A major proline carrier in *Escherichia coli* encoded by the *putP* gene mediates proline/Na⁺ or Li⁺ symport. Proline carrier mutants with altered cation specificity were obtained by mutagenesis with nitrous acid *in vitro* of a plasmid carrying the wild-type *putP* gene. Two mutant strains harboring plasmid pMOP4135 and pMOP4141 could transport proline efficiently only in the presence of an increased concentration of sodium ion. Mutations of these plasmids, *putP*4135 and *putP*4141, caused a reduced affinity for Na⁺ of proline transport and binding, without remarkable change in the affinity for proline or in production of the carriers. Consistent with the lower affinity of the *putP*4141 carrier for Na⁺, the mutant carrier was supersensitive to N-ethylmaleimide inhibition. The pH dependence of proline binding was also changed in these mutant carriers. The lesions of *putP*4135 and *putP*4141 were located in the N-terminal part of the *putP* gene (ClaI-PvuII fragment) by *in vitro* recombination and subsequent examination of the phenotype of the transformants. DNA sequencing of these fragments revealed one base alteration of G to A at nucleotides 299 and 656 in pMOP4141 and pMOP4135, respectively, which corresponded to amino acid changes from Gly²⁹ to glutamic acid and Cys¹⁴¹ to tyrosine, respectively.

Active transport of L-proline across the cytoplasmic membrane of *Escherichia coli* K-12 is mediated by a specific proline carrier encoded by the *putP* gene of the *put* regulon, *putP*-*putA*, at 23 min on the revised chromosome map (1–3). The DNA sequence of the *putP* gene has been determined, and the primary structure predicted from the sequence indicates that the carrier protein is very hydrophobic and has a molecular weight of 52,500 (2). Regulation of expression of the *putP*-encoded proline symporter is subject to amino acid changes from Gly²⁹ to glutamic acid and Cys¹⁴¹ to tyrosine, respectively.

The proline carrier catalyzes the symport of L-proline with reverse movement of sodium or lithium ions (8–13). In the presence of an electrochemical gradient of coupling cation, the carrier couples the downhill movement of the cation with the uphill movement of L-proline. Consistently with this coupling mechanism, the activity of the *putP* product for binding proline has been shown to have strict dependence on the coupling cations (14).

Previously, we isolated two *putP* mutants from a proline auxotroph that could not grow on minimal glucose medium with proline at low concentrations (5 or 20 µg/ml) but could grow on the medium with proline at high concentration (400 to 500 µg/ml) (1, 15). The *putP*32 mutation was especially interesting for study of the energy-coupling mechanism of proline transport because the *putP*32 carrier showed no activity for proline uptake but retained normal activity for binding proline (1). We concluded that the *putP*32 mutant carrier has acquired higher affinity for Na⁺, which resulted in lower efficiency of transport turnover, and found by DNA sequence analysis of the mutant *putP*32 that Arg⁵⁷ was replaced by Cys⁵⁷ in this mutant carrier (16).

Myers and Maloy (17) reported the isolation and genetic characterization of *putP* mutants of *Salmonella* with altered cation specificities, but they have not yet determined the sites of the mutations. Naturally we are interested in isolating mutants of a proline auxotroph which require a high concentration of Na⁺ for proline transport because such mutants should be useful in determining the cation-coupling domains in the symporter molecule.

This paper reports the isolation and characterization of a new type of cation-coupling mutant of the *putP* gene which requires higher concentrations of Na⁺ ion for efficient proline transport. The identifications of the mutations by DNA sequencing of the mutant carrier genes are also reported, and based on these and other observations from our laboratory, we attempt to map functional domains of the proline carrier in a putative folded structure predicted by hydropathy analysis.

**MATERIALS AND METHODS**

*Bacterial Strains*—The *E. coli* strains and plasmids used in this study are listed in Table I. All strains are derivatives of *E. coli* K-12. Wild type *E. coli* cells cannot grow well in a high concentration of LiCl because intracellular Li⁺ inhibits growth of cells on glucose (18). Therefore, in this study, we used a Li⁺-resistant strain ST3945 to circumvent the inhibitory effect of accumulated Li⁺ in the cells. Strain ST3945 was isolated from PT21R and could grow on B7 glycerol medium (19) supplemented with 400 µg/ml proline and 0.3 M LiCl. Thus, this strain is resistant to a high concentration of LiCl. All *putP* mutants isolated in this study were derived from strain ST3945.

