Identification of proline carrier in *Escherichia coli* K-12

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Proline carrier, a product of the *putP* gene of *Escherichia coli*, was identified as a 35 kDa cytoplasmic membrane protein by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Its identification was based on the following evidence: First, the density of the band corresponding to a 35 kDa protein correlated with the proline-binding activity of cytoplasmic membranes from *putP*-deficient and *putP*-amplified strains. Second, by the differential labeling method, the 35 kDa protein was specifically labeled with radioactive *N*-ethylmaleimide. The 35 kDa protein was found to aggregate on heat treatment and to show abnormal mobility on SDS-PAGE.

putP *gene* *Proline carrier* N-*[^{3}H]Ethylmaleimide labeling* *Heat aggregation* *Ferguson plot* *Hydrophobic protein*

1. INTRODUCTION

Proline transport in *E. coli*, which is mediated by a product of the *putP* gene, is a typical secondary active transport system [1]. ColE1-*putP*$^+$ hybrid plasmids were isolated [2–4] and the *putP* region was subcloned in vector pBR322 [5]. Cytoplasmic membranes from *putP* gene-amplified strains showed high activities of proline binding and transport [5]. However, so far, the product of the *putP* gene has been described as a protein of M_r 24000 or M_r 25000 only in a minicell system [3,6].

Here, we determined the apparent M_r of the proline carrier to be 35000 by analysis in 12.5% acrylamide SDS-PAGE of total cytoplasmic membrane proteins from carrier-overproducing strains. We also found that the apparent M_r of the carrier depends on the concentration of gel.

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Abbreviations: SDS-PAGE, SDS-polyacrylamide gel electrophoresis; MalNEt, *N*-ethylmaleimide; IPTG, isopropyl-β-D-thiogalactopyranoside

2. EXPERIMENTAL

The *E. coli* K-12 strains used are listed in table 1. Plasmids pLC4-45 and pTMP1 are ColE1-*putP*$^+$ and pBR322-*putP*$^+$ hybrid plasmids, respectively [2–5]. Plasmid pKHP1 is a pUC13-*putP*$^+$ hybrid plasmid, in which the *putP* gene is inserted downstream of the *lac* operator and promoter with the same transcriptional orientation (K. Hanada, unpublished). Cells were grown aerobically in DM medium [8] supplemented with 0.25% glycerol and 0.4% casamino acids (Difco). Ampicillin-resistant strains (ST3010 and ST3015) were grown in medium supplemented with ampicillin at 50 μg/ml. Cytoplasmic membranes from *E. coli* were prepared by a modification of the method of Yamato et al. [9]. The proline-binding activity of cytoplasmic membranes was measured as in [10] in assay medium consisting of 0.1 M Tris-maleate (pH 5.3), 0.38 M NaCl, 20 mM NaN_3, and 1 μM [^{14}C]proline (290 mCi/mmol). SDS-PAGE was performed as in [11]. Samples for SDS-PAGE were dissolved in SDS buffer by incubation at room temperature for 15 min, unless stated other-

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Table 1

Proline-binding activities of various cytoplasmic membranes

Strain	Relevant genotype	Proline binding[a]
JE2133	F⁻ *proA*	20
ST3501	F⁻ *proA proT putP proP recA*	<1
ST3009	ST3501/F′ *lacI*^{q} *pro*$^+$	<1
ST3010	ST3501/pTMP1	350
ST3014	ST3501/pLC4-45	160
ST3015[b]	ST3009/pKHP1	370
ST3015[c]	ST3009/pKHP1	540

[a] Proline-binding activities of cytoplasmic membranes from the indicated strains were measured as described in section 2 and expressed in pmol/mg protein

[b] ST3015 grown in the absence of IPTG

[c] ST3015 grown in the presence of 0.2 mM IPTG for 1 h

Strain JE2133 was described in [7]. ST3501 is a *recA* derivative of PT21, a *putP* mutant obtained from JE2133 (T. Nakao, unpublished). Other strains were constructed in this study

wise. Specific labeling with MalN[^{3}H]Et (50 Ci/mmol, New England Nuclear) was performed as described in the legend to fig.2 and fluorography of the gel was carried out as in [12].

