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Mechanism of Na^+/Proline Symport in *Escherichia coli:* Reappraisal of the Effect of Cation Binding to the Na^+/Proline Symport Carrier

Ichiro Yamato and Yasuhiro Anraku
Department of Biology, Faculty of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

Summary. The proton and sodium ion dependences of the proline binding and transport activities of the proline carrier in *Escherichia coli* were investigated in detail. The binding activity in cytoplasmic membrane vesicles from a carrier over-producing strain (PT21/*p*TMP5) was absolutely dependent on the presence of Na^+, but did not necessarily require protonation of the carrier, in contrast to the model previously reported (Mogi, T., Anraku, Y. 1984. *J. Biol. Chem.* **259**:7797–7801). Based on this and previous observations, we propose a modified model of the proline binding reaction of the proline carrier, in which a proton is supposed to be a regulatory factor for the binding activity. The apparent Michaelis constant of proline (*Kt*) of the transport activity of cytoplasmic membrane vesicles from the wild type *E. coli* strain driven by a respiratory substrate, ascorbate, showed dependence on a low concentration of sodium ion. The Michaelis constant of sodium ion for transport (Kt_{Na}) was estimated to be 25 μM. The proline transport activities in membrane vesicles and intact cells were modulated by H^+ concentration, the inhibitory effect of protons ($pK_a \approx 6$) being similar to that observed previously (Mogi, T., Anraku, Y. 1984. *J. Biol. Chem.* **259**:7802–7806). Based on these observations and the modified model of substrate binding to the proline carrier, a model of the proline/Na^+ symport mechanism is proposed, in which a proton is postulated to be a regulatory factor of the transport activity.

Key Words proline carrier · Na^+-symport · proline binding · *Escherichia coli* · transport model

Introduction

High affinity transport of proline in *E. coli* is mediated by the *putP* gene product, a major proline carrier in cytoplasmic membranes (Wood & Zadworny, 1979; Mogi et al., 1986). Proline transport has been thought to be a typical H^+/solute symport system (Hirata, Altendorf & Harold, 1973; Ramos & Kaback, 1977; Flagg & Wilson, 1978; Kaczorowski & Kaback, 1979; Bentaboulet, Robin & Kepes, 1979; Brink & Konings, 1980; Mogi & Anraku, 1984*c*), but recent studies in cells (Kayama-Gonda & Kawasaki, 1979; Stewart & Booth, 1983; Tsuchiya et al., 1984; Chen et al., 1985) and in a reconstituted membrane system (Chen & Wilson, 1986) suggested that proline transport is coupled with an electrochemical gradient of Na^+ or Li^+. Very recently, Hanada, Yamato and Anraku (1987, 1988*a,b*) purified the proline carrier to homogeneity, reconstituted it into proteoliposomes, and demonstrated definitely that it mediates electrogenic Na^+/proline symport.

The proline carrier is overproduced in a *putP* gene-amplified strain (Wood et al., 1979; Motojima et al., 1979), and the effects of Na^+ and H^+ on proline binding to the carrier in the cytoplasmic membranes from this strain have been examined extensively (Mogi & Anraku, 1984*b*). Proline binding activity was detected only in the presence of sodium ion, suggesting that proline binds only to the carrier/Na^+ complex. However, as proline uptake by cytoplasmic membranes is driven by a respiratory substrate without addition of sodium ion (Lombardi & Kaback, 1972; Mogi & Anraku, 1984*a*) and the addition of sodium ion at high concentration (>3 mM NaCl) and at low pH (<pH 6.0) inhibited the proline uptake activity in membrane vesicles (Lombardi & Kaback, 1972; Mogi & Anraku, 1984*c*), the mechanism of active proline transport has been interpreted in terms of a H^+/proline symport model.

On the basis of the recent findings that proline transport is coupled with Na^+ (Chen et al., 1985; Hanada et al., 1988*a*), the present study was undertaken to reevaluate previous experimental results and models for syncoupled transport of proline with H^+ and/or Na^+ by careful investigations on the effects of a low concentration of Na^+ and the pH dependence of the proline binding and transport activities in cytoplasmic membrane vesicles from the strain over-producing the *putP* gene product. The main problems examined were how the transport activity was stimulated by sodium ion and inhibited by a proton. Results showed that the stimulatory effect of sodium ion as a coupled cation was on the

Kt[1] values and was discernible at Na^+ concentrations of below 1 mM, in contrast to the observations by Stewart and Booth (1983) for *E. coli* proline carrier and by Cairney, Higgins and Booth (1984) for *Salmonella typhimurium* major proline permease. Based on these and previous results, especially on the re-evaluated characteristics of the proline binding activities, a kinetic model for Na^+/proline symport by the proline carrier is proposed in which the role of the proton is postulated to be as a regulator of the transport activity.

