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Sodium ion and proline binding sites in the Na^+/proline symport carrier of *Escherichia coli*

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Proline binding activity of the *Escherichia coli* Na^+/proline symport carrier is inhibited by a sulfhydryl reagent, *N*-ethylmaleimide (NEM). Proline and its analogs protected the carrier against the NEM-inactivation in a Na^+ (or Li^+)-dependent manner. Na^+ alone, even in the absence of proline, partially protected it from the NEM-inactivation. Mutant proline carriers, CS281, CS344 and CS349, which have a serine residue in place of Cys-281, Cys-344 and Cys-349, respectively (Yamato, I. and Anraku, Y. (1988) J. Biol. Chem. 263, 16055–16057) were also analyzed for cation-dependent proline binding and NEM-sensitivity. Proline binding activities of CS281 and CS344 were almost completely resistant to NEM, whereas that of CS349 was not. Furthermore, the proline binding activity of CS344 was remarkably lower than those of the wild-type, CS281 and CS349 carriers. These results indicate that Cys-344, which is located in the putative eighth membrane-spanning domain in the carrier, is a cysteine residue functionally involved in the high-affinity binding for sodium ion and proline.

Introduction

Proline carrier, the *putP* gene product in *Escherichia coli*, is the cytoplasmic membrane protein which mediates electrogenic Na^+/proline symport [1]. The carrier activity in cells and proteoliposomes is dependent on membrane potential and a chemical gradient of Na^+ or Li^+, either of which can act as a coupling cation [1–6]. The proline carrier consists of 502 amino acid residues, and is predicted to have twelve membrane-spanning domains [7]. It binds Na^+ or Li^+ and the carrier/cation binary complex binds proline with high affinity [8]. The transport cycle via the symport carrier consists of four elementary processes [9]. (i) Unloaded carrier exposing the binding site for a coupling cation to the *cis* side of the membrane first binds Na^+, and the carrier/Na^+ binary complex binds proline at the same side of the membrane. (ii) The carrier/Na^+/proline ternary complex changes its conformation so that the binding sites for substrate and coupling cation expose to the *trans* side of the membrane. (iii) At the *trans* side, proline first dissociates from the ternary complex and then Na^+ dissociates from the resultant binary complex. (iv) The unloaded carrier changes its conformation back to the initial state, to which Na^+ and proline are accessible from the *cis* side. All processes of the transport cycle are reversible, and the net flux of proline depends on the balance of electrochemical gradients of proline and Na^+ across the membrane. This model highlights that the bound Na^+ molecule affecting the affinity of the carrier for proline is the symporting Na^+ molecule.

The binding reaction of Na^+ and proline to the carrier protein primarily directs the coupling-specificity in the symport reaction. For manifestation of the molecular mechanism of the Na^+/proline symport, it is important to study the structure of binding sites for Na^+ and proline in the carrier protein. One way of doing this is by mutation analysis. Several mutants in which the Na^+/proline symport carrier have defects in substrate specificity and cation coupling have been isolated and the lesions of the mutations were located on the *putP* gene [10–13]. The other way is by selective protein modification with chemical reagents. Fox and Kennedy [14] developed an elegant 'differential labeling' experiment to identify the *E. coli lacY* gene product (a H^+/lactose symport carrier) where they utilized a protection activity of β-D-thiodigalactoside from the

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Abbreviations: NEM, *N*-ethylmaleimide; K_d, dissociation constant; BS_{max}, maximum number of binding sites.

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inactivation by a sulfhydryl reagent, NEM. Later, the cysteine residue protected by β-D-thiodigalactoside was identified to be Cys-148 [15]. These results indicate that Cys-148 is located near the substrate-binding site and is mainly relevant to the NEM sensitivity although it is not necessary for the H^+/lactose symport activity [16,17].

The proline carrier is inactivated by NEM [18,19], and can be differentially labeled with radioactive NEM [20]. Yamato and Anraku [21] prepared mutant proline carriers in which Cys-281, Cys-344 or Cys-349 in the carrier was replaced by a serine residue and showed that Cys-281 and Cys-344 were the NEM-reactive cysteine residues. In this work, we have examined whether coupling cations as well as substrate could protect the Na^+/proline symport carrier from inactivation by NEM, hoping to understand the location of the binding sites for Na^+ and proline in the carrier molecule.