*Media and Cell Growth*—L-broth was used as rich medium (20). For transformation, cells were grown in S05 (21). For transport assay and preparation of membrane vesicles, cells were grown to the exponential phase at 37 °C in DM medium (22) supplemented with 0.4% Bacto-casamino acid (Difco) and 0.25% glucose or 0.4% glycerol. For isolation of *putP* mutants, B7 medium (19), which consists of 40 mM K₂HPO₄, 22 mM KH₂PO₄, 1 mM K₂SO₄, 0.4 mM MgSO₄, 2 µM ferric citrate, and 50 mM NH₄Cl, was used. The sodium ion content

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of B7 medium was found to be 200–500 μM by atomic absorption spectroscopy (Perkin-Elmer 370 atomic absorption spectrometer). MT medium (17) was also used to check the growth phenotype of mutants. When required, 50 μg/ml ampicillin, 0.25 mM glycyll-L-proline, and 2 mM L-azetidine-2-carboxylic acid were added to the medium. 

**TABLE I**

<table>
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<th>Strain</th>
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<td>ST3143</td>
<td>ST3946/pMOP4135</td>
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<td>ST4102</td>
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<td>ST3949/pBR322</td>
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<td>ST4101</td>
<td>ST3949/pBR322</td>
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</tr>
<tr>
<td>JM108</td>
<td>recA1, endA1, yarA96, thi, hsdB7, supE44, relA, dcm, proA8, trdD6, proA8, lacF34F15</td>
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**Plasmid**

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</tr>
<tr>
<td>pMOP4141</td>
<td>pMOP7-pupP4141</td>
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<tr>
<td>pMOP7</td>
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</tbody>
</table>

**RESULTS**

Isolation of Mutants—We used the phenotype of L-azetidine-2-carboxylic acid resistance to isolate putP mutants as described under "Materials and Methods." The strain ST3945 was transformed with mutagenized pMOP7 and spread on B7 glucose plates containing 2 mM L-azetidine-2-carboxylic acid without NaCl or LiCl. About 400 mutants that were resistant to L-azetidine-2-carboxylic acid and seemed to have reduced activities of proline transport were obtained from the transformants with mutagenized pMOP7. In contrast, only a few L-azetidine-2-carboxylic acid-resistant transformants were obtained from nonmutagenized pMOP7. In the second screening of the 400 mutants on B7 glucose plates containing 5 μg/ml of proline, about 150 mutants that seemed to retain partial activities of proline transport were obtained.

These mutant colonies were picked up and restreaked on B7 glucose plates containing 2 mM L-azetidine-2-carboxylic acid and various concentrations of NaCl or LiCl (Table II). Two mutants, strains harboring plasmids pMOP4135 and pMOP4141, showed clear dependence of L-azetidine-2-carboxylic acid on NaCl concentration. Their growth phenotype was different from that of the strain harboring pTMP5-32, which carried the putP21 mutation (1, 16).

Myers and Maloy (17) used MT medium to characterize putP mutants. For comparison with our mutants, we also used MT plates to check the phenotype of the mutants isolated and obtained results essentially similar to those in Table II. In addition to the L-azetidine-2-carboxylic acid sensitivities of the mutants, we also examined whether they could grow on M1 plates containing 0.1% proline and found that most of them could not.

Proline Transport Properties of Strains Harboring pMOP4135 and pMOP4141—The proline transport activities in the absence and presence of NaCl and/or LiCl were examined in several mutants that showed cation dependence of L-azetidine-2-carboxylic acid sensitivity. Consistently with their growth phenotype (Table II), two of these mutants, strains harboring pMOP4135 and pMOP4141, showed sodium ion-dependent proline transport activities (Fig. 1).

Next, we examined the kinetic properties of proline transport in the two mutants in more detail (Fig. 2 and Table III). Double-reciprocal plots of their uptake activity versus the proline concentration were biphasic, as shown in Fig. 2. The apparent affinity for proline of the higher affinity components in both strains did not differ markedly from the affinity in a liquid scintillation counter. The initial rate of uptake was calculated from the amount of radioactive proline accumulated by cells in 10 or 30 s. All values are expressed in nmol/mg cell protein as determined by the method of Lowry et al. (25) using bovine serum albumin as a standard.