Materials and Methods

Strains and Medium

E. coli K-12 strain T184 (Teather et al., 1980) was cultured in M9 minimal salt medium supplemented with casamino acids, D,L-lactate, and succinate as described (Yamato & Rosenbusch, 1983). *E. coli* K-12 strain PT21/*p*TMP5 (Mogi et al., 1986) was cultured in DM minimal salt medium with casamino acids and glycerol as described (Hanada, Yamato & Anraku, 1985).

Membrane Preparation and Measurement of Proline Uptake Activity

Cytoplasmic membranes were obtained from a 10 liter culture as described (Yamato & Rosenbusch, 1983) and the uptake activity of proline in membrane vesicles was measured in 50 mM Tris-Mes buffer, pH 7.9, 7.1 or 6.4 containing 2 mM $MgSO_4$ at 25°C. Before use, the membranes were washed four times with 3 mM Tris-Mes buffer to remove contaminating Na^+. After addition of membranes, the reaction mixture was incubated for 3 min. Then, PMS and ascorbic acid-Tris, pH 6, were added to final concentrations of 200 μM and 5 mM, respectively, and after incubation for 15 sec, [^{14}C]proline and NaCl mixture were added to start the reaction. After incubation for 15 sec, an aliquot was taken, diluted in 2 ml of ice-cold 0.1 M LiCl, and applied to a nitrocellulose membrane filter (Sartorius, 0.45 μM). The filter was washed with 2 ml of 0.1 M LiCl, and then its radioactivity was counted. Plastic containers were used instead of glass containers in all experiments.

[1] *Abbrevations:* Mes, 2-(N-morpholino)ethanesulfonic acid; PMS, phenazine methosulfate; Kt, Michaelis constant of substrate for the transport reaction; Kt_{Na}, Michaelis constant of sodium ion during the transport reaction; K_{Na}, dissociation constant of sodium ion in the binding reaction; K_d, dissociation constant of substrate in the binding reaction; K'_s and K''_s, dissociation constants of proline from the proline/Na^+/carrier complex in the transport reaction; K_H, dissociation constant of H^+ from the H^+/carrier complex in the binding reaction; V_{max}, maximum velocity of transport; and $\Delta\phi$, membrane potential.

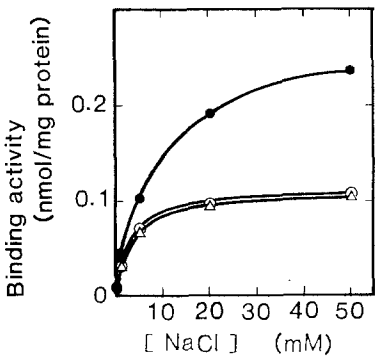

Fig. 1. NaCl concentration dependence of proline binding activity in cytoplasmic membrane vesicles at different pHs. The binding activity in cytoplasmic membrane vesicles from strain PT21/*p*TMP5 was measured by the centrifugation method as described in Materials and Methods in the presence of the indicated concentrations of NaCl at different pH values. –●–, pH 5.8; –○–, pH 7.1; –△–, pH 7.9

Determination of Sodium Ion Concentration

The sodium ion concentrations in various solutions and reaction mixtures were determined with an atomic absorption spectrometer (Perkin-Elmer 370). Protein was measured by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Assay of Binding Activity of Proline to Carriers

Specific binding of proline to carriers in cytoplasmic membrane vesicles from strain PT21/*p*TMP5 was measured by the centrifugation method described previously (Mogi & Anraku, 1984*b*). The reaction mixture (1 ml) in a centrifuge tube contained 1 mg of membrane protein, 50 mM Tris-Mes buffer (pH 5.8, 7.1 or 7.9), 1 μM L-[^{14}C]proline (290 mCi/mmol) and 0 to 100 mM NaCl. Addition of an uncoupler SF6847 or an ionophore monensin did not affect the binding activity, indicating that the binding activity of the proline carrier was measured without interference of the transport process in this assay condition (Amanuma, Itoh & Anraku, 1977; Mogi & Anraku, 1984*b*).