3. RESULTS

3.1. *Identification of proline carrier by SDS-PAGE analysis*

We compared the SDS-PAGE profiles of cytoplasmic membrane proteins from a proline carrier-deficient strain and proline carrier-overproducing strains. As shown in fig.1, a broad band at M_r 35000 was detected in the profiles on SDS-PAGE of cytoplasmic membranes from strains ST3014 (harboring pLC4-45), ST3010 (harboring pTMP1), and ST3015 (harboring pKHP1), but the band of M_r 35000 was undetectable in the profiles of strain ST3501 (*putP*) and wild (*putP*$^+$) strain JE2133. The profiles of protein bands other than the 35 kDa band were similar in all strains examined. In addition, the density of the 35 kDa band seemed to correlate with the proline-binding activity of the membranes (fig.1 and table 1). These results suggest that the 35 kDa band on 12.5% acrylamide SDS-PAGE is that of the proline carrier, the *putP* gene product.

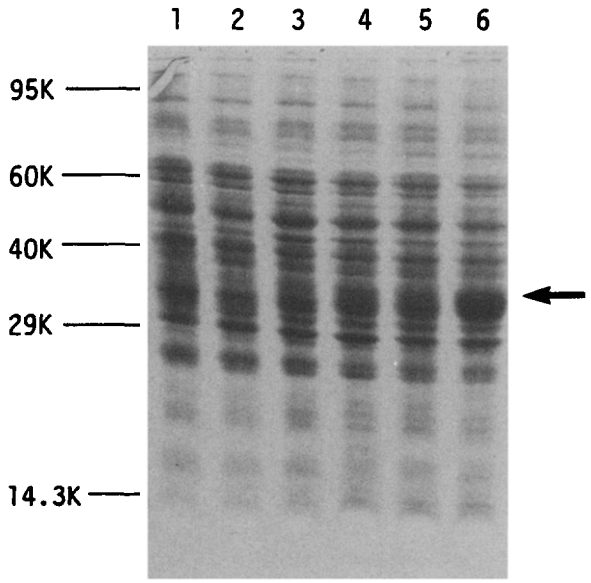

Fig.1. Detection of the *putP* product by 12.5% acrylamide SDS-PAGE of cytoplasmic membrane proteins. Cytoplasmic membranes (25 μg protein/lane) from the indicated strains were dissolved in SDS buffer by standing the suspensions for 15 min at room temperature. Lanes: 1, ST3501 (*putP*); 2, JE2133 (*putP*$^+$); 3, ST3014 (harboring pLC4-45); 4, ST3010 (harboring pTMP1); 5, ST3015 (harboring pKHP1) grown in the absence of IPTG; 6, ST3015 (harboring pKHP1) grown in the presence of 0.2 mM IPTG for 1 h. The marker proteins (in kDa) used were phosphorylase *b* (95), catalase (60), aldolase (40), bovine carbonic anhydrase B (29), and lysozyme (14.3).

3.2. *Specific labeling of the proline carrier with MalN[^{3}H]Et*

L-Proline effectively protected the proline carrier against inactivation by MalNEt ([13] and K. Hanada, unpublished). Making use of this property, the proline carrier was specifically labeled with radioactive MalNEt (fig.2). The specifically labeled protein and the 35 kDa protein of the membranes from the proline carrier-overproducing strains showed the same molecular mass values in 12.5% acrylamide SDS-PAGE (lane 6 in fig.1 and lane 1 in fig.2). When a sample of membranes for SDS-PAGE was dissolved in SDS buffer by boiling for 15 min, the specifically labeled band remained at the top of the gel (lane 2 in fig.2). Membranes from a proline carrier-deficient strain showed no specifically labeled proteins (not shown). These

results indicate that the 35 kDa protein has proline-binding activity and aggregates on heat treatment.

