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Solubilization and reconstitution of proline carrier in *Escherichia coli*; quantitative analysis and optimal conditions

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Proline carrier of *Escherichia coli* was extracted from the carrier-overproducing membranes with dodecylmaltoside in the presence of phospholipid. The solubilized carrier showed the same proline binding activity as that in normal membranes. As judged from determinations of the binding activity in the micellar state as a marker of active carrier and the radioactivity of *N*-[*ethyl*-2-^{3}H]ethylmaleimide-labeled carrier as a marker of carrier polypeptide, 80% of the carrier molecules in the membranes were extracted. Optimal conditions for reconstitution of the solubilized carrier were established. By a combination of freeze-thawing, sonication and dilution procedures, 70% of the solubilized carrier molecules were incorporated into proteoliposomes and the restored active transport of proline showed an apparent K_t of 1 μM and turnover number of 0.6 s^{-1}. The transport of proline was driven by a membrane potential in a Na^+ (or Li^+)-dependent manner.

Introduction

Proline transport in *Escherichia coli,* which is mediated by a product of the *putP* gene, is a typical secondary active transport system, ColE1-*putP*$^+$ hybrid plasmids were isolated from an *E. coli* DNA library [1,2], and recently, the nucleotide sequence of the *putP* gene was determined [3]. The proline carrier was identified as a cytoplasmic membrane protein by specific labeling of the carrier with *N*-[*ethyl*-2-^{3}H]ethylmaleimide [4]. The proline carrier binds H^+ and Na^+, and the ternary complex of the carirer-H^+-Na^+ in the membrane binds proline with high affinity [5]. Kinetic and thermodynamic analyses of the proline transport in membrane vesicles suggested the mechanism of $2H^+$/proline symport [6,7]. On the other hand, recent studies on proline transport in cells [8,9] and reconstituted membranes [10] demonstrated that the active transport of proline depended on Na^+ gradient across the membranes, and that Na^+ moved concomitantly with proline, suggesting a mechanism of Na^+/proline symport. For further elucidation of the mechanism of the proline transport, purification of the proline carrier is necessary.

For purification of a carrier protein, it is necessary to establish optimal conditions for efficient extraction of the carrier from the membranes without its appreciable inactivation and in a form that can be reconstituted into proteoliposomes for transport assay. For this purpose, it is necesary to determine how much of the population of the carrier molecules is active in the extract and the proteoliposomes. Amanuma et al. [11] reported solubilization and reconstitution of *E. coli* proline

Abbreviations: BS_{max}, maximum number of binding sites; $\Delta\psi$, membrane potential; ΔpNa, ΔpLi and ΔpH, chemical gradients of Na^+, Li^+ and H^+, respectively.

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carrier with acidic butanol, and Chen and Wilson [10] demonstrated that the proline carrier solubilized with cholate could be reconstituted by a dilution method, but neither of these previous reports quantitatively determined the amount of active carrier at each step of solubilization and reconstitution.

Specific labeling of target carrier proteins with chemical reagents [4,12,13] has been reported to be useful for measuring the amount of the carrier protein, although these chemical modifications of the carriers resulted in their inactivation. Previous studies in this laboratory indicated that the substrate-binding activities of the proline and glutamate carriers provide measures of the amounts of carriers in the membrane [5,14]. Wright and Overath [15] measured the *p*-nitrophenyl-α-D-galactopyranoside binding activity of the *E. coli* lactose carrier in a solubilized state. These findings suggest that proline binding activity will be a good quantitative marker of active proline carrier if the detergents used for solubilization do not significantly affect the binding activity and can be reversibly removed in the process of reconstitution.

This paper reports the optimal conditions for solubilization of proline carrier that maintains proline binding activity in a micellar state. In parallel, the total amount of carrier molecules recovered in the extract and redistributed into proteoliposomes was monitored based on radioactivity of *N*-[*ethyl*-2-^{3}H]ethylmaleimide-labeled proline carrier. Experimental conditions for reconstitution that resulted in high recovery of the proline binding activity and high efficiency of proline transport activity in proteoliposomes were also determined.