Measurement of Proline Uptake Activity in Intact Cells

Proline uptake activity in intact cells was determined by filtration assay as described (Motojima, Yamato & Anraku, 1978). The standard assay mixture (0.1 ml) contained about 15 μg cell protein, 2 mM $MgSO_4$, 40 μg/ml chloramphenicol, 5 mM glucose, 50 mM Tris-Mes buffer (pH 6.4, 7.1 or 7.9), 1 or 10 μM L-[^{14}C]proline, and 0 to 100 mM NaCl. After incubation at 25°C for 5 min, [^{14}C]proline was added to start the reaction. After incubation for 10 sec, the mixture was diluted in 5 ml of DM minimal salt medium and filtered on a Sartorius membrane filter. The filter was washed once with 5 ml of DM minimal salt medium and then its radioactivity was counted in a liquid scintillation counter.

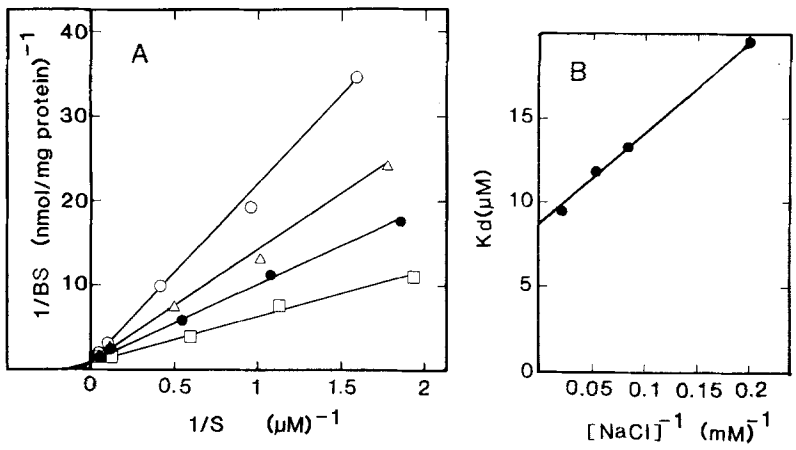

Fig. 2. Dependence of K_d on NaCl concentration. The binding activity of cytoplasmic membrane vesicles from PT21/*p*TMP5 was measured as described in Materials and Methods using various concentrations of [^{14}C]proline (from 0.5 to 100 μM) with various concentrations of NaCl (from 5 to 50 mM) at pH 7.9. (*A*) The double reciprocal plots of the binding activities *versus* free proline concentrations were obtained at various concentrations of NaCl. –○–, 5 mM; –△–, 12 mM; –●–, 15 mM; –□–, 50 mM. (*B*) The K_d values obtained from the double reciprocal plots in *A* were plotted against the reciprocals of NaCl concentrations

Chemicals

L-[^{14}C]Proline (290 mCi/mmol) was purchased from Amersham. Ascorbic acid was from Wako Pure Chem., Tokyo. Other chemicals were commercial products of analytical grade.

Results

Reappraisal of the pH dependence of the Proline Binding Activity and the Binding Model of the Carrier

We checked the results reported in a previous paper (Mogi & Anraku, 1984*b*) and found that the observed binding activities at alkaline pHs were not taken into account adequately in construction of the binding model (*cf.* Fig. 3*A*), especially that the binding activity at pH 8 (*cf.* Fig. 3 in the paper by Mogi & Anraku, 1984*b*) was too high comparing with the activities at other pHs to be consistent with the observation of Fig. 1 in the same paper (Mogi & Anraku, 1984*b*). Therefore, we re-examined the effect of pH on the binding activity, using cytoplasmic membrane vesicles from the strain in which the proline carrier is amplified (PT21/*p*TMP5) and examining the effect of Na^+ at various pHs.

Figure 1 shows the Na^+ ion dependence of the binding activity with 1 μM proline at pH 7.9, 7.1 and 5.8. Consistent with the results in Fig. 1 of the previous paper (Mogi & Anraku, 1984*b*), the binding activity at pH 8 was similar to that at pH 7. By performing similar stoichiometric measurements of proline binding at pH 7.9 (Fig. 2*A* and *B*) to those reported previously (Mogi & Anraku, 1984*b*), and taking into account the results obtained by Mogi and Anraku (1984*b*), we re-evaluated the binding model, as shown in Fig. 3*B*.