The N-ethylmaleimide (NEM) sensitivity of transport activity was examined as reported (26) except that the reaction mixture contained 60 mM NaCl.

**DNA Sequencing**—DNA fragments (Cial-Poull) from pMOP4135 and pMOP4141 were obtained by electrophoresis from agarose gel after digestion with Cial and Poull. The fragment itself or fragments after digestion with FagI were ligated with M13mp10 or 11 digested with Accl and Smal. Selection of M13 phages with an insertion, preparation of single-strand DNA, and DNA sequencing were carried out as described previously (2, 16). The primers used for sequencing were the M13 primer M3 (Takara Shuzo Co., Kyoto), oligonucleotide I (26), oligonucleotide III (26), and oligonucleotide IV (26). Oligonucleotide V (pGGCTTTGAGATAAAGGCG), which was synthesized in this study using an Applied Biosystems 381A DNA synthesizer, corresponds to nucleotides 582–601 of the putP gene (2).

"The abbreviations used are: NEM, N-ethylmaleimide; K_m, Michaelis constant for Na".
The mutant carriers have an altered conformation, resulting in lower affinity for sodium ion in the transport reaction without perturbation by this mutation of the environment near the substrate recognition site. This suggests that the mutant carriers cannot bind Na⁺ well, Na⁺ would not have a protective effect against NEM inactivation at the substrate recognition site.

The affinity of these components was reproducibly observed, but the origin of these components was not certain; we suppose that the sensitivity of the mutant carriers to this substrate analogue seemed to be similar to that of the wild-type carrier (26), suggesting that the mutations did not alter the substrate specificity of the substrate-binding site of the proline carrier (data not shown).

In contrast, the affinities for sodium ion of the two mutants were lower (Kₙa = 40 mM for the strain harboring pMOP4135 and Kₙa = 8 mM for the strain harboring pMOP4141) (Fig. 1 and Table III) than that of wild-type cells (Kₙa = about 30 mM) (8, 10). From these results, we concluded that these two mutants have an altered conformation, resulting in lower affinity for sodium ion in the transport reaction without remarkable change in the substrate specificity.

Na⁺ alone or Li⁺ plus proline is shown to protect the wild-type carrier against inactivation by NEM (24). Then the mutant carriers were expected to have altered NEM sensitivity because they have altered dependence on sodium ion concentration. As shown in Fig. 3, the carrier in ST4141 was supersensitive to NEM. This is consistent with the fact that this carrier has low affinity for Na⁺ because if this carrier cannot bind Na⁺ well, Na⁺ would not have a protective effect against NEM inactivation at the substrate recognition site. In contrast, the carrier in ST4141 was less sensitive than the wild-type carrier to NEM. This may be due to complex perturbation by this mutation of the environment near the sensitive cysteines (Cys²⁸⁶ and/or Cys⁴⁴ (26)) in the substrate recognition site.

**Cation Dependence of Proline Binding to the Mutant Carriers**—Previous studies showed that the proline carrier requires sodium (or lithium) ion for stoichiometric binding of

---

**TABLE II**

<table>
<thead>
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<tr>
<td>pMOP4141</td>
<td>+</td>
<td>±</td>
<td>±</td>
</tr>
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<td>pMOP7 (wild type)</td>
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<td>pBR322 (control)</td>
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**TABLE III**

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<tr>
<td>putP4141</td>
<td>17 mM</td>
<td>8 mM</td>
</tr>
<tr>
<td>putP32</td>
<td>&lt;100 μM</td>
<td>(&lt;50 μM)</td>
</tr>
</tbody>
</table>

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**FIG. 1.** Effects of cations on proline transport. Uptake of proline by intact cells from strains harboring plasmids pMOP7 (A), pMOP4135 (B), and pMOP4141 (C) was measured in the absence (○) and the presence of NaCl at 1 mM (●), 10 mM (▲), and 100 mM (■), or LiCl at 100 mM (△) and 500 mM (△). 1 nM [¹³C]proline was used as substrate.

**FIG. 2.** Double-reciprocal plots of proline transport activity. The initial rates of proline uptake by intact cells from strains harboring plasmids pMOP⁷ (□), pMOP⁴¹₃⁵ (△), and pMOP⁴¹₄¹ (■) were measured in the presence of 50 mM NaCl and various concentrations of L-[¹³C]proline.