Materials and Methods

Strains and growth of cells

Transformants of *Escherichia coli* K-12, ST3009 [relevant genotype: *proP proT putP recA* F′*lacI*^{q}], with a plasmid pKHP1 (carrying *putP*$^+$ gene) or a vector plasmid pUC13 [22], and transformants of ST3501 [*proP proT putP recA*], with plasmid pMOP7–223–13, pMOP7–325–23 or pMOP7–325–32 [21] were used. The *putP* products of pMOP7–223–13, pMOP7–325–23 and pMOP7–325–32 have replacements of Cys-281, Cys-344 and Cys-349 to a serine residue, respectively [21]. The mutant carriers of Cys-281 ⇒ Ser-281, Cys-344 ⇒ Ser-344 and Cys-349 ⇒ Ser-349 are designated as CS281, CS344 and CS349, respectively, in this paper. Cells were grown as described previously [20].

Assays of proline binding and transport activities

Cytoplasmic membrane vesicles were prepared by the method of Yamato et al. [23] with a modification [22]. Binding activity of proline to cytoplasmic membrane vesicles was determined in the reaction mixture (1 ml) containing 0.1 M Tris-maleate (pH 5.3), 0.1 M NaCl and 1 μM L-[^{14}C]proline (290 mCi/mmol, 1 Ci = $3.7 \cdot 10^{10}$ Bq, Amersham) by the ultracentrifuge method [8]. Transport activity of proline in cytoplasmic membrane vesicles was determined in the reaction mixture (0.2 ml) composed of 50 mM Tris-maleate (pH 8.0), 2 mM $MgSO_4$, 20 mM ascorbate-Tris (pH 8.0), 0.2 mM phenazine methosulfate and 1 μM L-[^{14}C]proline by the filtration method [24]. The reaction mixture for the transport assay contained 30 μM Na^+ [9]. The initial rate of transport was calculated from the amount of radioactive proline accumulated in membranes for the initial 10 s. The data represent the means from two or three experiments. The deviations were within ±10% of the means. Protein was determined by the method of Lowry et al. [25], using bovine serum albumin as a standard.

Treatment of membranes with NEM

Cytoplasmic membrane vesicles (1 to 2 mg of protein) were incubated in 1 ml of buffer (see below) with 10 μl ethanol solution of 50 mM NEM for 30 min at 25°C. After stopping the reaction with 5 mM dithiothreitol, membranes were washed with 8 ml of 0.1 M Tris-maleate (pH 7.0) two or three times by ultracentrifugation at 4°C, and then the binding or transport activity of the vesicles was determined. Compositions of the buffer used are indicated in legends to figures and tables. As a control, the vesicles were treated with 1% ethanol, which did not appreciably affect the activities of the proline carrier. NEM was purchased from Wako Pure Chemicals, Tokyo.

Results

Protective effects of substrate and substrate analogs on NEM-inactivation of the proline carrier

High-affinity binding of proline to the proline carrier requires the presence of Na^+ or Li^+ [8]. Consistent with the reaction model proposed [9], proline in the presence of Na^+ or Li^+ was found to protect proline carrier functions from NEM-inactivation; the binding and transport activities remained mostly unchanged (Table I). Protection efficiencies of substrate and substrate analogs increased in the order of their binding or transport affinities to the carrier [8,26]. These results indicate that the protection was due to the formation of a Na^+ (or Li^+)/proline/carrier ternary complex in the cytoplasmic membrane [9], so that NEM could not gain access to the cysteine residue(s) which is involved in proline binding of the carrier molecule. The fact that proline in the presence of K^+ or Rb^+ showed no protective effect (Table I) supports this conclusion.

The protective effects of coupling cations on NEM-inactivation of the carrier

We further found that coupling cations alone also showed partial protective effects. When membrane vesicles were treated with 0.5 mM NEM in the presence of Na^+ but in the absence of proline, residual binding and transport activities were 68% and 54%, respectively, of those of the control membranes (Table I). Scatchard analysis of the residual binding activity showed that the NEM-treatment in the presence of Na^+ reduced the BS_{max} value without any effect on the K_d value of the activity (Fig. 1), indicating that the inactivation and the protection of the carrier molecule were in an all-or-none fashion. The half-saturation concentration of Na^+ for protection was determined to be 3 mM (Fig. 2), which was consistent with the K_d for

TABLE I

Protection of the proline carrier against NEM inactivation by L-proline, proline analogs and coupling cations

Cytoplasmic membrane vesicles (2 mg of protein/ml) prepared from ST3069/pKHP1 were incubated with or without NEM (0.5 mM) and substrates (1 mM) in 0.1 M Tris-maleate (pH 7.0) containing 0.1 M alkaline metal cation (chloride-form) indicated, and washed three times with 0.1 M Tris-maleate (pH 7.0). Residual proline binding and transport activities in the membrane vesicles after NEM treatment were measured, and are represented as the percentages of the activities of control membranes. The control membranes were incubated without NEM in the absence of substrates. The proline binding and transport activities in the control membranes were 944 pmol/mg protein and 16.5 nmol/mg protein per min, respectively.

