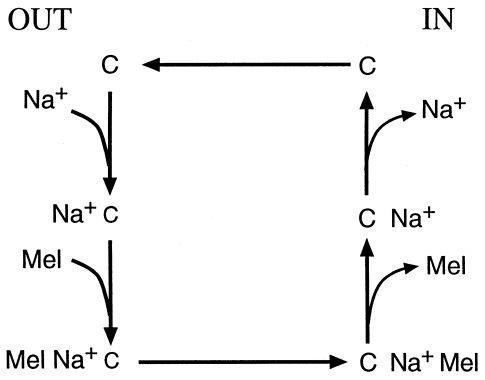

Fig. 1. Partial reactions of the melibiose carrier cycle during Na^+ coupled melibiose transport [4].

carrier. These observations were confirmed by Tokuda and Kaback [7] and by Niiya et al. [8]. A transport system found in *Escherichia coli* [9] also shows similar sodium melibiose cotransport [10].

The gene encoding the melibiose carrier (*melB*) in *E. coli* has been cloned [11] and sequenced [12]. The primary amino acid sequence predicts a highly hydrophobic protein (70% apolar) with a molecular mass of 52 kDa. Hydropathy analysis combined with information derived from *melB-phoA* fusions [12–14] and proteolytic digestion [15] predict a topological model in which the carrier forms 12 α-helical membrane spanning domains connected by hydrophilic loops with the carboxyl terminus and amino terminus located in the cytoplasm [13]. More recently a refined topological model has been derived from the amino acid sequencing of the N-terminal region [16].

2.1. Sugar and cation specificities

The melibiose carrier is present in *E. coli*, *S. typhimurium* and *Klebsiella pneumoniae*. In addition to melibiose several other α-galactosides are substrates for the melibiose carrier. These include: galactinol (*meso*-inositol-α-galactoside), α-methyl, ethyl or phenyl derivatives of galactose, 1-*O*- and 2-*O*-galactosylglycerol [9,17–23]. Among the β-galactosides tested, transport occurred with TMG, *o*-nitrophenyl-β-galactoside and lactose. The melibiose carrier also transports tri- and tetragalactosides (raffinose and stachyose) and several monosaccharides (galactose, fucose and arabinose) and α-methyl glucoside [9,17–23].

The cation specificity of the melibiose carrier is one of the most interesting features of this class of membrane transport systems. While many cotransport systems in bacteria use protons as the coupling cation, the melibiose carrier prefers Na^+. This carrier is most unusual in its ability to use H^+, Na^+ or Li^+ for cotransport [1,2]. The study of a variety of sugar substrates indicated that under the conditions of these experiments α-galactosides and galactose used H^+, Na^+ or Li^+ while β-galactosides used primarily Na^+ and Li^+ (protons have very little effect). Several monosaccharides (D-fucose, L-arabinose and D-galactosamine) used only Na^+ for cotransport [23].

One method of investigating the effect of cations is to study the role of various ions on sugar binding to the carrier protein [5,24]. For this purpose the chemical analogue *p*-nitrophenyl-α-D-galactopyranoside (NpaGal) has been used because of its high affinity for the sugar binding site. [^{3}H]NpαGal was exposed to right-side-out membrane vesicles and binding was assayed by flow dialysis. The K_d binding values for this sugar were 15, 2, 1 and 0.7 μM in the presence of 25 μM, 0.5, 1 and 10 mM sodium respectively [5]. Competition between the cations (Na^+ and H^+) during binding indicates the existence of a single cation binding site [5]. Using the total number of binding sites, one can calculate that each *E. coli* cell of RA11 possesses approx. 10 000 transport molecules.