**FIG. 3.** NEM sensitivity of the wild-type and mutant proline carrier activities. Uptake of proline by intact cells from strains harboring plasmids pMOP⁷ (○), pMOP⁴¹₃⁵ (▲), and pMOP⁴¹₄¹ (■) was measured in the presence of 50 mM NaCl after treatment with various concentrations of NEM as described under "Materials and Methods." Uptake activities are shown as percentages of the activity without NEM treatment. 1 μM [¹³C]proline was used as substrate. The control activity without NEM treatment was 6.8, 1.4, and 0.78 nmol/min/mg of protein for wild-type, putP⁴¹₃⁵, and putP⁴¹₄¹ carriers, respectively.
E. coli Proline Carrier Mutants with Altered Cation Specificity

Fig. 4. Double-reciprocal plots of proline-binding activity. Binding activities of cytoplasmic membranes from strains harboring plasmids pMOP4135 (○) and pMOP4141 (●) were measured at pH 8.0 with various concentrations of NaCl and 1 μM [14C]proline as substrate.

Fig. 5. Effect of pH on proline-binding activities. Binding activities of cytoplasmic membranes prepared from strains harboring plasmids pMOP7 (○, ●), pMOP4135 (△, ■), and pMOP4141 (□, ▣) were measured in the absence (closed symbols) and presence (open symbols) of 0.5 M NaCl. 1 M [14C]proline was used as substrate.

Fig. 6. Nucleotide and deduced amino acid sequences of the putP4135 and putP4141 mutants. Nucleotide sequences were determined as described under “Materials and Methods.” Nucleotide sequences and deduced amino acid sequences of the putP wild-type and putP4141 mutant genes around nucleotide position 299 (A) and of the wild type and putP4135 mutant genes around nucleotide 656 (B) are shown.

Fig. 7. Model of secondary structure of the proline carrier. Hydrophobic segments, shown in boxes as transmembrane domains with the numbers of amino acid residues at the beginning and end of each domain, are connected by hydrophilic loops. The locations of basic and acidic amino acid residues are shown by the symbols + and −, respectively. Amino acid residues that have been shown to be related in cation coupling or substrate recognition sites (Refs. 16 and 26; this study; and Footnote 5) are indicated with their residue number as a superscript.

Proline in nonenergized conditions (14) and that protons modify the binding properties of the carrier. Therefore, we examined the pH and NaCl dependence of the proline-binding activities of cytoplasmic membrane vesicles prepared from the mutant strains.

The affinities of the carriers for Na⁺ in the proline-binding reaction were measured under conditions in which both the concentration of substrate (1 μM) and the pH (pH 8.0) were constant (Fig. 4). The mutant carrier of pMOP4135 showed reduced affinity for Na⁺ (K_{Na} = 200 mM), but that of pMOP4141 showed little reduction in affinity for Na⁺ (K_{Na} = 17 mM) compared with that of wild-type carrier (K_{Na} = 10 mM) (Ref. 14, Fig. 4, and Table III). The maximum bindings of proline to the membranes from strains ST4141 and ST4135 were about 0.7 and 0.3 nmol/mg protein, respectively, which were similar to that of membranes from the strain harboring pMOP7. Consistent with this result, the production of mutant carrier in membranes, examined by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, was essentially normal (data not shown). Fig. 5 shows that the proline-binding activities of the mutant carriers from pMOP4135 and pMOP4141 had altered proton dependence. This suggests that the regulations of the mutant carriers and the wild-type carrier by protons are different.

Identification of Mutations in the Mutant putP Genes—Myers and Maloy (17) mapped cation sensitivity mutations in the N- and C-terminal parts of the putP gene genetically, whereas we identified one mutation of different cation dependence of substrate binding in the middle part of the gene (16). Therefore, it was of interest to compare the positions of mutations in our new mutants with those found previously (16, 17).