Salt	Substrate	Residual activities	
		binding (%)	transport (%)
NaCl	L-proline	97	96
LiCl	L-proline	88	100
KCl	L-proline	28	25
RbCl	L-proline	15	12
LiCl	D-proline	9.7	4.7
LiCl	DL-pipecolinic acid	9.1	4.8
LiCl	L-azetidine 2-carboxylic acid	28	26
LiCl	3,4-dehydro-DL-proline	50	51
NaCl	None	68	54
LiCl	None	7.6	5.3
KCl	None	3.9	7.9
RbCl	None	5.5	3.5

Na^+ of the proline carrier [8]. Although the K_d value for Li^+ of the carrier was estimated to be 31 mM (Mogi, T., unpublished results), 0.1 M Li^+ did not show significant protective effect under these conditions (Table I and Fig. 2). However, Li^+ exhibited a

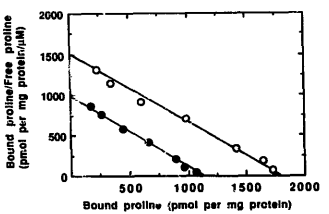

Fig. 1. Effect of NEM treatment on the Na^+-dependent proline binding activity in cytoplasmic membrane vesicles. Cytoplasmic membrane vesicles were incubated in the presence of 0.1 M NaCl with (●) or without (○) 0.5 mM NEM as indicated in the legend to Table I. Then, proline binding activities of the membranes were measured at various proline concentrations. The data obtained are represented in the form of Scatchard plots. The K_d and BS_{max} values of proline binding were 1.2 μM and 1.2 nmol/mg protein, respectively, in the NEM-treated membranes and 1.2 μM and 1.8 nmol/mg protein, respectively, in the untreated membranes.

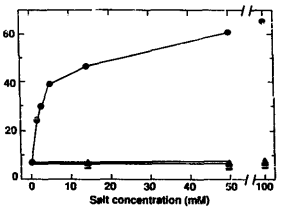

Fig. 2. The Na^+ dependence of protection of the proline carrier against NEM-inactivation. Cytoplasmic membrane vesicles were incubated with 0.5 mM NEM in the presence of salts (NaCl, ●; LiCl, ▲; KCl, ■) at various concentrations, and then proline binding activities were measured. Residual activities are shown as percentages of the activity of NEM-untreated membranes.

slight protective effect when concentrations of NEM were much lower (see below). K^+ or Rb^+ did not show any protective effect at all.

Effects of alkaline metal cations on the NEM-inhibition were examined in more detail at various concentrations of NEM. NEM-concentrations required for 50% inhibition of the binding activity were 10 μM, 20 μM and 1.3 mM in the presence of 0.1 M K^+, Li^+ and Na^+, respectively (Fig. 3). This result confirmed the effective protection of Na^+, and further showed that Li^+ also had a slight protective effect, compared with K^+. The fact that not only proline but also coupling cations protected the carrier from NEM-inactivation suggests that the binding sites for coupling cations and proline are spatially close in the carrier molecule and that the two binding sites involve an NEM-reactive cysteine residue(s).

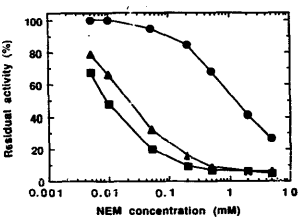

Fig. 3. Residual proline binding activity of cytoplasmic membranes after treatment with various concentrations of NEM. Cytoplasmic membrane vesicles were incubated in the presence of 0.1 M salts (NaCl, ●; LiCl, ▲; KCl, ■) with various concentrations of NEM. Residual activities are shown as percentages of the activity of NEM-untreated membranes.