The uptake of radioactive Na^+ during cotransport with melibiose has been demonstrated [4,6,7]. Alternatively one may use a sodium electrode and measure the Na^+ concentration in the external medium

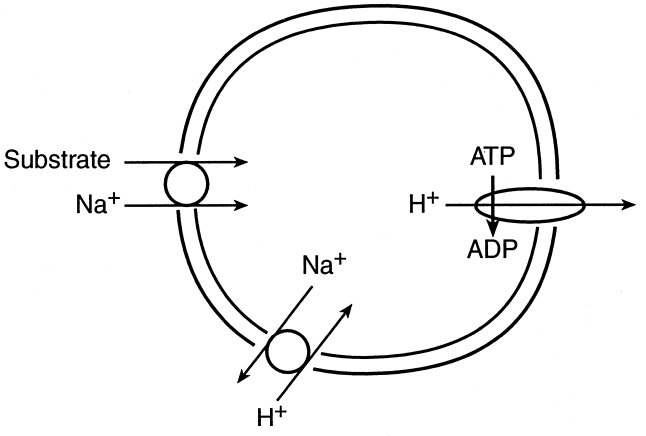

Fig. 2. Model of the pathway of Na^+ entry via the melibiose carrier and exit via the H^+/Na^+ exchanger.

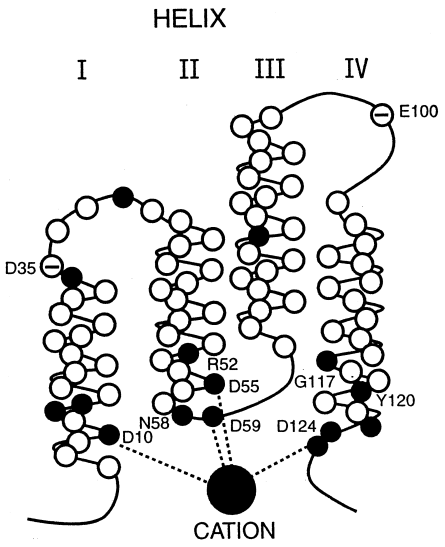

Fig. 3. Hypothetical sodium ion binding site [1].

of a cell suspension. The addition of melibiose results in a rapid reduction in the Na^+ concentration of the medium [23,25]. The uptake of protons by cells exposed to melibiose may be measured with a pH electrode [26,27].

2.2. Temperature sensitivity of the carrier in E. coli

An unexpected characteristic of the melibiose carrier of *E. coli* K12 was its thermal lability. Prestige and Pardee [19] first noted that cells grown at 37°C lacked melibiose carrier activity, while cells grown at 30°C possessed full activity. Subsequent work identified the heat labile component to be the *melB* carrier protein [28]. It was found that cells grown at 30°C lost 50% of their transport activity when incubated at 37°C for 30 min and greater than 90% of their activity when incubated at 37°C for 2 h [28]. For this reason temperature stable *melB* genes were isolated for studies in intact cells [29] and in plasmids [30,31].

2.3. The importance of charged residues in transmembrane α-helices

The first observation concerning the negatively charged residues was by Pourcher et al. [32] who showed that changing Asp59 to Cys resulted in the loss of the ability of the carrier to couple either H^+ or Na^+ to melibiose transport. In addition, this mutant failed to show Na^+ stimulated substrate binding. Substitution of Asp55 or Asp124 by Ser, Gln, Tyr or Leu led to complete loss of ability to transport melibiose [33]. In another study [34,35] substitution of Asp 55, Asp59 or Asp124 by Asn or Cys resulted in the complete loss of Na^+ coupled accumulation of TMG or melibiose.

The carrier in which glutamic acid was substituted for Asp35, Asp55 or Asp59 retained H^+/melibiose cotransport but lost much of the Na^+ coupled transport [36,37]. Glutamic acid substitution for Asp124, on the other hand, resulted in loss of proton/melibiose cotransport but retained most of the Na^+ cotransport [37]. TMG transport was severely defective in D55E, D59E, and D124E but showed 40% of normal transport in D35E. Thus, the four aspartic acid residues in helix I, helix II and helix IV are important for Na^+/melibiose transport activity. The hypothetical cation binding site is shown in Fig. 3.