Materials and Methods

Preparation of cytoplasmic membrane vesicles

Transformants of *Escherichia coli* K-12 strain ST3009 (relevant genotype, *putP proP proT recA*/F′ *lacI*^{q}) with pKHP1 (a multi-copy plasmid carrying the *putP* gene) [16] and pUC13 (the vector plasmid for pKHP1) were grown as described previously [4]. Cytoplasmic membrane vesicles were prepared as described in Ref. 17 with a modification [16].

E. coli phospholipid

Total lipid of *E. coli* was extracted from strain W3110 ($F^-\lambda^-$) grown in minimal salt medium containing 0.5% glucose by the method of Bligh and Dyer [18]. The phospholipid was separated from neutral lipids by silica-gel (Clarkson Chemicals, Unisil) chromatography, and stored in a dried form at −80 °C. For use in solubilization and reconstitution experiments, phospholipid was dispersed in water or appropriate buffer at a concentration of 50 mg phospholipid per ml with a bath-type sonicator (Laboratory Supplies) as described in Ref. 19.

Solubilization

All procedures for solubilization and reconstitution were done at 4 °C or on ice unless otherwise mentioned. When needed, the membrane vesicles were mixed with *N*-[*ethyl*-2-^{3}H]ethylmaleimide-labeled membranes in a ratio of 70 : 1 (w/w of protein). In a typical experiment, cytoplasmic membrane vesicles (1.8 mg protein) from ST3009/pKHP1 were suspended in 756 μl of 30 mM Tris-HCl (pH 7.5) containing 1 mM dithiothreitol. After addition of 84 μl of phospholipid (50 mg/ml) to the suspension, 360 μl of 5% dodecylmaltoside was added to the mixture with blending to give a final concentration of the detergent of 1.5%. The detergent-treated mixture was incubated for 20 min and an aliquot of 0.6 ml was centrifuged at 130000 × *g* for 1 h. The supernatant was recovered as the solubilized fraction.

Reconstitution

In the standard method, 25 μl of the solubilized fraction was added to a mixture of 100 μl of *E. coli* phospholipid (50 mg/ml in 50 mM potassium phosphate (pH 7.5) containing 1 mM dithiothreitol) and 11.4 μl of 15% (w/v) octylglucoside. The resulting mixture was frozen in dry solid CO_2/acetone, thawed at room temperature and sonicated for 2 to 3 s as described in Ref. 19. Then the mixture was dilute 200-fold into 50 mM potassium phosphate (pH 7.5) containing 0.2 mM dithiothreitol. Proteoliposomes were precipitated by centrifugation (100000 × *g*, 30 min). The solution in the tube was drained off, and the precipitate was suspended in 20 to 40 μl of 50 mM potassium phosphate (pH 7.5) containing 2 mM $MgSO_4$. The

standard buffer systems used for freeze-thaw, sonication, dilution and suspension steps were changed to alter the buffer compositions in proteoliposomes as indicated below.

The proline transport reaction was started by 100-fold dilution of the proteoliposome suspension into reaction mixture at 25 °C. The standard reaction mixture was composed of 40 mM Tris-phosphate (pH 7.5), 10 mM sodium phosphate (pH 7.5), 2 mM $MgSO_4$, 10 μM valinomycin, and 1 μM L-[^{14}C]proline. At appropriate intervals, 95 μl samples of reaction mixture were filtered on a nitrocellulose filter (0.3 μm pore size) with suction. The filter was washed with 6 ml of ice cold 0.1 M LiCl and radioactivity trapped on the filter was measured as described in Ref. 6. The initial rate of proline transport was determined by measuring the amount of proline taken up in 15 or 20 s of incubation.

Assay of proline binding activity

Proline binding activity in the presence of detergent was determined by an equilibrium dialysis method [20] in assay mixture composed of 50 mM sodium phosphate (pH 7.0) and 1 μM L-[^{14}C]proline. Equilibrium was attained in 3 h at 4 °C. Background radioactivity was determined in assay mixture containing a large excess (1 mM) of non-radioactive L-proline. The BS_{max} value of proline binding in proteoliposomes was determined as described in Ref. 5.

