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Overexpression of the Alanine Carrier Protein Gene from Thermophilic Bacterium PS3 in *Escherichia coli*[1]

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The alanine transporter (alanine carrier protein, ACP) gene of thermophilic bacterium PS3 was previously cloned and expressed in a functionally active form in *Escherichia coli* cells. To achieve controlled overproduction of the ACP protein, we designed a plasmid encoding a fusion protein comprising ACP joined to the carboxyl terminus of the maltose binding protein (MBP-ACP). Upon transduction of the plasmid into *E. coli* RM1 cells defective in alanine/glycine transport, the transport activity was expressed even before induction with 1-thio-β-D-galacto-pyranoside (IPTG), and increased slightly on induction with IPTG at low concentrations. However, overexpression of the MBP-ACP gene, induced by higher concentrations of IPTG, resulted in death of the host cells. Hence we screened other host cells and found that the MBP-ACP fusion protein was produced in a large quantity in *E. coli* TB1 cells 3 h after IPTG induction. The MBP-ACP fusion protein was accumulated in cytoplasmic membranes in an amount reaching more than 20% of the total membrane protein. The affinity-purified MBP-ACP exhibited very low transport activity when reconstituted into proteoliposomes.

Key words: amino acid transporter, fusion protein, overexpression, sodium alanine symporter family, thermophilic alanine carrier.

Bacterial cells take up various organic substrates from the surrounding medium in a manner known as secondary active transport, which is driven by an electrochemical potential of either H^+ or Na^+ ions (for a recent review, see Ref. *1*). The genes encoding these transporter proteins have been cloned, and it has been suggested that these proteins are very hydrophobic and composed of multi-membrane spanning domains (10-12 membrane spanning domains). These membrane proteins have variable characteristics in terms of solute specificity and/or ion coupling, but are thought to share common structural properties (*1, 2*). However, the molecular structures of these transporter proteins have not yet been elucidated as they are present in relatively small amounts in membranes, and thus sufficiently large amounts for structural analysis by means of physicochemical studies, such as X-ray crystallography, cannot be obtained. Large quantities of a protein are often obtained by overproduction of the protein in an artificial system such as an *Escherichia coli* expression system, using appropriate expression vectors. A number of successful examples have been reported, the genes of soluble proteins being produced, but there have only been a few reports of successful overproduction. Problems encountered in the attempted overexpression of membrane proteins included poor overexpression, severe host cell death caused by overexpression and the tendency of the overexpressed proteins to form inclusion bodies (*3*).

The $Na^+(H^+)$-coupled alanine transporter (ACP) has been isolated from thermophilic bacterium PS3 membranes (*4*), and the ACP gene (*acp*) has been cloned from a genomic library of PS3 and expressed in a functionally active form in *E. coli* cells (*5, 6*). The purified ACP transports alanine, glycine, serine, and other small neutral amino acids, the transport being driven by either a H^+ or Na^+ ion gradient (*4*). In addition, ACP has been classified into the sodium alanine symporter family (SAF) (*2*) with relatives in *E. coli* (*7*), *Bacillus subtilis* (*8*), *Haemophilus influenzae* (*9*), and *Alteromonas haloplanktis* (*10*). These proteins exhibit high identity with each other and are predicted to contain eight to twelve transmembrane spanning domains. Among these SAF proteins, ACP has been the most analyzed biochemically in detail, but molecular structural studies have not yet been performed.

The aim of this study was to obtain ACP in a sufficiently large quantity for crystallization. For this, we constructed a fusion gene (pAC6268) comprising the maltose binding protein (MBP) gene (pMALc2) fused with the PS3 ACP gene. We report here the achievement of successful overexpression of the fusion gene in *E. coli* cells.

MATERIALS AND METHODS

Expression Plasmids and Bacterial Strains—Plasmid

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Abbreviations: ACP, alanine carrier protein; IPTG, 1-thio-β-D-galacto-pyranoside; MBP, maltose binding protein.

pAC9701 encoding the *acp* gene (*5*) was digested with *Hin*dIII and *Bam*HI, blunt-ended with T4 DNA polymerase, and then ligated to pMAL-c2 (New England BioLabs), which had been digested with *Pst*I and blunt-ended with T4 DNA polymerase. The plasmid encoding the MBP-ACP fusion protein was designated as pAC6268. *E. coli* strain RM1 (kindly donated by Prof. MacLeod, R.A., McGill Univ., Canada), which lacks active alanine and glycine transport activity (*11*), was used as the host strain and transformed with various plasmids for the transport assay in this study. For overexpression of MBP-ACP, the *E. coli* TB1 strain (New England Biolabs), harboring expression plasmid pAC6268, was used.