Positively charged residues in membrane spanning α-helices are also important. When Arg52 was changed to Ala52 [36] or to Ser52, Gln52 or Val52 [38] activity was reduced to low levels. Revertants to higher activity were obtained. Among the revertants occurring at a position other than the original mutation (second site revertants) was R52S/D55N which suggests an intrahelical salt bridge between R52 and D55. When Lys377 was changed to Val it gave low

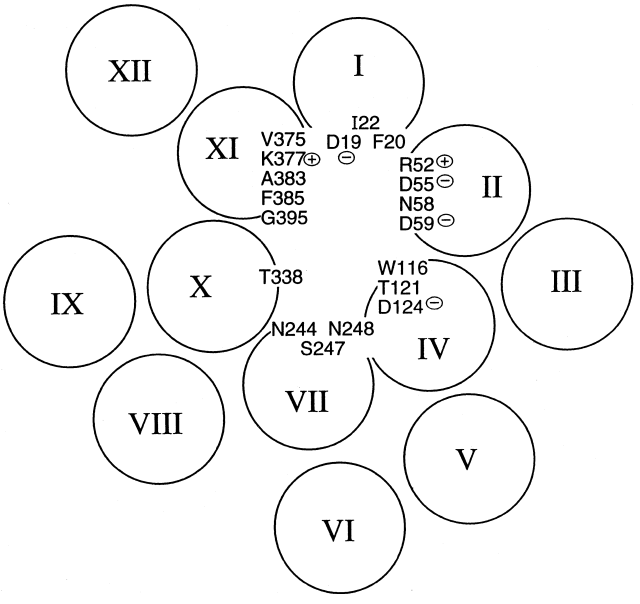

Fig. 4. Proposed arrangement of helices based on studies with second site revertants.

transport activity. One of the second site revertants was D377V/D59V which indicates an interaction (perhaps a salt bridge) between K377 in helix XI and D59 in helix II [39] (see Fig. 4). None of the seven histidines in the carrier molecule are essential for transport activity [40,41]. His98 mutations, however, alter the expression and stability of the carrier.

Several water soluble cation binding proteins have been studied by X-ray crystallography and the exact interaction with cations has been identified. In some of these proteins two negatively charged residues are among the five or six amino acids that constitute the binding site [42,43].

2.4. Cation recognition for melibiose transport in *K. pneumoniae*

The cloning and sequencing of the *K. pneumoniae* melibiose carrier [44] allowed a comparative physiological approach to identifying elements of the cation recognition site. Despite a 79% amino acid identity to the *E. coli* melibiose carrier the *K. pneumoniae* carrier does not use Na^+ as a coupling ion but will use H^+ or Li^+ depending on the substrate. Hama and Wilson [45] constructed chimeric carriers in which amino-terminal portions of *K. pneumoniae* carrier were replaced with the equivalent *E. coli* sequence. It was found that replacement of the first 81 amino acids of the *K. pneumoniae* carrier with those of the *E. coli melB* was sufficient to allow the *K. pneumoniae* carrier to couple Na^+ and sugar transport. In this amino-terminal region only five residues are not conserved between the two carriers. These five non-conserved amino acids on the *K. pneumoniae* carrier were individually changed to the corresponding *E. coli* residue [46]. The striking observation was that a single mutation from Ala58 to Asn allowed the *K. pneumoniae* carrier to use Na^+ as a coupling ion for sugar transport. This implied that Asn58 is crucial for Na^+ recognition.

2.5. Relative positions of helices

One technique that has been used to obtain information concerning the relative position of helices to each other has been the isolation of second site revertants from functionally inactive mutants. For example, K377V was prepared by site directed mutagenesis [39]. Cells carrying this mutation on a plasmid showed reduced transport activity and gave white clones on melibiose MacConkey indicator plates. Clones from normal control cells gave a bright red color on these plates. The MacConkey plates containing white clones of K377V were incubated for several days and a few small red papilli appeared on the surface of the white clones. These red papilli were restreaked on similar plates to purify the red colony. The plasmid DNA from cells derived from the red clone was sequenced. Frequently the cell contained the original mutation (K377V) plus a second site mutation [39].

A number of interesting observations may be made from these data. For example, one of the second site mutations from K377 was D59V [39]. Since the revertant was an aspartic acid it seemed reasonable that K377 and D59 were salt bridged in the normal protein. Loss of the positive charge in K377V led to an uncompensated negative charge at position 59 which caused a structural change in the protein resulting in loss of function. When this negative charge at position 59 was mutated to a neutral amino acid the structure of the carrier became more stable and regained activity. This second site revertant did not show normal activity but regained considerably more activity than the original K377V. It is reasonable to conclude from these experiments that K377 and D59 are salt bridged. This indicates that helix XI must be very close to helix II.