Miscellaneous

The proline carrier in membrane vesicles was labeled with *N*-[*ethyl*-2-^{3}H]ethylmaleimide as described previously [4,16]. Proteins in proteoliposomes were determined by a modification of Schaffner and Weissmann's method [21,22] using bovine serum albumin as a standard. 1-*O*-*n*-Dodecyl β-D-glucopyranosyl (1 → 4)-α-D-glucopyranoside (dodecylmaltoside) was purchased from Calbiochem-Behring, 1-*O*-*n*-octyl β-D-glucopyranoside (octylglucoside) from Dojin Laboratories, and Triton X-100 from Wako Pure Chemical Ind. Valinomycin was obtained from Sigma Chemicals. L-[^{14}C]Proline (290 mCi/mmol) was purchased from Amersham, and *N*-[*ethyl*-2-^{3}H]ethylmaleimide (50 Ci/mmol) from New England Nuclear.

Results

Solubilization of proline carrier

Equilibrium binding of proline to proline carrier has been shown to be a measure for the amount of the proline carrier in membranes [5]. We examined several detergents in terms of their effects on the binding activity and solubilization of the proline carrier. Three non-ionic detergents, dodecylmaltoside, octylglucoside and Triton X-100, were used, since they have been useful in purification of carrier proteins [15,19,22]. Table I shows that dodecylmaltoside was effective for both preservation and recovery of the binding activity in the solubilization step. In the presence of exogenous *E. coli* phospholipid, dodecylmaltoside did not significantly affect the binding activity, at least, in the assay conditions used, and extracted both the binding activity and the polypeptide of proline carrier (labeled with *N*-[*ethyl*-2-^{3}H]ethylmaleimide was an internal marker) equally well with maximal efficiencies of about 80%. These parallel solubilizations by treatment with the detergent indicated that almost all the carrier molecules in the detergent/phospholipid/carrier mixed micellar state are active in terms of binding activity. Dodecylmaltoside-treated membranes from ST3009/pUC13, a *putP*$^{-}$ strain, showed no appreciable proline binding (data not shown). In the absence of exogenous phospholipid, dodecylmaltoside inhibited the activity, showing an absolute requirement for exogenous phospholipid for preservation of an active form of the carrier in the micellar state, but solubilized the carrier molecule as efficiently as in the presence of phospholipid, as judged by recovery of *N*-[*ethyl*-2-^{3}H]ethylmaleimide-labeled carrier. Octylglucoside and Triton X-100 strongly inhibited the binding activity, even in the presence of phospholipid, and were less efficient in solubilizing carrier molecule.

Fig. 1 shows the effect of pH on the preservation of the proline binding activity in the dodecylmaltoside-treated mixture. The binding activity was preserved almost completely and kept constant at pH from 6.0 to 7.5. Addition of 50 mM KCl, NaCl or LiCl in the mixture did not significantly affect the preservation of the activity (data not shown).

TABLE I

EFFECTS OF DETERGENTS ON BINDING ACTIVITY AND SOLUBILIZATION EFFICIENCY OF PROLINE CARRIER

Cytoplasmic membranes (or membranes mixed with *N*-[*ethyl*-2-^{3}H]ethylmaleimide-labeled membranes) from ST3009/pKHP1 were incubated in the presence or absence of the indicated detergent (1.5%) and exogenous *E. coli* phospholipid (3.5 mg/ml) in a total volume of 1.2 ml for 20 min. Half the detergent-treated mixture was centrifuged (130000 × *g*, 1 h). The protein content, proline binding activity and radioactivity of *N*-[*ethyl*-2-^{3}H]ethylmaleimide-labeled carrier in the detergent-treated mixture and the supernatant were determined. n.d., not determined.

Detergent	Spec. act. of proline binding in detergent-treated mixture (pmol/mg protein)	Recovery in supernatant [b]		
		Protein (%)	Proline binding activity (%)	*N*-[*ethyl*-2-^{3}H]-ethylmaleimide-labeled carrier(%)
None	370	–	–	–
Dodecylmaltoside	320	77	77	81
Octylglucoside	< 40	59	n.d.	59
Triton X-100	< 40	69	n.d.	57
Dodecylmaltoside [a]	< 40	82	n.d.	90

[a] No phospholipid was added.

[b] Percentages recoveries were calculated from amounts of total protein (mg/ml), *N*-[*ethyl*-2-^{3}H]ethylmaleimide-labeled carrier (cpm/ml) and proline binding activity (pmol proline/ml) in the supernatant and the detergent-treated mixture.