Medium and Growth Condition—*E. coli* RM1 harboring pAC6268 was grown in LB broth medium at 37°C as described (*6*). *E. coli* TB1 harboring pAC6268 was grown at 37°C in LB broth overnight, and the seed culture was then diluted 1:100 or 1:200 with fresh NZCY medium containing 50 μg of ampicillin per ml (*12*). Incubation was continued until the optical density of the culture deviated from that in the logarithmic phase of growth and then 1-thio-β-D-galacto-pyranoside (IPTG) was added to 1.0 mM. Three hours after IPTG administration, the culture broth was chilled on ice, and the cells were harvested by centrifugation and washed twice with 25 mM Tris-HCl (pH 8.0) containing 1 mM EDTA.

Subcellular Fractionation—Cell lysates were prepared by sonication using a Bioruptor sonifier (Cosmo Bio), and then centrifuged at 10,000 × *g* for 10 min at 4°C to remove the undisrupted cell debris. The supernatants were centrifuged at 18,000 × *g* for 30 min at 4°C, and the pellets were resuspended in 25 mM Tris-HCl (pH 8.0) containing 1 mM EDTA. This was designated as the crude membrane fraction. Alternatively, cells resuspended in 25 mM Tris-HCl (pH 8.0) containing 1 mM EDTA and 0.5 M sucrose were treated with lysozyme (0.5 mg/ml), and the resulting spheroplasts were disrupted with a French pressure cell disrupter (Ohtake 5501-M) and then fractionated by sucrose density gradient centrifugation as described by Yamada *et al.* (*13*). The fractionated samples were diluted with 25 mM Tris-HCl (pH 8.0) containing 1 mM EDTA, precipitated and then resuspended in the same buffer. The resulting membrane vesicle suspensions were used for transport assays.

Purification of the Recombinant MBP-ACP Fusion Protein and Reconstitution into Proteoliposomes—The recombinant MBP-ACP fusion protein was purified after subcellular fractionation as described above. The enriched fractions were combined, diluted with 25 mM Tris-HCl (pH 8.0) containing 1 mM EDTA, and then centrifuged at 18,000 × *g* for 30 min at 4°C. The resulting pellets were solubilized with 1% Triton X-100, and the solubilized sample was mixed with an amylose resin slurry (2 mg of protein/1 ml of slurry). After incubation with slow mixing for 1 h at 4°C, the resin was washed thoroughly with 25 mM Tris-HCl (pH 8.0) containing 1 mM EDTA and 0.1% Triton X-100, followed by extraction with the same buffer mixture containing 10 mM maltose and 100 mM NaCl. The purified MBP-ACP (0.5 mg protein) was mixed with 10 mg soybean phospholipids (asolectin; Woodside, NY) and then treated with BioBeads SM-2 (Bio Rad Laboratories, CA) to remove Triton X-100 for 16 h at 4°C. The resulting proteoliposomes were collected, washed with 25 mM Tris-HCl (pH 8.0) containing 5 mM $MgCl_2$, and then loaded with 0.5 M K-phosphate buffer (pH 8.0) as described (*4*).

Transport Assays—Transport by intact cells was measured at 37°C as described previously (*6*) except that the incubation medium comprised 10 mM Tris-HCl (pH 7.4), 300 mM choline-Cl, and 5 mM $MgCl_2$. Proline and alanine transport driven by D-lactate oxidation in membrane vesicles obtained as above was assayed in a mixture (500 μl) comprising 25 mM Tris-HEPES (pH 8.0), 10 mM $MgCl_2$, 5 mM D-lactate, and 20 μM [^{14}C]proline (3.8 kBq) or 20 μM [^{14}C]alanine (3.7 kBq) with vigorous shaking. Reactions were started by adding D-lactate, and at various times 100 μl aliquots of the suspension were withdrawn and filtered through membrane filters (Schleicher & Schuell, NH; pore size, 0.45 μm). The filters were washed with 2 ml of 25 mM Tris-HEPES (pH 8.0) containing 10 mM $MgCl_2$ and then radioactivity was measured with a scintillation counter (Beckman, LS6000-TA-2). Alanine transport by reconstituted proteoliposomes dependent on an artificial membrane potential was assayed as described (*4*).

SDS-PAGE and Immunoblotting—Samples were subjected to SDS-PAGE using 10.0% acrylamide, and then transferred to a nitrocellulose membrane (ECL membrane, Amersham) following the manufacturer's instructions. Immunoblotting was carried out using polyclonal anti-MBP (New England BioLab) and anti-ACP (*6*) antibodies.