A similar experiment suggested a salt bridge between R52 and D19. R52S has low activity and a second site revertant showed D19S [38]. Similarly other revertants suggest that a salt bridge exists between R52 and D55 [38].

A variety of second site revertants have been isolated from inactive single mutants. Although the explanation for the interaction of the residue at the second site mutation to the position of the initial mutation is frequently not understood it seems reasonable to postulate that the helix containing the original mutation is probably physically close to the helix containing the second site revertant. From such data it is postulated that helices I, II, IV, VII, X and XI are close together [38,39,47,48] (Fig. 4).

2.6. Purification and reconstitution of the melibiose carrier of E. coli

The membrane carrier can be solubilized with nonionic detergents such as *n*-octyl glucoside and functionally reconstituted into liposomes [49–51]. French press membranes from cells overexpressing the melibiose carrier were solubilized in octyl glucoside in the presence of exogenous *E. coli* phospholipid. The addition of phospholipid was necessary to stabilize the hydrophobic protein during the solubilization process; the failure to include phospholipid dramatically reduces the yield of the solubilization steps [51]. Following extraction, the carrier was reconstituted into proteoliposomes via the octyl glucoside technique described by Racker et al. [52]. Morphological analysis of the resultant proteoliposomes indicated a homogeneous population of 80–200 nm unilamellar vesicles [51]. Other detergents have been successfully utilized: nonanoyl-*N*-methylglucamide [53], *n*-octyl β-D-thioglucoside [54], heptyl β-D-thioglucoside [55] and 3-(laurylamido)-*N*,*N*′-dimethylaminopropylamine oxide (LAPO) [56].

A very attractive method for purification was suggested by Hochuli and colleagues [57,58]. They suggested attachment of a polyhistidine peptide to the N- or C-terminal region of the protein. This results in a high affinity of the protein for a nickel column which facilitates purification. The melibiose carrier was constructed with six histidines at the C-terminal region and used for a large scale purification [56]. This purified protein was reconstituted into proteoliposomes and found to show sugar binding and melibiose transport. The carrier protein was purified by this method and two-dimensional crystals were formed [59]. The authors emphasize that 'although this paper is not concerned with high resolution analysis per se, the methods developed here will later be important in determining the conditions needed to get large and well-organized two-dimensional crystals suitable for electron crystallography and further three-dimensional reconstruction'.

2.7. Fluorescence resonance and active site photolabeling

Recent studies from the Leblanc laboratory [60–63] have investigated the role of tryptophan residues. First, they replaced each of the six N-terminal tryptophans by phenylalanine and studied the effect on transport. Two mutations, W116F (located in helix IV) and W128F (located in the cytoplasmic loop 4-5) result in loss of activity. W116 and W128 are mostly responsible for the cation induced fluorescence variations. These two tryptophans are also responsible for the sugar induced fluorescence changes observed in the N-terminal domain of the transporter. The results suggest that these two residues may play a critical role in the mechanism of Na/sugar symport. These studies are consistent with the view that the sugar binding site includes helix IV of the carrier. In addition the cytoplasmic loop 2-3 appears to be an N-terminal region close to the sugar site [62].

Active site photolabeling studies have shown that one of the cleavage products is a peptide between Asp124 and Met181. This includes cytoplasmic loop 4-5, helix V and periplasmic loop 5-6. This suggests that this region plays a significant role in melibiose transport [64].

2.8. Na^+/sugar transport in marine bacteria

The rate of glucose uptake into *Vibrio fisherii* was stimulated by sodium ion [65]. Galactose uptake by *Alteromonas haloplanktis* was also stimulated by Na^+. A Na^+ concentration of 300 mM gave the maximal stimulation of galactose uptake.

Na^+ was found to be essential for sucrose accumulation by *Vibrio alginolyticus*. Sucrose uptake was completely inhibited by the addition of a proton conductor. It was concluded that sucrose transport is driven by the electrochemical potential of Na^+ in this organism [66].

