Volume 256, number 1,2, 135–138 FEB 07683 October 1989

Orientation of the carboxyl terminus of the Na^+/proline symport carrier in *Escherichia coli*

Yuto Komeiji, Kentaro Hanada*, Ichiro Yamato+ and Yasuhiro Anraku

Department of Biology, Faculty of Science, University of Tokyo, Hongo, Tokyo 113, Japan

Received 17 August 1989

The orientation of the carboxyl terminal region of the *Escherichia coli* proline carrier in the cytoplasmic membrane was studied. The β-galactosidase moiety of the *PutP-LacZ* fusion protein [(1987) J. Biol. Chem. 262, 14100–14104] was exposed outside the inside-out vesicles and inside the right-side-out vesicles. A site-directed antibody raised against a synthetic peptide corresponding to the putative carboxyl terminal region of the carrier reacted preferentially with the inside-out vesicles prepared from a wild-type proline carrier overproducing strain and less with the right-side-out vesicles. These results indicate that the carboxyl terminus of the proline carrier is exposed to the cytoplasmic side of the membrane.

Proline carrier, orientation; Carboxyl terminus; Fusion protein, *PutP-LacZ*; Antibody, site-directed; ELISA, competitive

1. INTRODUCTION

The high-affinity transport of proline in *E. coli* is mediated by a major proline carrier identified as an integral protein in the cytoplasmic membrane [1]. This is a typical secondary active transport system which couples a downhill movement of Na^+ (or Li^+) to an uphill movement of proline across the membrane [2–5]. Also, the carrier binds proline in an H^+- and Na^+ (or Li^+)-dependent manner under non-energized conditions [6,7]. The proline carrier is encoded by the *putP* gene [8]. The nucleotide sequence of the *putP* gene was determined, and the deduced amino acid sequence indicated that the carrier consists of a single polypeptide with an M_r of 54343 and may contain 12 membrane spanning segments [9].

The proline carrier was purified by taking advantage of a site-cleavable fusion protein of proline carrier and β-galactosidase linked through a collagen linker (*PutP-LacZ* fusion protein) in a functional form [5,10]. The β-galactosidase activity of the fusion protein was cryptic in intact cells and could be detected upon permeabilization, suggesting that the carboxyl terminus of the proline carrier is exposed to the cytoplasmic side of the membrane [10]. However, a possible mislocation of the carboxyl terminus due to the bulky β-galactosidase moiety could still be argued. In this study we demonstrated the orientation of the carboxyl terminus of the wild-type proline carrier by an immunochemical approach.

Correspondence address: Y. Anraku, Department of Biology, Faculty of Science, University of Tokyo, Hongo, Tokyo 113, Japan

* *Present address:* Department of Chemistry, National Institute of Health, Kamiosaki, Tokyo 141, Japan

\+ *Present address:* Department of Biological Science and Technology, Science University of Tokyo, Yamasaki, Chiba 278, Japan

Abbreviations: RSOV, right-side-out vesicles; ISOV, inside-out vesicles; oNPG, *o*-nitrophenyl β-D-galactopyranoside; Ct, carboxyl terminus; KLH, keyhole limpet hemocyanin; IgG, immunoglobulin; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; BS_{max}, number of maximum binding sites; K_d, dissociation constant

2. EXPERIMENTAL

2.1. *Bacterial strains and preparation of membrane vesicles*

ST3019, a *putP*$^-$ strain, and ST3015, a proline carrier over-

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

producing strain, were grown as described [1]. ST3039, which produces the collagen-linked *PutP-LacZ* fusion protein, was grown as described [10]. Cells were harvested at late log phase. RSOV [11] and ISOV [12] were prepared and stored at −80°C until use. A syringe filled with a 20-gauge needle was used to suspend the vesicles during preparation. The amount of the proline carrier in each vesicle preparation from ST3015 was estimated by proline binding assay as described [10].