First, as shown previously (Mogi & Anraku, 1984*b*), binding of Na^+ to the carrier is absolutely necessary for the carrier to acquire proline binding ability. Second, the expression of binding activity at pH 8 (Fig. 1) suggests that the deprotonated form of the carrier in the presence of Na^+ (CNa^-) is capable of binding proline with high affinity, a fact that was not crucially taken into account in the previous model (Fig. 3*A*). Third, the increase of the binding activity at lower pH suggests that the protonation of the carrier results in higher activity of the proline binding. Thus, from Fig. 3*B*, we can easily formulate the kinetic parameters, such as the K_d, of the binding activity. The dissociation constants K_H, K_{Na} and K_S, and constants α and β are as defined (Fig. 3*B*). Then the K_d for substrate is formulated as follows:

$$K_d = \frac{1 + [H^+]/K_H + [Na^+]/K_{Na} + [H^+][Na^+]/\alpha K_H K_{Na}}{[Na^+]/K_{Na}K_S(1 + [H^+]/\alpha\,\beta\,K_H)}. \quad (1)$$

The binding parameters satisfying the results obtained in this study and the previous paper (Mogi & Anraku, 1984*b*) were estimated as follows: Fig. 1 shows that at pH 8 the effect of protonation of the carrier on the binding activity was negligible. Therefore, the terms containing $[H^+]$ in the formula for K_d can be neglected and the formula becomes a function of $[Na^+]$ alone, which is shown in Fig. 2*B*. From this result, the values for K_S and K_{Na} were estimated to be 8 μM and 5 mM, respectively. At lower pH (higher proton concentration), the binding reaction will be mainly represented by the protonated form of the carrier. Thus, the values for $\alpha\,K_{Na}$ and $\beta\,K_S$ should be the same as those obtained at low pHs in the previous study (Mogi & Anraku, 1984*b*) and α and β can be calculated as 4 and 0.08, respectively. Since the values for β and $\alpha\,\beta$ are

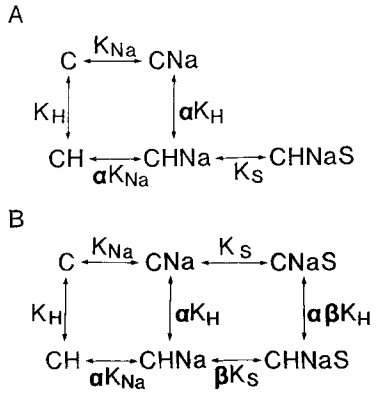

Fig. 3. Model of the binding reaction of the proline carrier. C, H, Na and S represent the carrier, proton, sodium ion and substrate, respectively. K_H, K_{Na} and K_S indicate the dissociation constants of H^+, Na^+ and substrate from carrier/H^+, carrier/Na^+ and carrier/Na^+/substrate complexes, respectively. α and β are constants. (*A*) Model of the proline binding reaction of the proline carrier reported previously (Mogi & Anraku, 1984*b*). (*B*) Model for the proline binding reaction described in this paper

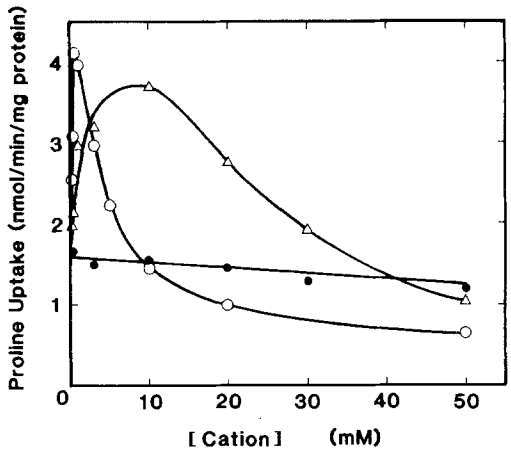

Fig. 4. Cation concentration dependence of proline transport in *E. coli* cytoplasmic membrane vesicles. The initial rate of proline uptake by cytoplasmic membrane vesicles from strain T184 was measured with or without addition of the indicated concentrations of cations at pH 7.9 as described in Materials and Methods. The concentration of radioactive proline used was 1 μM with 66 μg protein of cytoplasmic membranes in 100 μl of reaction mixture. Cation added: -○-, NaCl; -●-, KCl; -△-, LiCl

small enough, the binding reaction at about pH 6 can be approximated to that shown in the model in Fig. 3*A* by neglecting the contribution of the CNaS species. Then, the value for K_H can be calculated from the results in the previous report to be 1.2 μM as described (Mogi & Anraku, 1984*b*) when we take K_S, K_{Na}, α and β as 8 μM, 5 mM, 4 and 0.08.

This model implies that the deprotonated form of the carrier has a low but finite affinity for proline binding and that the protonated form exhibits a lower affinity for Na^+ but a higher affinity for proline. Thus the proton is supposed to act as a regulator in the binding of proline *via* the proline carrier as the Na^+/proline symporter.