Cys-344 is a candidate residue involved in cation and proline binding

The nucleotide sequence of the *putP* gene predicts that the proline carrier contains five cysteine residues [7]. We previously prepared mutant proline carriers CS281, CS344 and CS349 and examined their transport activities and NEM-sensitivities [21]; the CS344 carrier showed lower activity than the other mutant carriers and the wild-type carrier, and the CS281 and CS344 carriers are resistant to NEM. Here, we examined the cation-dependence and NEM-sensitivity of proline binding to the three mutant carriers.

Table II shows that the CS281 and CS349 carriers had Na^+ (and Li^+)-dependent proline binding activities as high as those of the wild-type carrier. The Na^+-dependent proline binding of the CS344 carrier, however, was found to be remarkably low and its Li^+-dependence was less than the Na^+-dependence.

The Na^+-dependent proline binding activities of CS281 and CS344 carriers were resistant to NEM treatment whereas that of CS349 carrier was not (Table II). Inactivation of the CS349 carrier by NEM was protected by the presence of Na^+ and proline (data not shown). Thus, it is concluded that chemical modification by NEM of a reactive cysteine residue(s) in the proline carrier inhibits the Na^+-dependent proline binding activity and results in the inhibition of the Na^+/proline symport cycle (Tables I and II; also see Ref. 21). We suggest that Cys-344 may be one of candidate residues which are functionally involved in operation of the symport cycle or located near or spatially close to the binding sites for proline and sodium ion.

TABLE II

Cation-dependence and NEM-sensitivity of proline binding activity of mutant carriers

Carrier	Binding activity [a] (pmol/mg protein)				Residual activity [b] (%) after NEM-treatment
	None	LiCl	NaCl	KCl	
CS281	150	440	470	170	90
CS344	4	60	110	7	80
CS349	200	450	470	260	10
Wild-type [c]	100	1000	940	140	5

[a] Proline binding activity in membrane vesicles was measured in 0.1 M Tris-maleate (pH 5.5) containing 1 μM [^{14}C]proline in the absence or presence of 0.1 M LiCl, NaCl or KCl.

[b] Membranes were treated with 0.5 mM NEM in the absence of proline and Na^+, and the residual activities of proline binding were measured as described in Materials and Methods, and are represented as the percentage of the activities of control (NEM-untreated) membranes.

[c] Wild-type carrier was about twice more overproduced than mutant carriers because plasmid pKHP1 has tandem promoters for the expression of the *putP*$^+$ gene [20,22].

Discussion

The ordered binding of coupling cation and proline to their specific sites in the proline carrier is an elementary process in the Na^+/proline symport cycle [9]. In this study, to obtain insights into domains of these binding sites, we characterized protective effects of proline and coupling cations against the inactivation of wild-type and mutant carriers by NEM.

The proline binding activity of the wild-type carrier was inactivated by NEM, and the inactivation was protected by proline and its analogs in an Na^+- or Li^+-dependent manner (Table I). Furthermore, Na^+ alone showed the protective effect against the NEM-inactivation (Table I). From these results, we suggest that the reactive cysteine residue(s), to which NEM binds covalently, is located near binding sites for proline and coupling cation, and that NEM molecule is less accessible to the cysteine residue(s) in the carrier/Na^+ or the carrier/Na^+ (Li^+)/proline complex than in the unloaded carrier. Li^+ alone also showed a slight protective effect but the efficiency was much lower than that expected from the binding affinity of Li^+ to the carrier (Fig. 3), even though both cations are the coupling cations [1–6]. We do not know why Na^+ and Li^+ render such different levels of protection efficiency. The difference in their ionic radii or the conformations of cation/carrier binary complexes may cause the difference in the accessibility of NEM to a reactive cysteine residue(s) in the carrier.

The proline and coupling cation binding sites were further clarified by analysis of the mutant carriers CS281, CS344 and CS349. Proline binding activities of the CS281 and CS344 carriers were resistant to NEM (Table II). Furthermore, the proline binding activity of the CS344 carrier was remarkably lower than those of CS281, CS349 and wild-type carriers (Table II). The differential dependence of the CS344 carrier on Na^+ and Li^+ (Table II) suggests that this mutant carrier has an altered conformation at the cation binding site. Thus, it is reasonable to conclude that Cys-344 is a cysteine residue functionally involved in the Na^+ and proline binding. We propose that the proline binding site and cation binding site are close to Cys-344, which is located in the putative eighth membrane-spanning domain in the proline carrier (Fig. 4). Although Cys-281 was shown to be relevant to NEM-sensitivity (Table II; Ref. 21), its actual function remains unsolved. Cys-349 does not seem to play a role in the Na^+/proline symport reaction because the replacement of Cys-349 by a serine residue showed no significant change in carrier activities and NEM-sensitivity (Table II; Ref. 21).