2.2. *Orientation of the* PutP-LacZ *fusion protein*

The orientation of the β-galactosidase moiety of the *PutP-LacZ* fusion protein was examined by using RSOV and ISOV from ST3039 essentially as described [10]. The suspension of RSOV or ISOV (0.25 mg protein) in 0.5 ml 25 mM Tris-HCl (pH 7.5) containing 2.5 mM $CaCl_2$ was treated with 5 units of *Clostridium histolyticum* collagenase (Sigma) to release the β-galactosidase moiety exposed outside the vesicles. The reaction was stopped with 6 mM EGTA, and the reaction mixture was centrifuged at 360000 × *g* for 20 min. The precipitate was resuspended in 0.5 ml 25 mM Tris-HCl (pH 7.5) and permeabilized with 0.01% of dodecyl-β-D-maltoside (Calbiochem-Behring). Then β-galactosidase activities of the supernatant and the precipitate were measured with oNPG as substrate.

2.3. *Preparation of anti-Ct-antibodies*

A synthetic peptide corresponding to the putative Ct (YHSAPPSRLQES) of the proline carrier with a cysteine residue added to the amino terminus and the Ct-peptide conjugated to KLH were kindly supplied by Fujiya Co. Ltd. (Tokyo). The conjugate (0.3 mg) was emulsified with Freund's complete adjuvant (Wako Pure Chemicals) and injected into lymph nodes of male Japanese white rabbits [13]. They received intradermal booster injection of the conjugate (0.8 mg) emulsified with Freund's incomplete adjuvant (Wako Pure Chemicals) every 10 days for 5–10 times and bled. Immunoglobulin (IgG) fraction was precipitated from the antisera with ammonium sulfate and affinity-purified with immunosorbent prepared by conjugation of the Ct-peptide to activated CH-Sepharose 4B (Pharmacia). The anti-Ct-IgG obtained was concentrated by ultrafiltration (Centricon 30, Amicon) and dialyzed against 0.15 M PBS [14].

2.4. *Assay of antibodies*

The antisera and purified IgG were tested by dot-blot and Western blot analyses. The Ct-peptide and Ct-KLH conjugate were spotted on nitrocellulose membrane filters for dot-blot analysis. The membrane proteins in RSOV or ISOV from ST3015 or ST3019 were blotted onto a nitrocellulose membrane filter [15] in the presence of 0.05% SDS for Western blot analysis. Then ELISA was performed with alkaline phosphatase-conjugated anti-rabbit (Fc) IgG according to the instruction of the manufacturer (ProtoBlot immunoscreening system, Promega).

2.5. *Determination of orientation of Ct by competitive ELISA*

The purified anti-Ct-IgG was biotinylated as described [16]. Disrupted vesicles were prepared by two cycles of freeze-thaw of vesicles (1 mg/ml of protein) in 50 mM KOH containing 5 mM EDTA; then the pH was neutralized with HCl. Serial dilutions of intact and disrupted vesicles from ST3015 or ST3019 in PBS containing 0.1% BSA were preincubated with 0.3 μg/ml of the biotinylated IgG for 1 h at 37°C. Each well of a microtiter plate (Immulon 2, Dinatec laboratories) was coated with 50 μl of the Ct peptide (1 μg/ml in 50 mM sodium carbonate, pH 9.6) for overnight at 4°C and blocked by incubation with 10% skimmed milk–PBS for 1 h at 37°C. After washing with PBS, each well was incubated with 50 μl of the mixture of the IgG and the vesicles for 1 h at 37°C. The IgG bound to each well was assayed as described [16] with avidine-peroxidase conjugate (Vector). Absorbance at 492 nm was determined in Chromo Scan ELISA reader (MTP32, Corona). There was a linearity between the amount of the IgG used below 0.3 μg/ml and the resultant coloration.

2.6. *Protein measurement*

IgG was determined by measurement of absorbance at 280 nm. Other proteins were measured as described [17] with BSA as a standard.

3. RESULTS AND DISCUSSION

3.1. *Orientation of the* PutP-LacZ *fusion protein*

The orientation of the *PutP-LacZ* fusion protein was determined taking advantage of the cleavability of the collagen linker. As in table 1, 84% of the β-galactosidase activity of the fusion protein in ISOV was released by collagenase from ISOV but only 14% of that was released from RSOV, indicating that the β-galactosidase moiety resides in the cytoplasmic side of the membrane. This finding was consistent with the previous observation that the β-galactosidase activity was cryptic in the cell [10]. It also suggested that the fusion protein seldom undergoes dislocation during vesicle formation.