Cation Dependence of Proline Transport in Membrane Vesicles

The proline transport in intact cells was shown to be stimulated in the presence of NaCl or LiCl (Kayama-Gonda & Kawasaki, 1979; Stewart & Booth, 1983). However, the proline transport activity in the cytoplasmic membrane vesicles was inhibited by the presence of more than 3 mM NaCl, which on the other hand, stimulated proline binding to the carrier (Mogi & Anraku, 1984*b*,*c*, also Fig. 1). Therefore, we examined the effect of sodium ion at below 3 mM on the proline uptake activity in cytoplasmic membrane vesicles from the wild type *E. coli* strain.

As shown in Fig. 4, the addition of NaCl or LiCl stimulated the uptake activity while addition of KCl had no effect. Without added NaCl or LiCl, about half the maximum uptake activity was detected, and this was shown by atomic absorption analysis of Na^+ to be due eventually to contaminating sodium ion (about 15 μM) in the reaction mixture. The major contamination came from 5 mM ascorbic acid-Tris and the membrane preparation, which were found to contribute about 5 and 4 μM, respectively.

This result led us to the conclusion that cations (Na^+ or Li^+) which stimulate the proline binding activity of the carrier also stimulate the uptake activity within a limited range of concentration. This conclusion is consistent with the notion that proline is symported with sodium ion in intact cells (Stewart & Booth, 1983; Chen et al., 1985).

Inhibition of Proline Transport in Membrane Vesicles and Intact Cells by H^+ and Na^+ Ions

Mogi and Anraku (1984*c*) observed inhibition of proline transport in membrane vesciles by high concentration of H^+ and Na^+. To explain this inhibition and the results described in the previous section, we re-examined the inhibitory effects of H^+ and Na^+ in detail.

As shown in Fig. 5, the proline uptake activities in membrane vesicles and intact cells were inhibited progressively by increase in H^+ concentration. This suggests that H^+, which affects the binding activity, inhibits the translocation reaction from outside, because intact cells are known to maintain their inter-

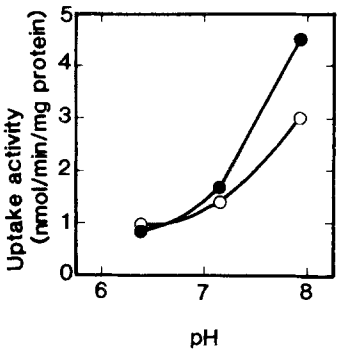

Fig. 5. Inhibition by H^+ of proline uptake activity in cytoplasmic membrane vesicles and intact cells. Kinetic experiments using cytoplasmic membrane vesicles from strain T184 as in Fig. 7*A* were performed at pH 7.9, 7.1 and 6.4. The initial rates of uptake of different concentrations of proline in the presence of various concentrations of NaCl in cytoplasmic membrane vesicles from strain T184 were measured as described in Materials and Methods. From double reciprocal plots of the uptake rates *versus* proline concentrations, the V_{max} values were obtained at pH 7.9, 7.1 and 6.4 (–●–). The uptake activity in intact cells of strain T184 was measured as described in Materials and Methods with radioactive proline at a concentration of 10 μM in the presence of 40 mM NaCl (–○–)

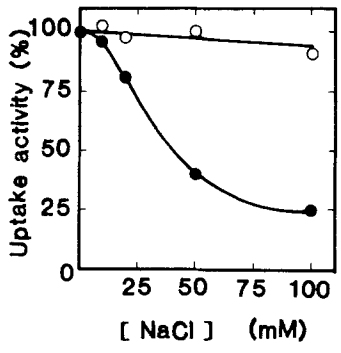

Fig. 6. Effect of NaCl on proline uptake activity by cytoplasmic membrane vesicles and intact cells. Proline uptake activity in cytoplasmic membrane vesicles from strain T184 was measured at pH 7.9 as described in Materials and Methods and in the legend to Fig. 4 in the presence of different concentrations of NaCl (–●–). The activity in intact cells of strain T184 was measured at pH 7.9 as described in the legend to Fig. 5 in the presence of 1 μM [^{14}C]proline and the indicated concentrations of NaCl (–○–). The uptake activity in intact cells without addition of NaCl was 1.2 nmol/min/mg protein

nal pH at about 7.5 and the electrochemical proton gradient across the membrane is thought to be constant at about this pH (Mogi & Anraku, 1984*a*,*c*). From this observation, we concluded that the proton was not a coupling cation, but rather that it played a regulatory role in the transport reaction.