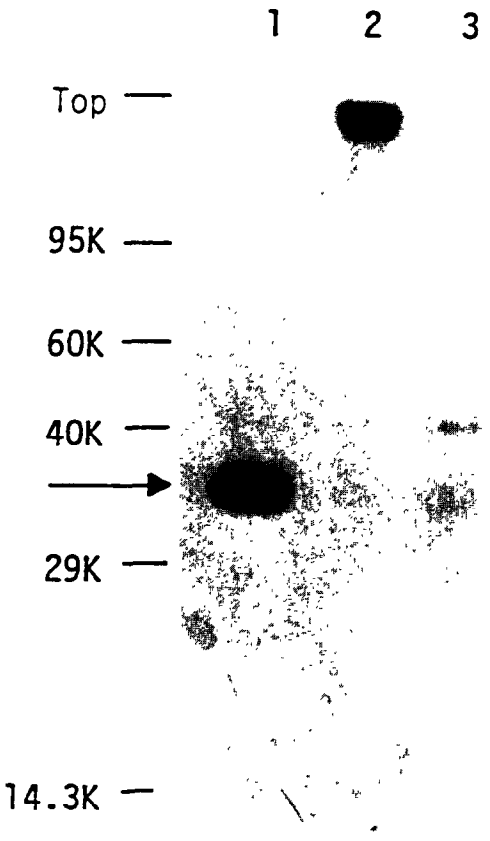

Fig.2. Specific labeling of the proline carrier with MalN[^{3}H]Et. Cytoplasmic membranes from strain ST3015 were preincubated with 0.5 mM nonradioactive MalNEt for 1 h at 25°C in a reaction medium consisting of 0.1 M Tris-maleate (pH 7.0) and 0.1 M LiCl in the presence of 1 mM L-proline. The pretreated membranes were washed and incubated with MalN[^{3}H]Et for 3 h at 25°C in the same reaction medium in the absence of L-proline (this membrane fraction is referred to as specifically labeled membranes). As a control, the pretreated membranes were incubated with MalN[^{3}H]Et in the presence of 1 mM L-proline. Labeled proteins were analyzed by fluorography of SDS-PAGE gel. The membranes treated with MalN[^{3}H]Et were dissolved in SDS-buffer by standing the suspensions for 15 min at room temperature (lanes 1,3) or by boiling it for 15 min (lane 2). Lanes: 1,2, specifically labeled membranes (100 μg protein (3720 cpm)/lane); 3, control membranes (100 μg protein (3250 cpm)/lane).

3.3. *Abnormal mobility of the proline carrier on SDS-PAGE*

Some membrane proteins such as the *lacY* protein are known to behave abnormally on SDS-PAGE [14]. We examined the mobility of the proline carrier on SDS-PAGE by a Ferguson plot [15]. As shown in fig.3A, the proline carrier migrated faster than marker proteins in gels of lower concentration, and so its apparent M_r increased with increase in concentration of the gel (fig.3B). The