To determine whether the mutations were in the N-terminal half or C-terminal half, we first isolated the ClaI-PvuII (N-terminal part) and PvuII-MluI (C-terminal part) DNA fragments from the plasmids pMOP4135 and 4141 and introduced them into the wild-type putP gene on the plasmid pMOP7 in place of the corresponding wild-type fragments. The resultant recombinant plasmids obtained were transformed with strain ST3945, and the phenotype of L-azetidine-2-carboxylic acid sensitivity was examined. In this way, both mutations were shown to be on ClaI-PvuII fragments. Therefore, the ClaI-PvuII fragments of DNA from the two mutant plasmids were sequenced as described under “Materials and Methods.”

I. Yamato and Y. Anraku, manuscript in preparation.
In pMOP4141, only one alteration of the nucleotide G at position 299 (2) to A was detected. This mutation caused an amino acid change from glycine to glutamic acid at position 22 in the predicted amino acid sequence of the putP gene (Figs. 6 and 7). In pMOP4135, an alteration from G to A at position 656 was identified, corresponding to a change from cysteine to tyrosine at position 141 in the predicted amino acid sequence (Figs. 6 and 7).

**DISCUSSION**

In this study, we isolated putP mutants with defects of cation coupling by *in vitro* mutagenesis with nitrous acid. Nitrous acid was chosen as a mutagen because it is known to induce both G:C to A:T, and A:T to G:C transitions (29, 30), and so there was a good number of possible mutational mispairing events. By device of a new selection method, we could isolate mutants showing altered cation coupling in the symport cycle.

Previous studies in this and other laboratories have indicated that the proline carrier utilizes either Li⁺ or Na⁺ as a coupling cation (8, 9, 13). The properties of the two mutant strains harboring pMOP4135 and pMOP4141 strongly suggested that the carriers had lost ability to utilize Li⁺ effectively in the symport cycle while maintaining ability to couple proline transport to an electrochemical gradient of Na⁺, although with reduced efficiency. As summarized in Table III, the mutant carriers showed reduced affinities for Na⁺ in proline transport and binding without change in specificity or affinity for proline, and this specific defect in cation coupling caused their reductions in proline transport activity.

Kinetic analysis of proline uptake showed a biphasic mode of transport (Fig. 3). We did not examine the low affinity components further, as we supposed that they corresponded to a facilitated diffusion mode of the wild-type proline carrier activity (27). In fact, the null-type mutant strain ST3945 did not show this low affinity component, and so we concluded that these components were due to the transport activities encoded by the putP4135 and putP4141 genes.

Previously, Mogi et al. (1) isolated a putP22 mutant that is defective in proline transport but retains normal proline-binding activity (1). The proline-binding activity of the carrier in this mutant showed low dependence on Na⁺, and the putP22 mutation was interpreted to cause higher affinity for Na⁺ in proline binding (16). On the contrary, the two mutant strains harboring pMOP4135 and pMOP4141 isolated in this study could transport proline only in the presence of higher concentration of Na⁺, and the mutant carriers had reduced affinities for Na⁺ in proline transport. Furthermore, the mutant carrier of pMOP4135 shows markedly reduced affinity for Na⁺ (Kₐ = 200 mM) in the proline-binding reaction, but that of pMOP4141 showed only slightly less affinity for Na⁺ (Kₐ = 17 mM) than the wild-type carrier (Kₐ = 10 mM) (Fig. 4 and Table III). In addition, the mutant carriers had similar affinities for Na⁺ in the transport and binding reactions, whereas the wild-type carrier has about 100 times higher affinity for Na⁺ in the transport reaction than in the binding reaction (Table III). This difference in affinities for Na⁺ of the proline transport and binding reactions of the wild-type carrier may be interpreted as due to a difference between the carrier conformations outside and inside the membrane. On the basis of this model, the mutant carriers are thought to have acquired altered conformations that specifically reduced their affinities for Na⁺ outside the membrane. The observations that the putP4141 carrier was supersensitive to inactivation by NEM is consistent with the phenotype of requirement of a high concentration of Na⁺ for proline transport because the wild-type carrier was shown to be protected against NEM inactivation by Na⁺ (24), and the putP4141 mutation resulted in reduced affinity for Na⁺ of the transport reaction by the mutant carrier (Table III). On the other hand, the reduced sensitivity to NEM of the carrier in ST4135 may reflect some conformational perturbation around the NEM-sensitive Cys³⁴⁵ and/or Cys³⁴⁴ (26), possibly due to the replacement of Cys¹⁴¹ by tyrosine. These 3 cysteines may be located in close proximity in the folded carrier conformation.

