PROTON-DEPENDENT BINDING OF PROLINE TO CARRIER IN *ESCHERICHIA COLI* MEMBRANE

Hiroshi AMANUMA, Jindow ITOH and Yasuhiro ANRAKU
Department of Botany, Faculty of Science, University of Tokyo, Bunkyo-Ku, Tokyo 113, Japan

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1. Introduction

Hirata et al. [1] showed that membrane vesicles from *Escherichia coli* took up proline actively on generation of an artificial membrane potential (negative inside). Recently we solubilized a proline carrier from *E. coli* membranes with acidic *n*-butanol, and found that it could catalyze stereospecific uphill uptake of proline when reconstituted into vesicles with *E. coli* phospholipids and supplied with energy in the form of a membrane potential (negative inside) [2]. These findings and observations on intact cells [3,4] strongly support the idea that the driving force for active transport of proline is an electrochemical gradient of protons across the membranes and that the uptake occurs via a proton-symport carrier [5].

Thus the proline carrier should interact directly with protons before and/or during translocation of proline across the membranes. It therefore seemed interesting to examine the effect of pH on the binding of proline to the carrier, avoiding the complication of uptake by using sonicated membranes of *E. coli*. This paper shows that binding of proline to the carrier occurred without a supply of energy, but that it was greatly affected by the pH of the medium. These findings can be explained by assuming formation of a ternary proline–proton-carrier complex.

2. Materials and methods

2.1. *Materials*

E. coli K12 strain W1-1 (Leu$^-$) was used unless otherwise stated. Strain JE2133 (*proA*), and its proline transport defective mutants PT21 and PT22 [2] were also used. Cells were grown, harvested and stored as described previously [2]. Uniformly labeled L-[^{14}C] proline (290 mCi/mmol) and L-[^{14}C]serine (130 mCi/mmol) were purchased from the Radiochemical Centre, Amersham. D-Lactate (lithium salt) was from Calbiochem. SF6847 (3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile) [6,7] was kindly supplied by Dr Y. Nishizawa, Sumitomo Chemicals, Osaka.

2.2. *Preparation of the sonicated membranes*

Sonicated membranes were prepared as described previously [2]. Washed membranes were suspended in buffer A (10 mM Tris–HCl, pH 7.3, 2.5 mM EDTA, 2.9 mM β-mercaptoethanol) at 40–50 mg protein/ml and either used immediately for binding experiments or stored at 4°C. For experiments on the effects of sulfhydryl reagents the washed membranes were washed once more and suspended in buffer A without β-mercaptoethanol. Protein was determined by the method of Lowry et al. [8] with bovine serum albumin as a standard.

2.3. *Assay of the binding of proline to carrier*

Binding of proline to the carrier in the membranes was measured by the centrifugation method originally developed by Kennedy et al. [9] for measuring binding of sugar to *lac* carrier M protein.

The assay mixture in the sample tube (1 ml total in a centrifuge tube) consisted of sonicated membranes (about 10 mg protein) and [^{14}C]proline (final concentration, 0.05–15 μM) in buffer of the indicated pH containing 2.5 mM EDTA and 2.9 mM β-mercaptoethanol. Other additions were as indicated in the text.

The control tube contained 1 mM of unlabeled L-proline in addition to the constituents of the sample tube. After incubation at 18°C for 20 min, the membranes were precipitated by centrifugation for 20 min at 145 000 X *g* and 17–20°C. Aliquots of the supernatant were mixed with 10 ml of Triton–toluene liquid scintillator [10] and counted. Then the rest of the supernatant was removed, and the inner surface of the tube was carefully wiped without disturbing the precipitate. The precipitate was dissolved in 1 ml 5% Triton X-100 and counted. From the difference in the radioactivities of the precipitates in the sample and control tubes, the amount of proline bound to the saturable binding sites of carrier in the membranes was calculated.

Various control experiments were done to verify this method for assay of binding using buffers of pH 7.0 and pH 5.3 and 0.1 μM [^{14}C]proline and 0.2 μM [^{14}C]serine as follows:

(i) When $^{32}P_i$ was added to the assay mixtures together with [^{14}C]proline and centrifuged, the amount of $^{32}P_i$ in the precipitate in the control tube was equal to that of the precipitate in the sample tube, indicating that the volume of the precipitate was not changed by 1 mM unlabeled proline.

(ii) The binding was not affected by the time (15–90 min) or temperature (0°C and 25°C) of incubation.

(iii) The binding was not stimulated by D-lactate (20 mM), or inhibited by the potent proton conducting uncoupler SF6847 (5 μM) [6,7], indicating that the binding was not the result of energy-dependent uptake of proline into the membrane vesicles.

(iv) The binding was inhibited by DL-3,4-dehydroproline (2 mM), but not by L-isoleucine (1 mM), as expected from the specificity of proline transport in intact cells and isolated membrane vesicles [11,12].

(v) The binding was almost completely blocked by preincubating the membranes for 10 min with 0.2 mM of either *p*-chloromercuribenzoate, *N*-ethylmaleimide or $HgCl_2$ before adding [^{14}C]proline and unlabeled proline. However, it was not inhibited by $ZnCl_2$ (0.2 mM), which inhibits proline transport by blocking oxidation of respiratory substrates [11].

(vi) The binding was not observed with membranes from proline transport defective mutants, although these membranes bound almost as much [^{14}C]serine (1.1 ~ 1.4 pmol/mg protein at pH 7.0) as membranes from the parent cells.

All these results clearly indicated that the method can be used for measuring specific binding of proline to the carrier in the membranes.