In contrast, as shown in Fig. 6, Na^+ which inhibited the uptake activity in membrane vesicles did not affect the activity in intact cells. This indicates that the inhibition by Na^+ in membrane vesicles may be due to the sodium ion inside vesicles. The concentration of Na^+ for half inhibition seemed to be about 15 mM, which coincided with the value reported previously (Mogi & Anraku, 1984*c*). This inhibitory activity of Na^+ inside the vesicles is well explained by the concept of product inhibition in the Na^+/proline symport reaction (Stewart & Booth, 1983).

Sodium Ion Concentration Dependence of Kinetic Parameters of the Uptake Activity

Proline transport activity was examined kinetically in the presence of various concentrations of NaCl. Double reciprocal plots of the proline uptake activities at pH 7.9 are shown in Fig. 7*A*. The results clearly show that sodium ion affects the *Kt* values of proline transport rather than the V_{max} values, consistent with the proline binding properties of the carrier in membrane vesicles (Mogi & Anraku, 1984*b*). Assuming that a carrier/Na^+ binary complex can bind proline and that the resultant carrier/Na^+/proline ternary complex can carry out the symport reaction, the Kt_{Na} for Na^+ during the transport reaction is deducible from the plot of *Kt* values *versus* sodium ion concentrations (Fig. 7*B*) as described previously for glutamate/Na^+ symport (Fujimura, Yamato & Anraku, 1983*a*,*b*). From the data in Fig. 7*B*, the Kt_{Na} was calculated to be 25 μM, which is in good agreement with the value obtained for proline transport in intact cells (Chen et al., 1985) and which is quite small (about 1/100) compared with the value for the K_{Na} of the proline binding reaction in membrane vesicles (Mogi & Anraku, 1984*b*). In contrast, the *Kt* values for substrate transport are comparable with the K_d values for the substrate binding reaction (Mogi & Anraku, 1984*b*).

Discussion

In a detailed study of the binding reaction of the proline carrier, Mogi and Anraku (1984*b*) postulated that the binding of a proton was essential for the carrier to bind proline (Fig. 3*A*). However, they did not take the observed binding activity at pH 8 (Fig. 1 in the paper by Mogi & Anraku, 1984*b*) into account in their construction of their model (Fig. 3*A*). Furthermore, the results of Fig. 3 in the same paper by Mogi and Anraku (1984*b*) were inconsistent with their model and the results (Figs. 1 and 4

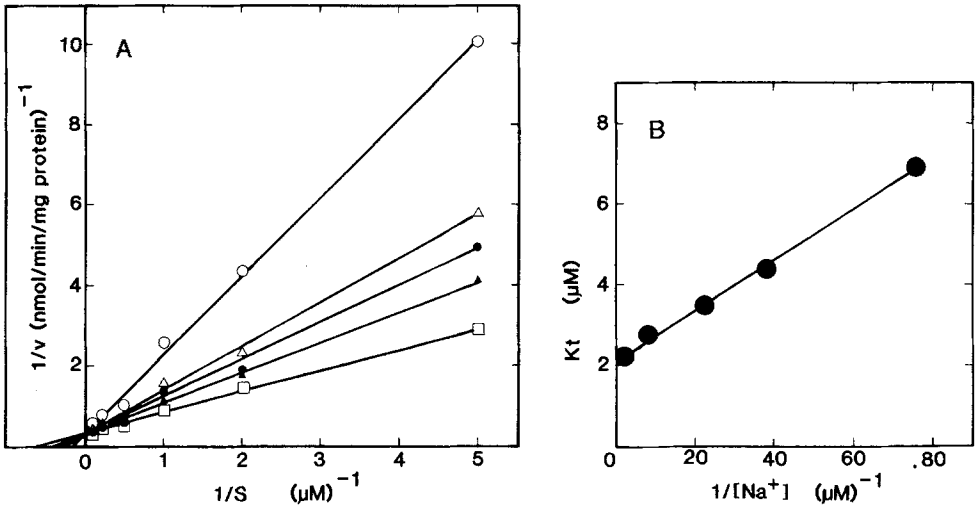

Fig. 7. Kinetics of proline uptake at various concentrations of sodium ion. The initial rates of uptake of different concentrations of proline in the presence of various concentrations of NaCl by cytoplasmic membrane vesicles from strain T184 were measured at pH 7.9 as described in Materials and Methods. The concentration of sodium ion in each reaction mixture was determined by atomic absorption analysis; –○–, 13 μM; –△–, 28 μM; –●–, 42 μM; –▲–, 110 μM; –□–, 500 μM. (*A*) The double reciprocal plots of uptake rates *versus* proline concentrations are shown. (*B*) The *Kt* values at various sodium ion concentrations are plotted against the reciprocals of sodium ion concentrations