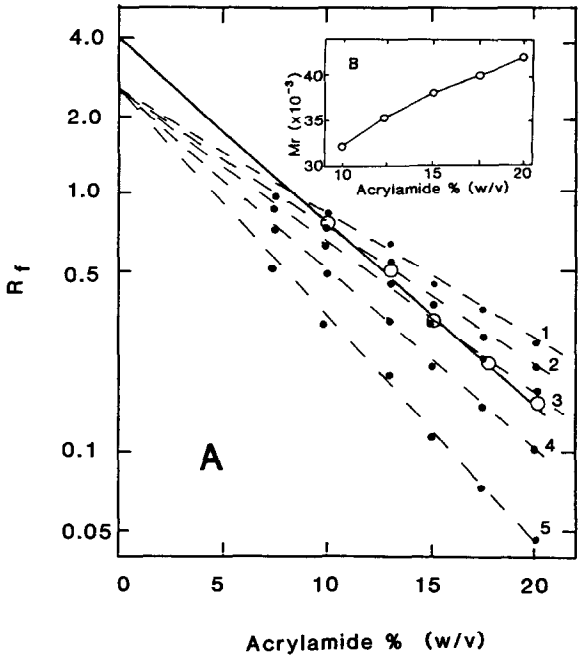

Fig.3. Abnormal mobility of the proline carrier on SDS-PAGE; (A) Ferguson plot of the proline carrier. The dependence of the relative mobility (R_f) on the percentage of acrylamide in gels was analyzed. Gels with an acrylamide:*N,N'*-methylenebisacrylamide ratio of 75:2 were used. The solid line represents the plot for the proline carrier. The band of the proline carrier was identified by comparison of profiles from strain ST3015 (harboring pKHP1) and ST3501 (*putP*). Dashed lines (1–5) are plots for marker proteins (in kDa): 1, bovine carbonic anhydrase B (29); 2, aspartate transcarbamylase (35); 3, aldolase (40); 4, catalase (60); 5, phosphorylase *b* (95). (B) Dependence of the apparent M_r of the proline carrier on the gel concentration in SDS-PAGE. Apparent M_r values of the proline carrier were determined by comparing the mobilities of the proline carrier in gels with those of marker proteins of known M_r.

M_r of the proline carrier estimated from the slope of the Ferguson plot [16] was 60000. When the SDS-PAGE system of Weber and Osborn [17] was used, the apparent M_r of the proline carrier was 35000 on 12.5% acrylamide gel and 32000 on 10% acrylamide gel (not shown).

4. DISCUSSION

Here we have shown that the density of a 35 kDa band correlates with the proline-binding activity of cytoplasmic membranes (fig.1 and table 1) and that this 35 kDa protein is specifically labeled with MalN[^{3}H]Et by the differential labeling method (fig.2). These results indicate that the proline carrier, a product of the *putP* gene, has an apparent M_r of 35000 on 12.5% SDS-PAGE.

In a Ferguson plot, the value of the intercept on the ordinate was considerably larger for the carrier than for marker proteins (fig.3A). This suggests that more SDS molecules bound to the proline carrier than to the marker proteins. Similar abnormal mobilities of hydrophobic proteins such as the lactose carrier of *E. coli* have been reported [14].

Plasmids pLC43-41 and pLC4-45 from an *E. coli* gene library complement both *putP* and *putA* mutations [4]. Wood and Zadworny [6] reported that pLC43-41 codes for 25, 36, 38 and 130 kDa proteins as proteins derived from an *E. coli* chromosome DNA fragment by the minicell method. A product of the *putA* gene, proline dehydrogenase, was purified and its M_r estimated to be 124000 [18]. Therefore, Wood and Zadworny [6] concluded that the band at M_r 25000 was that of the *putP* product, since gene products other than that of the *putA* gene were estimated to be no larger than M_r 26000 by DNA contour length measurement. However, their criterion for concluding that the 25 kDa protein was the *putP* product among the 25, 36 and 38 kDa proteins is not adequate, since it is difficult to determine the sizes of gene products only by DNA contour length measurement [19]. Accordingly, we consider that the 25, 36 and 38 kDa proteins detected by the minicell method are all candidates for the *putP* gene product. Wood [20] also analyzed the membrane proteins from *putP* gene-deficient and *putP* gene-amplified strains by 10–14% linear gradient SDS-PAGE. A broad band at about M_r 35000 is detectable in her SDS-PAGE profiles of membranes from *putP* gene-amplified strains, although she did not discuss this particular result [20].

Motojima et al. [3] found by a minicell method that a 24 kDa protein separated by 12% acrylamide SDS-PAGE is a major membrane protein encoded by pLC4-45 and concluded that this protein was a proline transport carrier. But under their experimental conditions, all membrane samples for SDS-PAGE were prepared in SDS buffer by heat treatment [3]. We have shown here that when membranes for SDS-PAGE are dissolved in SDS buffer with heating, the proline carrier aggregates and so does not migrate on the gel (fig.2). Thus, the previous experimental conditions were probably unsuitable for detection of the *putP* product by SDS-PAGE.

All these facts show that the discrepancy between the apparent molecular masses of the proline carrier observed in this work and in previous studies [3,6] cannot be simply explained by its abnormal mobility on SDS-PAGE (fig.3A,B) and that the *putP* product is the 35 kDa protein, while the 25 and 24 kDa proteins described in previous reports are unknown products of pLC43-41 and pLC4-45.

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