Reconstitution of solubilzied carrier into proteoliposomes

The dodecylmaltoside extract containing proline binding activity (see Table I) was used for reconstitution of proline transport in proteoliposomes. We examined the conditions for freeze-thawing sonication [19] and dilution [23] to establish an optimal procedure for reconstitution in terms of transport activity. For this purpose, the effect of octylglucoside in these reconstitution steps was also examined, since several papers have reported that addition of octylglucoside to phospholipid in the reconstitution steps is critical to

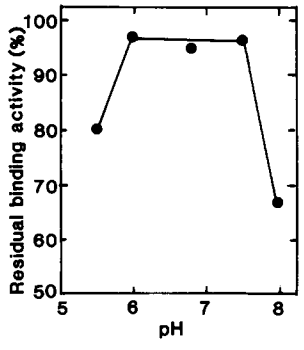

Fig. 1. Effect of pH on the preservation of proline binding activity in dodecylmaltoside-treated mixture. Cytoplasmic membrane vesicles were incubated in 25 mM 4-morpholineethanesulphonate-Tris of various pH containing 1.5% dodecylmaltoside, 1 mM dithiothreitol and 3.5 mg/ml phospholipid for 3 h at 4° C. Residual binding activities were shown as the percentage of those of intact membrane vesicles.

TABLE II

EFFECTS OF ADDITION OF OCTYLGLUCOSIDE, FREEZE-THAWING, SONICATION AND DILUTION ON RECONSTITUTION OF PROLINE TRANSPORT ACTIVITY

Various combinations of reconstitution procedures were examined with (+) or without (−) addition of 1.25% octylglucoside (OG) and with (+) or without (−) steps of freeze-thawing (FT), sonication (S) and dilution (Dil). Proline transport activities in the resulting proteoliposomes were determined (see Materials and Methods). Maximum accumulation was determined from the time-course (0–10 min) of proline uptake. Values are means ± S.E. for three experiments.

Step				Proline transport	
OG	FT	S	Dil	initial rate (nmol/mg protein per min)	maximum accumulation (nmol/mg protein)
+	+	+	+	26 ± 5	29 ± 5
−	+	+	+	13 ± 2	16 ± 1
+	+	−	+	18 ± 1	12 ± 1
−	+	+	−	12 ± 2	16 ± 1
+	−	+	+	< 1	< 1
+	+	+	−	< 1	< 1
+	−	−	+	< 1	< 1

successful reconstitution of carrier proteins [22,24–27].

We found that the proline transport activity could be reconstituted into proteoliposomes efficiently by a combination of freeze-thawing, sonication, and octylglucoside dilution. In this method (Table II), the freeze-thawing step was indispensable. The sonication and dilution steps with addition of octylglucoside in the reconstitution mixture enhanced the proline transport activity about 2-fold. In test at concentration of 0 to 2.0%, this stimulatory effect of octylglucoside was highest at 1.25%. Based on these results, we adopted the standard conditions for reconstitution shown in the first line in Table II. In the standard conditions, about 70% of the solubilized carrier molecules were incorporated in proteoliposomes, as judged from the recovery of *N*-[*ethyl*-2-^{3}H]ethylmaleimide-labeled carrier and the proline binding activity.

Properties of proline transport in proteoliposomes

Proline transport in proteoliposomes driven by a $\Delta\psi$ in the presence of Na^+ showed higher activity than that in the absence of Na^+, and imposition of ΔpNa further stimulated the activity (Fig. 2A). The imposition of ΔpLi was also stimulatory (Fig. 2B), but proline transport driven by ΔpNa alone was very low in our experimental conditions (Fig. 2A). The imposition of ΔpH (outside acidic) was rather inhibitory (data not shown).