in the paper by Mogi & Anraku, 1984*b*). Therefore, we re-examined the binding activity at high pH carefully in this study (Fig. 2). At present, we do not know the reason why Mogi and Anraku (1984*b*) obtained such high activities at pH 8 as shown in Fig. 3 in their paper. The binding activities at pH 5.8 and 6.6 were similar to those reported by Mogi and Anraku (1984*b*) (*data not shown*). In the control experiments for the binding measurements, the addition of an uncoupler SF6847 or an ionophore monensin did not affect the observed activities as reported previously (Amanuma et al., 1977; Mogi & Anraku, 1984*b*), indicating that the binding activity was specifically measured without the interference of the transport process in this assay condition.

The present results (Figs. 1 and 2) imply that the deprotonated form of the carrier can still bind Na^+ and proline in an ordered manner with lower affinity, suggesting that a proton is not essential for the binding reaction. Therefore, the binding properties are now best represented by the model in Fig. 3*B*, in which the carrier first binds Na^+ and then the resulting binary complex binds the substrate with high affinity, and every species of carrier can be protonated, resulting in decrease in affinity for Na^+ and increase in affinity for proline. Moreover, the protonated carriers are unable to translocate the substrate across the membrane, as shown in the present study by the inhibitory effect of protons on the transport reaction (Fig. 5). This suggests that proton affecting the binding activity is not involved in the transport reaction and rather that it regulates the transport reaction by changing the V_{max} value of the carrier cycle.

As shown in Figs. 4 and 7, we observed that Na^+ at very low concentrations (<1 mM) stimulated active transport by decreasing the *Kt* for proline, which is consistent with the Na^+-symport mechanism. We consider that this transport property of the carrier coincides well with the binding property of the carrier (Fig. 3*B*) assuming that the Na^+ affecting the binding is the coupling Na^+. A similar molecular mechanism has been proposed for glutamate transport (Fujimura et al., 1983*a*,*b*). Thus we postulate that the transport model of the proline carrier is as shown in Fig. 8, where only the deprotonated form of the carrier is thought to have transport activity. In Fig. 8, the binding reactions at the surfaces of the membrane are postulated to be according to the model shown in Fig. 3*B*: Na^+ binds to the carrier forming an Na^+/carrier binary complex, and only this binary complex can bind proline. The unloaded carrier and the Na^+/proline/carrier ternary complex can be translocated across the membrane either inward or outward, and so that transport cycle can operate in reverse. Our finding that Na^+ affects the *Kt* of proline transport is not consistent with the observation by Stewart and Booth (1983) for *E. coli* proline carrier and by Cairney et al. (1984) for *Salmonella typhimurium* proline permease; they reported that in intact cells, Na^+ in the external medium increased the V_{max} of entry with-

out affecting the Kt. However, the Na^+ concentration they used was to high to detect the dependence of Kt on the cosubstrate concentration as will be easily calculated from the data in Fig. 7. And the dependence of V_{max} of proline transport activities on Na^+ concentration that they observed (Stewart & Booth, 1983; Cairney et al., 1984) may be due to the disturbance of the sodium ion electrochemical potential in cells by the different concentrations of Na^+ outside of the cells.

The failure to detect the stimulatory effect of sodium ion on the uptake activity in the previous study (Mogi & Anraku, 1984*c*) was partly due to the high contamination of the reaction mixture with sodium ion owing to the use of 20 mM ascorbic acid-Tris, and partly to the assay procedure in which the membranes were preincubated with sodium ion and then the reaction was started by adding labeled proline. In fact, as suggested by Stewart and Booth (1983), a higher concentration of NaCl inside vesicles would inhibit the transport cycle by product inhibition. In this work this inhibition was in fact observed with membrane vesicles (Fig. 6).