3. Proline

Early studies by Harold [67] in *E. coli* suggested that this cell used exclusively H^+ cotransport systems. The proline carrier of *E. coli* was one of these carriers. Indeed, one paper [68] suggested that two protons were transported for each proline molecule. In l976 Kayama and Kawasaki [69] reported that 10 mM Li^+ stimulated proline transport. Stewart and Booth [70] clarified many conflicting views by showing that addition of Na^+ to the external medium

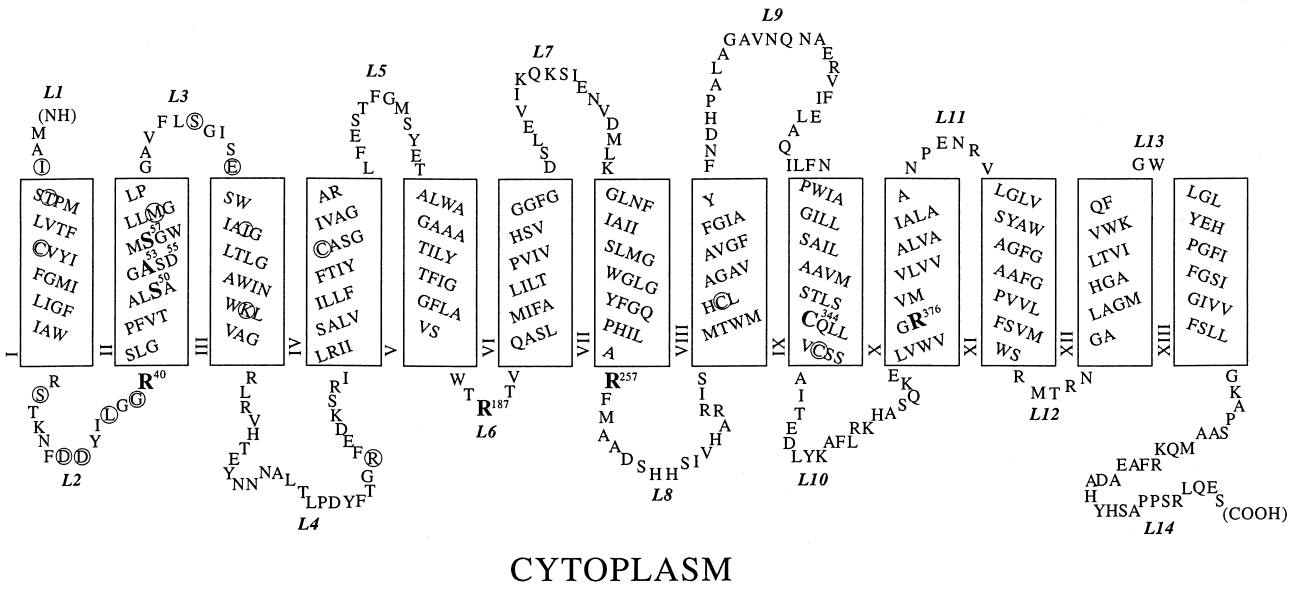

Fig. 5. Model of the proline carrier. The circled residues are those that have been altered by site directed mutagenesis and no change in function was found. The large letters with numbers are those residues whose alteration led to a marked change in function of the carrier [78,91].

simultaneously with radioactive proline led to stimulation of amino acid uptake. Na^+ increased the V_{max} of entry without affecting the K_m. These authors concluded that their data were consistent with Na^+/proline cotransport. Tsuchiya et al. [71] showed that addition of proline induced the uptake of Li^+ into the cell. Thus, proline uptake is associated with uptake of either Na^+ or Li^+ (but not H^+) [69,72–74]. Na^+ coupled proline transport is also present in *S. typhimurium* [75]. The previous results which failed to show Na^+ stimulation of transport were due to the presence of 50–100 μM Na^+ as a contamination in the buffers used. Only with the use of Na^+-free buffers and plastic containers could one clearly demonstrate the Na^+ requirement for transport.

3.1. Structure

The *putP* gene encoding the Na^+/proline transporter has been cloned [76] and sequenced [77]. The hydropathy plot suggested that the structure consisted of 12 membrane spanning segments. Later Jung [78] analyzed *putP-phoA* and *putP-lacZ* fusions and site directed labeling of the transporter and concluded that there were 13 helices with the N-terminus on the periplasmic side and the C-terminus facing the cytoplasm (Fig. 5).