3. Results and discussion

3.1. *pH-Dependent binding of proline to the carrier*

Binding of proline (0.1 μM) to the carrier in the membranes was measured as described in Materials and methods using buffers of different pH-values. Figure 1 shows that the binding had a sharp pH-optimum at about pH 5.3 and that it scarcely occurred at pH 4.3, pH 7.8 or pH 8.9.

The effect of pH on the binding of proline was next analyzed kinetically. Figure 2 shows double reciprocal plots of the bindings at pH 7.0, pH 6.2 and pH 5.3. At each pH the values fell on a straight line and extrapolation of the three lines to the ordinate gave the same value of 9.4 pmol proline bound/mg protein for the maximum amount of proline bound to the carrier. On the other hand, the dissociation constant (K_d) of the proline–carrier complex was significantly affected by the pH of the assay medium; the K_d decreased from 10 μM at pH 7.0, through 1.6 μM at pH 6.2, to 0.50 μM at pH 5.3.

3.2. *Mechanism of interaction between proton, proline and the carrier*

Since proline exists predominantly as a dipolar ion between pH 5.3 and pH 7.0, it is unlikely that the sharp pH-dependence of binding is due to change of

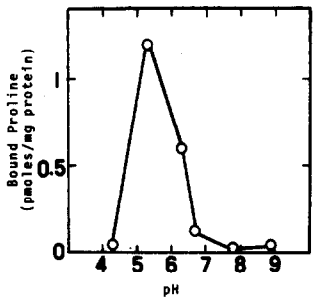

Fig.1. Effect of pH on the binding of proline to the carrier. Binding of [^{14}C]proline (0.1 μM) was determined as described in Materials and methods. The buffers (0.05 M) used were Tris–HCl (pH 8.9, pH 7.8 and pH 6.7), sodium phosphate (pH 6.3) and sodium acetate (pH 5.3 and pH 4.3).

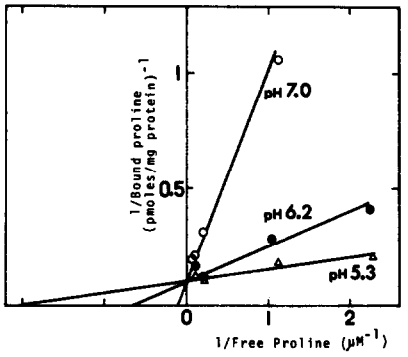

Fig.2. Kinetics of binding of proline to the carrier at various pH-values. At the pH-values indicated, binding of proline was measured as described in the Materials and methods using various concentrations of [^{14}C]proline. Concentrations of free proline were calculated from the radioactivities of the supernatants of the sample tubes. The buffers (0.05 M) used were Tris–HCl (pH 7.0), sodium phosphate (pH 6.2) and sodium acetate (pH 5.3).

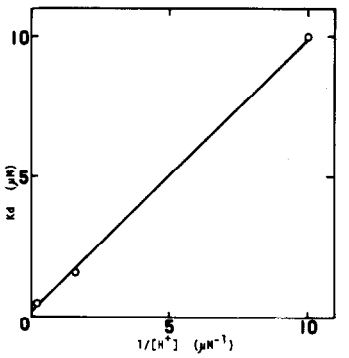

Fig.3. Linear relation between the observed dissociation constants (K_d) of the proline–carrier complex and $1/[H^+]$. The values of K_d are those determined by extrapolation of the lines in fig.2 and are plotted against the reciprocals of the proton concentrations at which the binding measurements were done. For details, see text.

its ionic form. Thus, protons must affect the binding of proline to the carrier by reacting directly with a particular site(s) of the carrier. This means that protons are co-substrates of the carrier and that the binding of proline is affected significantly by the binding of protons to the carrier. The simplest mechanism for this type of binding reaction is as follows:

$$C + H^+ \xleftrightarrow{K_1} CH^+$$
$$CH^+ + S \xleftrightarrow{K_2} CH^+S \qquad (1)$$

where C and S represent the carrier and the substrate, respectively and K_1 and K_2 are dissociation constants.

From eq. (1) the amount of proline bound to the carrier (BS) can be described as follows:

$$BS = CH^+S = \frac{C_t[S]}{K_2 + K_1K_2/[H^+] + [S]} \qquad (2)$$

where C_t is the total amount of carrier and $[S]$ the concentration of free substrate. Equation (2) indicates that at each pH a plot of $1/BS$ versus $1/[S]$ is linear and that the BS_{max} is equal to C_t and independent of pH.

In eq. (2) the term $K_2 + K_1K_2/[H^+]$ corresponds to the apparent dissociation constant. Thus there should be a linear relationship between the observed K_d-values and $1/[H^+]$. Figure 3 shows that this is the case and that K_1 and K_2 are 4.8 μM and 0.2 μM, respectively.

These results clearly indicate that the observed pH-dependence of binding of proline to the carrier can be explained quantitatively by the mechanism of eq. (1). The calculated value for K_1 of 4.8 μM suggests that some residue with a pK-value of 5.3, possibly a γ-carboxylic group of glutamic acid or the imidazole ring of a histidine in the carrier molecule, participates in binding of a proton which results in a binding site for the substrate with a dissociation constant of 0.2 μM. The reason why no binding could be detected at pH 4.3 (fig.1) may be that pH has some nonspecific effect on the carrier.

The mechanism of binding of substrate to carrier postulated in eq. (1) is useful for analyzing the mechanism of substrate–proton symport. Equation (1) indicates that the carrier activity in binding of the substrate is regulated by the concentration of protons around the carrier molecule. This means that the proportion of carrier having a high affinity for substrate differs at different surfaces of the membranes, depending on the local pH-difference across the membranes. Further studies are in progress on these effects of protons on the carrier, which may lead to establishment of a concentration gradient of proline.

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