Proteoliposomes with membrane proteins of

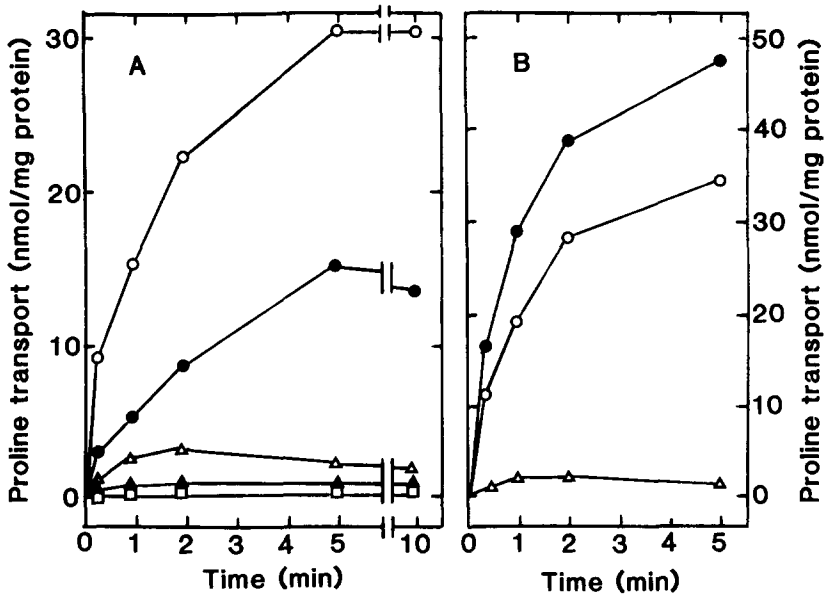

Fig. 2. Proline transport in reconstituted proteoliposomes. The membrane proteins from ST3009/pKHP1 were solubilized and reconstituted as described in Materials and Methods. Proline transport was measured by 100-fold dilution of the proteoliposome suspension into assay solution (pH 7.5) of various compositions containing 2 mM $MgSO_4$, 10 μM valinomycin and 1 μM L-[^{14}C]proline. (A) Effect of $\Delta\psi$ and Na^+ on the proline transport. The buffer systems of the internal space of proteoliposomes/the assay solution were: ●, 40 mM potassium phosphate and 10 mM sodium phosphate/40 mM Tris-phosphate and 10 mM sodium phosphate (imposing a K^+-diffusion potential, inside negative, in the presence of Na^+ on both sides); ○, 50 mM potassium phosphate/40 mM Tris-phosphate and 10 mM sodium phosphate (imposing $\Delta\psi$ and ΔpNa); ▲, 50 mM potassium phosphate/40 mM potassium phosphate and 10 mM sodium phosphate (imposing ΔpNa); △, 50 mM potassium phosphate/50 mM Tris-phosphate (imposing $\Delta\psi$ in the absence of Na^+); □, the membrane proteins from ST3009/pUC13 were solubilized and reconstituted. The buffer systems were 50 mM potassium phosphate/40 mM Tris-phosphate and 10 mM sodium phosphate (imposing $\Delta\psi$ and ΔpNa). (B) Effect of Li^+. ●, 50 mM potassium phosphate/40 mM Tris-maleate and 10 mM LiCl (imposing $\Delta\psi$ and ΔpLi); ○, 50 mM potassium phosphate/ mM Tris-maleate and 10 mM NaCl (imposing $\Delta\psi$ and ΔpNa); △, 50 mM potassium phosphate/50 mM Tris-maleate (imposing $\Delta\psi$ in the absence of Na^+ nor Li^+).

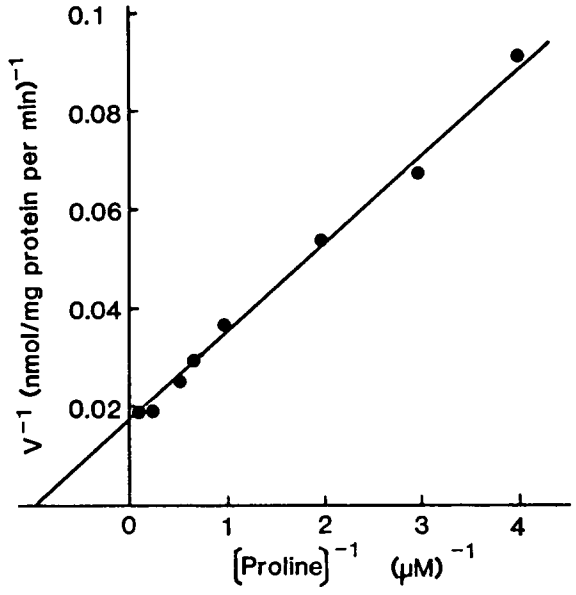

Fig. 3. Kinetics of proline transport in proteoliposomes. The initial rate of proline transport in proteoliposomes driven by both $\Delta\psi$ and ΔpNa was measured in the presence of 0.25 to 10 μM L-[^{14}C]proline as described in Materials and Methods. $K_t = 1.0$ μM. $V_{max} = 58$ nmol/mg protein per min.