3.2. Solubilization and reconstitution

Solubilization and functional reconstitution of the proline carrier was reported by Chen and Wilson [79]. Cells were disrupted by a French pressure cell at 9000 psi. The carrier protein was solubilized with sodium cholate followed by octyl glucoside by the method of Racker [80]. When reconstituted into phospholipid membranes the carrier transported proline in response to an electrochemical potential difference for Na^+ across the membrane. The stoichiometry of the cotransport system was approximately one Na^+ for one proline [79]. Hanada et al. [81] purified the carrier by first generating a fusion protein of the proline carrier and β-galactosidase. This fusion protein was purified with the use of an antibody against β-galactosidase. They obtained more than 95% homogeneity of the protein [82]. Proline transport in proteoliposomes reconstituted with the purified carrier was dependent on the membrane potential and chemical gradient of Na^+ across the membrane. The apparent K_m for proline transport

was 3.6 μM and Na^+ was 31 μM. These results confirmed that the proline carrier mediates electrogenic Na^+/proline cotransport [79].

3.3. Effect of mutations of putP on transport

Site directed mutagenesis has been utilized in an attempt to identify amino acid residues in *putP* involved in ligand binding and transport. Out of the acidic residues in the N-terminal domain that have been studied only Asp55 proved to be essential. Glu75, Asp33 and Asp34 appeared dispensable [83]. Analysis of D55E showed a 50-fold decrease in affinity of the carrier for Na^+ compared with normal although little change in the affinity of proline. This suggested that Asp55 is at or close to the cation binding site. Replacement of Asp187 by cysteine resulted in a Na^+ independent high affinity conformation for proline but very low rate of transport [84]. It was suggested that Asp187 is located close to the pathway for cation transport through the membrane.

Three positively charged residues (Arg40, Arg257 and Arg376) have been identified as having functional roles in the transport process. Arg40 is immediately adjacent to the cytoplasmic end of helix II. Removal of the positive charge at position 40 (R40C) leads to a dramatic decrease in V_{max} of Na^+ coupled proline uptake [85]. This is associated with a reduction in the affinity of the transporter for Na^+ and Li^+ while the apparent affinity of proline is only slightly altered. These results suggest that Arg40 is located close to the site for ion binding and is important for the coupling of ion and proline transport.

Arg257 (which is immediately adjacent to the cytoplasmic end of helix III) when changed to cysteine (R257C) shows no proline transport [86]. It does, however, bind proline with altered sodium ion and proton dependences. Arg376 (in helix X) is also important. The R376K mutant carrier showed no transport or binding of proline [87]. Curiously enough changing Arg376 to either glutamine or glutamic acid resulted in almost normal transport. These results are surprising since this is the only arginine in the middle of a membrane bound helix and the charge would be expected to be important.

In addition to charged residues Ser57 was found to be critical for transport [88]. Study of *putP* containing Ala, Cys, Gly or Thr in place of Ser57 showed a reduction in the apparent affinity of Na^+ and for proline up to two orders of magnitude with little effect on V_{max}. It was proposed that Ser57 is located at or close to the binding sites for Na^+ and for proline. This region of the carrier in helix II has additional residues of importance. Alterations in Arg40, Ser50 or Ala53 dramatically alter transport kinetics [89] so that helix II is critical for transport function.

When Cys344 (in helix VIII) was changed to serine proline transport was not lost but proline binding was reduced to very low levels [90]. It was concluded that Cys344 is functionally involved in the high affinity binding site for sodium ion and proline.

4. Glutamate

An early observation of Na^+ stimulated glutamate transport in *E. coli* was reported by Frank and Hopkins [92], in which they studied the increased uptake of glutamate by *E. coli* in the presence of Na^+, and suggested that a transport process was involved. The observation was extended to membrane vesicles [93]. The phenomenon was interpreted in terms of an electrochemical potential gradient of Na^+ across the cell membrane which drives the glutamate entry [94]. The gene for the glutamate carrier *gltS* was identified in *E. coli* and the protein sequence of 401 amino acids was deduced [95]. The carrier protein was predicted to be highly hydrophobic, with 73% non-polar amino acid composition. Hydropathy indicated that it has 12 transmembrane segments. It also has a conserved common motif among Na^+ symport carrier proteins in the form of Gly42-Ala82-X-X-X-X-Leu87-X-X-X-Gly91-Arg92, the putative binding site for cation [95].