3.2. *Specificity of the antibodies*

The antisera and the purified anti-Ct-IgG cross reacted with the Ct-peptide and the Ct-peptide-

Table 1

Localization of β-galactosidase activity of the fusion protein in membrane vesicles

Membrane vesicles	β-Galactosidase activity (nmol oNPG hydrolyzed/fraction^{-1} per min^{-1})		Ratio sup:ppt)
	Supernatant	Precipitate	
RSOV	370	2280	14:86
ISOV	1430	280	84:16

The vesicles from ST3039 were treated with collagenase and centrifuged. The β-galactosidase activities of the supernatant and the precipitate (permeabilized) were measured. See section 2 for details

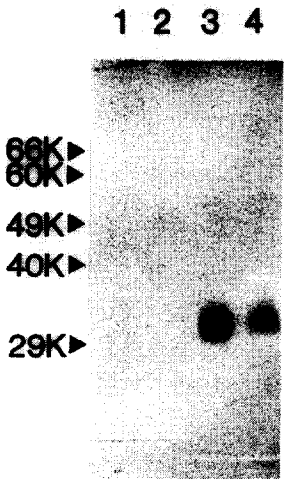

Fig.1. The specificity of the affinity-purified anti-Ct-IgG. Assays were made by Western blotting: lane 1, ST3019 RSOV; lane 2, ST3019 ISOV; lane 3, ST3015 RSOV; lane 4, ST3015 ISOV. Each lane contained 10 μg protein. Wild-type proline carrier shows an apparent M_r of 32000 on 10% acrylamide gel [1].

KLH conjugate on dot-blot analysis (data not shown). The affinity purified anti-Ct-IgG was highly specific to the proline carrier in the membrane from ST3015 and did not react with proteins from ST3019 (fig.1).

The fact that the antibody raised against the synthetic peptide reacted with the proline carrier both in denatured state and, as mentioned later, in membrane-bound state suggests that the carboxyl terminal region of the carrier takes a relaxed conformation similar to that of the synthetic peptide. It is consistent with the previous observation that the bulky β-galactosidase moiety attached to the proline carrier had no effect on transport or binding activity of the carrier [10].

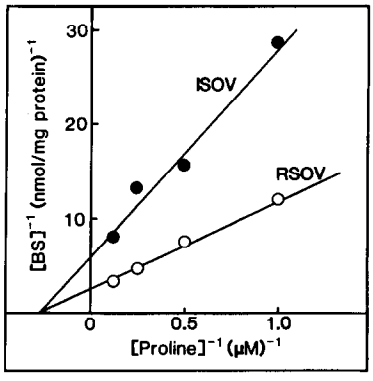

Fig.2. Double reciprocal plots of proline binding to membrane vesicles. Proline binding activity of vesicles prepared from ST3015 was assayed by a centrifugation method. BS_{max} for proline of each preparation was estimated: 0.40 for RSOV and 0.17 nmol/mg of protein for ISOV.

3.3. *Proline binding assay of the vesicles*

The amounts of proline carrier in RSOV and ISOV from ST3015 were estimated from the numbers of BS_{max} for proline (legend to fig.2). The difference in the amounts of proline carrier between the two vesicle preparations was also seen on Western blot (fig.1, lanes 3 and 4). This must be due to a relative content of outer membrane, since RSOV were partially purified by isopycnic sucrose