Recently, Hediger et al. [27] found that the human intestinal Na^+/glucose symport carrier and the *E. coli* Na^+/proline symport carrier are highly homologous in

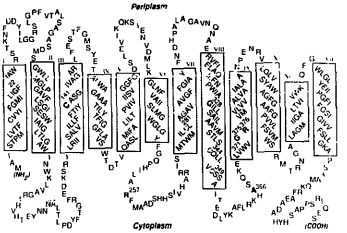

Fig. 4. A topological model of the Na^+/proline symport carrier in the cytoplasmic membrane. Potential membrane-spanning regions (represented as rectangles with Roman numerals) were predicted from hydropathic analysis of the carrier [7]. The carrier does not have a cleavable signal sequence * and the carboxyl terminus of the carrier is exposed to the cytoplasmic side of the cytoplasmic membrane [22,31]. Locations of amino acid residues in the carrier are shown as one-letter symbol, and Cys-281, Cys-344 and Cys-349 are highlighted. Mutation studies indicate that Gly-22, Cys-141 and Arg-257 (bold-face characters) play important roles in cation binding [10, 11]. The SOB-motif, a proposed binding motif for Na^+ (Gly-328, Ala-366, Leu-371, Gly-375 and Arg-376), is also shown.

their predicted amino acid sequences. Mammalian intestinal Na^+/glucose carriers are inhibited by sulfhydryl reagents, but not protected from the inhibition by their substrates, suggesting that the reactive cysteine residue(s) does not reside near the substrate-binding site [28,29]. This apparent contradiction between homology of the primary structure and the difference in protective effects of substrates in the *E. coli* Na^+/proline and human intestinal Na^+/glucose symport carriers is not surprising, because there is no conservative cysteine residues between the two carriers [7,27]. Thus, the locations of reactive cysteine residues may be quite different between these carriers. Probably, the cysteine residue(s), including Cys-344, in the proline carrier is very close to its substrate/cosubstrate-binding sites, but the cysteine residue(s) in the glucose carrier is not.

Proline binding activity was completely inhibited by treatment of cytoplasmic membrane vesicles with 3 mM diethyl pyrocarbonate or 0.5 mM dicyclohexylcarbodiimide, and the activity was protected from these inhibitions by proline in the presence of Na^+ (or Li^+) or partially by Na^+ alone (unpublished observation), suggesting that histidine and glutamate/aspartate residues are also located near the binding sites.

Recently, Deguchi et al. [30] reported that there is a conservative amino acid sequence (G----AXXXXLXXXGR) in molecules of the *E. coli* Na^+/glutamate, Na^+/proline and mammalian Na^+/glucose symport carriers, which is suggested to be a sodium ion binding-motif (SOB-motif). In a topological model of the proline carrier, this putative SOB-motif exists in the region between Gly-328 to Arg-376 and is mapped on the eighth and ninth membrane-spanning domains (Fig. 4). In addition, we have shown that mutations at Gly-22 to Glu-22 or Cys-141 to Tyr-141 caused a remarkable decrease of affinity for Na^+ to the proline carrier, and suggested that these residues are related to the binding for coupling cation [9]. In summary, we suggest that the first, third, eighth and ninth membrane-spanning domains as well as the cytoplasmic loops between sixth and seventh, and eighth and ninth membrane-spanning domains are involved to form binding sites for the symport carrier (Fig. 4).

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* The proline carrier was purified near homogeneity as described previously [1]. Prior to the determination of the N-terminal amino acid sequence, phospholipids and dodecyl maltoside contained in the purified preparation were removed by electrophoresis in sodium dodecylsulfate-polyacrylamide gel followed by electroelution [32]. The sample thus obtained was analyzed with a gas phase sequencer (model 477A/120A, Applied Biosystems) and a sequence of six N-terminal amino acid residues (MAISTP) was determined. The sequence was identical to a sequence predicted from the DNA sequence of the *putP* gene [7], indicating that the proline carrier has no cleavable signal sequence (Hanada, K. and Anraku, Y., unpublished results).

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