In the model shown in Fig. 8, proline is postulated to be symported with Na^+, where binding reactions at the surfaces of the membrane are in local equilibrium. Then the initial velocity of transport (v) is formulated as:

$$\frac{1}{v} = \frac{k_{-c} + k_s + k_s Na_i/K''_{Na} + K'_S K'_{Na} (k_c + k_{-c} + k_{-c}Na_o/K'_{Na} + k_c Na_i/K''_{Na})/Na_o S_o}{k_{-c}k_s Ct} \quad (2)$$

Here, Na_i, and Na_o and S_o represent the concentrations of Na^+ inside and outside the vesicles and of substrate outside, respectively, and Ct represents the total concentration of carrier. From this formulation, Kt and V_{max} are derivated as:

$$Kt = \frac{K'_S K'_{Na}\{(k_c + k_{-c})K''_{Na} + k_{-c}Na_o K''_{Na}/K'_{Na} + k_c Na_i\}}{(k_{-c} + k_s)K''_{Na}Na_o + k_s Na_i Na_o} \quad (3)$$

$$V_{max} = \frac{k_{-c}k_s Ct}{k_{-c} + k_s + k_s Na_i/K''_{Na}}. \quad (4)$$

From the formulation of Kt, the Kt value at infinite concentration of Na_o and zero concentration of Na_i can be expressed as $K'_S k_{-c}/(k_{-c} + k_s)$, and this was estimated to be 2 μM from the data in Fig. 7*B*. This $Kt(Na_o \to \infty)$ value was in the same order of magnitude as the K_S value for the substrate binding reaction (8 μM). Therefore, we suppose that the K_S well

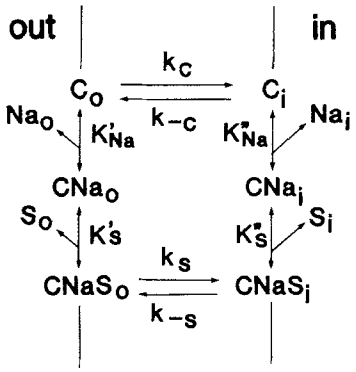

Fig. 8. Model for the transport reaction of the proline carrier. C, Na and S represent the carrier, sodium ion and substrate, respectively. Subscripts o and i represent species outside and inside the membrane. K'_{Na}, K''_{Na}, K'_S and K''_S indicate the dissociation constants of sodium ion and substrate for the transport reaction at the outer and inner surfaces of the membrane, as shown in the figure. k_c, k_{-c}, k_s and k_{-s} are the translocation rate constants for the forward and backward reaction of the unloaded carrier and carrier/Na^+/substrate complex

represents the K'_S for the transport reaction. The Kt_{Na} can be expressed as $(k_{-c} + k_c)K'_{Na}/k_{-c}$ assuming that Na_i is zero, and this Kt_{Na} value (an approximate value for K'_{Na}) was estimated to be 25 μM from the data in Fig. 7*B*. However, the dissociation constant of Na^+ in the binding reaction (K_{Na} in Fig. 3*B*) was about 5 mM, which was about 200 times more than the Kt_{Na} value. The discrepancy between the values of Kt_{Na} for the transport reaction and K_{Na} for the binding reaction (Mogi & Anraku, 1984*b*) may be due to (*i*) conformational change of the carrier by $\Delta\phi$, as suggested in the case of the lactose carrier (Wright, Seckler & Overath, 1986); (*ii*) a kinetical effect due to different rate-limiting steps other than the translocation steps (Sanders et al., 1984), or (*iii*) functional asymmetry of the carrier. We consider that the third possibility is the most likely. Evidence in support of this possibility is as follows: (*i*) From the formulation for V_{max}, the concentration of Na^+ for half inhibition of the transport reaction can be expressed as $(k_{-c} + k_s)K''_{Na}/k_s$, which can be approximated to be in the order of K''_{Na}. (*ii*) We observed that this concentration of Na^+ for half inhibition of the transport reaction was about 15 mM (Fig. 6; *see also* Mogi & Anraku, 1984*c*). (*iii*) The measured value of K_{Na} in the binding reaction should be the average of the values of K'_{Na} and K''_{Na}. In fact, the observed K_{Na} value was 5 mM, which was intermediate between these two values.

Recently another asymmetrical carrier model, that of the melibiose carrier, has been proposed (Bassilana, Pourcher & Leblanc, 1987, 1988). This model was based on detailed study of influx and

efflux activities in active transport and facilitated diffusion modes of the carrier. In the case of the proline carrier, it was difficult to measure facilitated transport activity quantitatively. Therefore, we did not take a mechanistical asymmetrical model of the carrier cycle into account, but instead, based on binding studies, we constructed a classical model with a minor deviation; asymmetry of the binding constants (K'_{Na} and K''_{Na}) with the same mechanistical mode of the carrier cycle. Further studies of the transport and binding properties of the proline carrier in proteoliposomes suitably reconstituted with purified carrier and in definite orientation are necessary, first of all to demonstrate the concomitant movement of proline and Na^+ as a direct evidence for the proline/Na^+ symport and secondly to substantiate the possibilities of the transport mechanism discussed here.

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