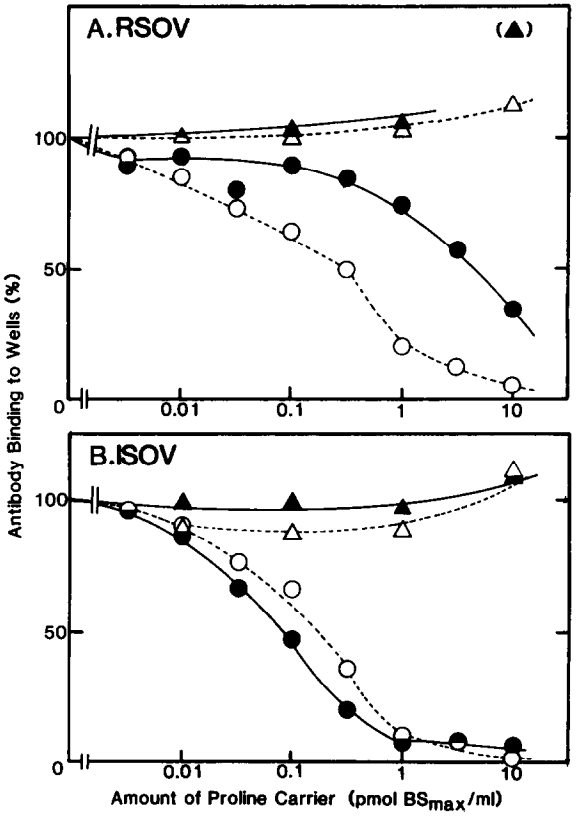

Fig.3. Ability of membrane vesicles to inhibit the antibody binding to the Ct-peptide. Competitive ELISA was carried out as in section 2. 100% of the y-axis is defined as the absorbance at 492 nm when no membrane vesicles were added. Membrane vesicles used were: ST3015 intact (●); ST3015 disrupted (○); ST3019 intact (▲); and ST3019 disrupted (△). The same amount of protein of vesicles from ST3019 as those from ST3015 were used for control experiments.

centrifugation but ISOV were not. The apparent K_d of each preparation calculated from fig.2 was the same (3.7 μM). Under our assay condition the proline concentration was equilibrated inside and outside the vesicles by a carrier-mediated facilitated diffusion; thus we may not see a difference in the affinity for proline between these two preparations.

3.4. *Orientation of wild-type proline carrier*

Degree of the exposure of the carboxyl terminus of proline carrier in different vesicle preparations was estimated by competitive ELISA in which the ability of vesicle preparations to inhibit the antibody binding to the Ct-peptide coated on the plates was measured. Fig.3B shows that the ISOV from ST3015 bound the antibody equally well in either intact or disrupted state. The RSOV from ST3015 bound far less IgG than the ISOV, but upon disruption they bound more IgG (fig.3A). The antibody binding to the vesicles was specific to the proline carrier, because the vesicles from ST3019 did not show antibody binding at all (fig.3A,B). These results indicated that the carboxyl terminal region of the proline carrier resides outside the ISOV and inside the RSOV; we therefore concluded that it is located in the cytoplasmic side of the membrane.

Acknowledgements: We are grateful to Dr T. Mogi for critical reading of the manuscript. We thank Drs M. Umeda and K. Inoue for their kind guidance in the ELISA experiment. This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science, and Culture of Japan.

REFERENCES

[1] Hanada, K., Yamato, I. and Anraku, Y. (1985) FEBS Lett. 191, 278–282.
[2] Tsuchiya, T., Yamane, Y., Shiota, S. and Kawasaki, T. (1984) FEBS Lett. 168, 327–330.
[3] Chen, C.-C., Tsuchiya, T., Yamane, Y., Wood, J.M. and Wilson, T.H. (1985) J. Membr. Biol. 84, 157–164.
[4] Hanada, K., Yamato, I. and Anraku, Y. (1988) Biochim. Biophys. Acta 939, 282–288.
[5] Hanada, K., Yamato, I. and Anraku, Y. (1988) J. Biol. Chem. 263, 7181–7185.
[6] Amanuma, H., Itoh, J. and Anraku, Y. (1977) FEBS Lett. 78, 173–176.
[7] Mogi, T. and Anraku, Y. (1984) J. Biol. Chem. 259, 7797–7801.
[8] Mogi, T., Yamamoto, H., Nakao, T., Yamato, I. and Anraku, Y. (1986) Mol. Gen. Genet. 202, 35–41.
[9] Nakao, T., Yamato, I. and Anraku, Y. (1987) Mol. Gen. Genet. 208, 70–75.
[10] Hanada, K., Yamato, I. and Anraku, Y. (1987) J. Biol. Chem. 262, 14100–14104.
[11] Kaback, H.R. (1971) Methods Enzymol. 22, 99–120.
[12] Futai, M. (1974) J. Membr. Biol. 15, 15–28.
[13] Sigel, M.B., Sinha, Y.N. and VanderLaan, W.P. (1983) Methods Enzymol. 93, 3–12.
[14] Hudson, L. and Frank, C.H. (1976) Practical Immunology, Blackwell, Oxford.
[15] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[16] Umeda, M., Diego, I., Ball, E.D. and Marcus, D.M. (1986) J. Immunol. 136, 2562–2567.
[17] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.