ST3009/pKHP1, which over-produces the proline carrier, exhibited high proline transport activity while those of ST3009/pUC13, which lacks a product of the *putP* gene, did not (Fig. 2A), indicating that proline transport in proteoliposomes is mediated by the product of the *putP* gene.

Kinetic studies indicated that the apparent K_t value for the active transport driven by both $\Delta\psi$ and ΔpNa was 1 μM (Fig. 3). The BS_{max} value of proline binding in the proteoliposomes was determined to be 1.7 nmol/mg protein, and the turnover number of proline transport in the proteoliposomes, which is defined as V_{max}/BS_{max}, was estimated to be 0.6 s^{-1}.

Discussion

It is generally difficult to assess whether solubilize carrier proteins are reconstitutively active or irreversibly inactivated. In this study, we demonstrated that the proline carrier was solubilized efficiently with dodecylmaltoside in a form preserving the proline binding activity (Table I), which has been shown to be a good marker for an active proline carrier [5]. Essentially, the similar results were reported by Wright and Overath [15] who demonstrated that the *E. coli* lactose carrier retains the galactoside binding activity even in the presence of dodecylmaltoside.

We also shown that the addition of phospholipid in the dodecylmaltoside-treated mixture was critical for preservation of the proline binding activity of the solubilized carrier (Table I). Several reports described that in solubilization of carrier proteins with octylglucoside, the addition of exogenous phospholipid was important for protection of the solubilized carriers against irreversible inactivation [22,24–27].

Although dodecylmaltoside is better than octylglucoside and Triton X-100 for solubilization of an active proline carrier in a mixed micellar form (Table I), the extract may not be a good sample for reconstitution of the carrier into liposomes because dodecylmaltoside has low critical micellar concentration and large micellar size [28]. In fact, Wright and Overath [15] reported that for reconstitution of the dodecylmaltoside-solubilized lactose carrier, removal of the detergent with Bio-Bead SM-2 was the first critical steps to restore the active transport in large unilamellar proteoliposomes. We found that the dodecylmaltoside-solubilized proline carrier could be reconstituted by a combination of freezing-thawing and sonication [19], and octylglucoside-dilution [23] methods. Under optimal conditions, our standard method is simple and highly efficient in recovery of the proline carrier and restores the activity of proline transport. As shown in Table II, the proline transport activity was reconstituted to some extent only by the freezing, thawing and sonication method, while the activity was not appreciably reconstituted only by octylglucoside-dilution method, and a combination of the two method resulted in enhancement of the proline transport activity in the proteoliposomes. It was reported that the presence of octylglucoside of concentration around 1.25% in phospholipid is critical to reconstitute the lactose [22,24], melibiose [25,26] and galactose [27] carriers by octylglucoside-dilution method. Freezing-thawing in the presence of 1.25% octylglucoside may additively stimulate in-

tegration of the carrier proteins in a phospholipid environment.

The apparent K_t value of proline transport in the proteoliposomes (Fig. 3) was consistent with those in whole cells [8,16] and cytoplasmic membrane vesicles [6,7]. Furthermore, the apparent turnover number in the proteoliposomes was in the same order of magnitud as that in respiring membrane vesicles, estimated from the data described in Refs. 6 and 7, suggesting that the proteoliposomes fully restored a proline transport activity as in respiring membrane vesicles. However, it should be pointed out that the relevant turnover number in whole cells [16] was about 40-fold higher than that in the proteoliposomes. The discrepancy may be partly due to technical difficulty in measuring the number of proline binding sites in whole cells or to difference in capabilities of vesicles and intact cells to maintain and regenerate $\Delta\psi$ and ΔpNa. Overath and co-workers [15,29] reported that the turnover numbers of transport via the lactose carrier in membrane vesicles and proteoliposomes were much smaller than that in intact cells, suggesting that this discrepancy was partly due to difficulty in measuring true initial rates in the vesicles with relatively small size.

The proline carrier in proteoliposomes was dependent on both $\Delta\psi$ and ΔpNa (or ΔpLi) (Fig. 2). This observation suggests the operation of a mechanism of Na^+ (or Li^+)/proline symport [8–10].

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