Studies on membrane vesicle transport of glutamate [96] showed that either membrane potential, or pH gradient or sodium ion gradient can drive the transport. By testing structurally similar or different amino acids, the study indicated high specificity of the glutamate carrier for its substrate. It also suggested that glutamate is cotransported with one sodium ion and one proton. It was believed that the Na^+ (or H^+) binds to the carrier first to stimulate the binding of glutamate to the cation loaded carrier [97].

5. Serine and threonine

Early observations of Na^+ coupled serine or threonine cotransport in *E. coli* [98] showed competition between the two structurally similar amino acids. This suggests that serine and threonine are cotransported by the same transporter. This Na^+ coupled bacterial transport system utilizes only Na^+ as the coupling cation (whereas melibiose and glutamate transport systems may also use H^+ and Li^+). Na^+ increases the V_{max} of transport without much affecting the K_m (whereas Na^+ decreases the K_m without affecting the V_{max} in the melibiose and glutamate system).

Recently the gene (*sstT*) for the major serine transporter, the Na^+ coupled serine transporter has been cloned [99]. The gene encodes a highly hydrophobic protein with 414 amino acid residues, having a calculated molecular mass of 43.5 kDa. Hydropathy shows nine transmembrane segments.

6. Citrate

Under anaerobic conditions, *K. pneumoniae* has an inducible citrate transport system when citrate is present as a carbon source. This citrate transporter was found be to Na^+ dependent [100,101]. The carrier has been solubilized and reconstituted into proteoliposomes to study the transport [100]. The amount of citrate uptake increased as the outside Na^+ concentration increased from 0 to 100 mM. Citrate enters the cell in symport with Na^+, and is cleaved to acetate and oxaloacetate by citrate lyase. Oxaloacetate then undergoes decarboxylation to pyruvate by a membrane bound enzyme oxaloacetate decarboxylase. This enzyme is a biotin containing Na^+ pump that pumps out two Na^+ out of the cell while one oxaloacetate is decarboxylated, thus regenerating the Na^+ gradient [102].

The gene for the Na^+ dependent citrate transport system from *K. pneumoniae* (*citS*) has been identified [103]. It encodes a hydrophobic protein with 446 amino acid residues. The molecular mass was predicted to be 47.5 kDa. Study of hydropathy shows 12 transmembrane segments. One interesting feature of the structure is the presence of a long cytoplasmic loop between helix VII and helix VIII.

Because citrate has three carboxyl groups, with different pK_a values of 3.14, 4.77 and 5.40, it exists in different protonated forms, dependent on the medium pH. Mechanistic studies [101] showed that H-citrate^{2-} is the species transported, in symport with one Na^+ and at least two H^+. However, another report [104] based on rates of uptake indicated a Na^+ stoichiometry of 2.

7. Branched chain amino acids

Sodium dependent transport of branched chain amino acids (leucine, isoleucine and valine) was observed in membrane vesicles [105] and proteoliposomes reconstituted with purified carrier [106] from *Pseudomonas aeruginosa*. The mutually inhibitory effect among the uptakes of these amino acids suggested a common carrier for all of them. The gene for the sodium coupled branched chain amino acid carrier (*braB*) in *P. aeruginosa* was cloned and sequenced [107]. It encodes a hydrophobic protein with 437 residues and a calculated molecular mass of 45.3 kDa. Hydropathy indicated 12 transmembrane segments. Genes for very similar carriers (*brnQ*) were also identified in *S. typhimurium* [108] and *Staphylococcus aureus* [109].

8. Sequence similarities

Eleven families of sodium cotransport carriers have been defined by Saier [110,111] based on their degree of sequence similarity. The sodium galactoside family (SGF) is composed of several carriers, including the melibiose carrier (*melB*) from *E. coli*, *S. typhimurium* and *K. pneumonia*. There are ten identical residues in a 40 amino acid sequence near the N-terminal region (residues 336–376). The sodium citrate carriers from *K. pneumonia*, *S. pullorum* and *Salmonella dublin* are all 92% identical. On the other hand, there is little similarity between the amino acid sequences of the different families of sodium cotransport carriers from bacteria or eukaryotes.

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