The pH dependence of the proline-binding activities of the putP4135 and putP4141 carriers was also different from that of the putP22 carrier. This finding is consistent with the concept that the proton is a modulating regulatory factor of the transport activity rather than a coupling cation (4). The physiological significance of this modulation by protons is not yet clear, but further biochemical and molecular biological studies on these mutant carriers should give clues to the role of H⁺.

In the cases of the lactose carrier and melibiose carrier of *E. coli*, functional amino acid residues have been identified by mutation analysis or site-directed mutagenesis (29-35). From extensive studies on the H⁺-coupling mechanism of the lactose carrier using site-directed mutagenesis, a charge-relay mechanism of H⁺ translocation has been proposed in which Arg⁵⁴⁵, His⁵⁵⁵, and Glu⁵⁶⁵ are postulated to form a functional proton pore (30, 31, 36-38). Fewer but similar studies have been made on the melibiose carrier (34, 35). In the case of the proline carrier, Arg⁵⁷⁵ was the first amino acid residue suggested to be functional (18). Myers and Maloy (17) reported that cation specificity mutations of the gene of *Salmonella typhimurium* were located in the N and C termini. These regions are different from the site at which substrate specificity mutations were mapped genetically (39). Sequencing of pMOP4141 and pMOP4135 revealed lesions in the N-terminal part of the putP gene, resulting in amino acid alterations of Gly²² to glutamic acid and Cys¹⁴¹ to tyrosine, respectively. These locations are consistent with a previous report (17) that the N-terminal part is related to the binding site for coupling cation. The change from a neutral glycine to a negatively charged glutamic acid in pMOP4141 seems to be advantageous for the binding of cation and could be expected to result in increased affinity for Na⁺, which is obviously contradictory to the present finding. Thus, the present finding suggests that Gly²² is not directly involved in cation binding but rather participates in conformational stabilization for the high affinity binding of Na⁺. However, the NEM supersensitivity of this carrier is apparently consistent with low affinity to Na⁺. On the contrary, the change from Cys¹⁴¹ to tyrosine is consistent with the property of low affinity for Na⁺, because the pKₒ of tyrosine is higher than that of cysteine, and so this change would decrease the negative charge, which should be disadvantageous for Na⁺ binding. The partial resistance of this carrier to NEM may indicate that this cysteine is close to Cys³⁴⁵ and Cys³⁴⁴, which are responsible for the NEM sensitivity of the carrier, and interacts with them (26).

As summarized in Fig. 7, these results suggest us that Gly²² in helix I, Cys¹⁴¹ in helix III, Arg²²⁷ between helix VI and VII (16), Cys³⁴⁵ in helix VII, and Cys³⁴⁴ in helix VIII (26) are in close contact, forming a major cation-binding site of the proline carrier. Hediger et al. (40) reported close homology between the primary amino acid sequences of the proline/Na⁺ symporter in *E. coli* and the glucose/Na⁺ co-transporter in human intestine. The amino acid residues Gly²² and Arg²²⁷ in the proline/Na⁺ symporter identified as forming a cation-binding site are conserved in the glucose/Na⁺ co-transporter (40). Independent homology search in our laboratory of the primary amino acid sequences between the glutamate/Na⁺
symporter in *E. coli* and the glucose/Na⁺ co-transporter in rabbit intestine (41) showed weak homology. Interestingly, we found a distinct consensus sequence motif, Gly-Ala-Leu-GlyArg, in the primary amino acid sequences of these three Na⁺/substrate symporters (2, 41). This unique consensus sequence motif can be located in domains VIII and IX, and a loop in between the two in the amino acid sequence of the proline/Na⁺ symporter (Gly²²⁸-Ala³⁸⁵-Leu³⁷¹-Gly³⁷²Arg³⁷⁶ in Fig. 7) and may be relevant to the Na⁺ symport process. We do not yet know the functional role of this sequence motif or whether it is essential for a cation-coupling domain in close contact with NEM-reactive cysteines (proximal Cys²⁸¹/Cys³⁴⁴ or distal Cys¹⁴¹) and cation specificity determinant residues, Gly²² and Arg³⁷². Our current work is addressed to these interesting problems for further understanding of the cation-coupling mechanism of the symport reaction.

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I Yamato, M Ohsawa and Y Anraku


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