RESULTS

Expression of the MBP-ACP Fusion Protein in E. coli RM1—A plasmid (pAC6268) was constructed in which the *acp* gene was fused to the *malE* gene, so the ACP protein was expressed as a fusion protein, MBP-ACP. Since the object of this study was to develop a high level expression system for the *acp* gene product in an active form, we studied first whether or not the MBP-ACP fusion protein showed functional activity in *E. coli* cells. For this, *E. coli* RM1, defective in alanine/glycine transport, was used as the host strain. As reported previously, RM1 transformed with the pAC5629 plasmid which contains the *acp* gene coding region with its 5′ regulatory sequences, exhibited high alanine/glycine transport activity (*6*). Figure 1a shows that RM1 transformed with the pAC6268 plasmid [RM1-(pAC6268)] showed glycine transport activity even before IPTG induction. The transport activity increased slightly upon induction with IPTG at 5 μM, but a higher concentration of IPTG (1 mM) had a deleterious effect on the transport activity indicating that the overproduction of MBP-ACP was highly toxic to the host cells. In fact, the addition of 1 mM IPTG to the cell culture at the logarithmic phase of growth resulted in instantaneous cessation of growth (data not shown).

The apparent K_t values for alanine and glycine transport in the cells without IPTG induction were determined to be 10 and 5 μM, respectively, which were very similar to those reported previously for the RM1 transformant (pAC-5629) (*6*). Furthermore, the substrate specificity of the alanine transport by RM1(pAC6268) determined by means of competition studies was also very similar to that of RM1(pAC5629) (Table I). The results indicate that the MBP-ACP fusion protein is functionally active in *E. coli* cells and that the catalytic activity of ACP is not influenced

greatly by the fusion of the MBP protein to its N-terminal.

Expression of the MBP-ACP fusion protein, whose molecular weight was estimated to be 90 kDa, *i.e.* the sum of those of MBP (45 kDa) and ACP (43 kDa), was examined by immunoblotting of cell lysate of RM1(pAC6268). As shown in Fig. 1b, although a 90 kDa band cross-reacting with both anti-MBP and anti-ACP antisera was barely detectable for the cells before IPTG induction, such a band became apparent after the administration of 5 μM IPTG. In addition, anti-ACP failed to cross-react with any appropriate proteins in RM1(pAC5629) (data not shown), suggesting that the transport activity was supported by a barely detectable amount of protein.

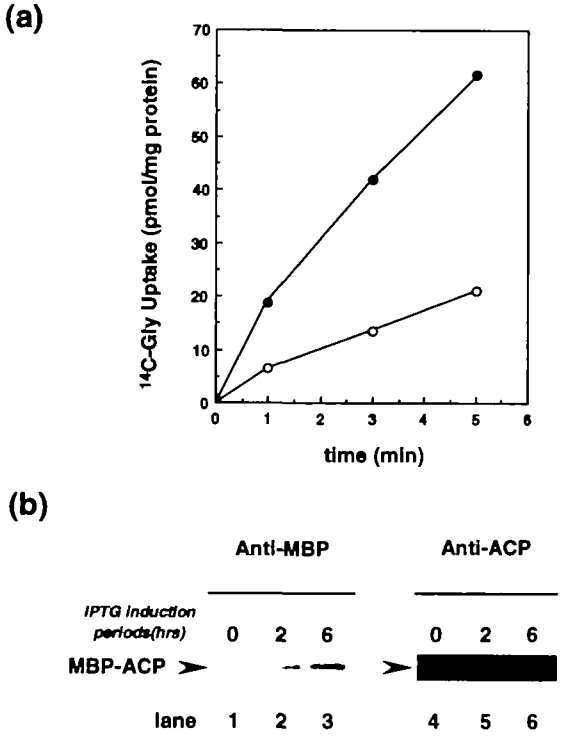

Fig. 1. **Expression of MBP-ACP in *E. coli* RM1(pAC6268).** (a) Glycine transport in RM1 cells harboring the pAC6268 plasmid or mock pMALc2. [^{14}C]Glycine transport was measured in RM1(pAC6268) (●) or RM1(pMALc2) (○) as described under "MATERIALS AND METHODS." (b) Immunoblotting of cell lysates of RM1 harboring the pAC6268 plasmid or mock pMALc2 before induction with IPTG (lanes 1 and 4), and after induction with 5 μM IPTG for 2 h (lanes 2 and 5) and 6 h (lanes 3 and 6). Cell lysates were prepared, subjected to SDS-PAGE and blotted onto ECL membranes, and then the membranes were reacted with anti-MBP (lanes 1-3) and anti-ACP (lanes 4-6) antisera as described under "MATERIALS AND METHODS."

Re-Evaluation of the N-Terminal Sequence of ACP—Previously we proposed that the structural gene of ACP consisted of 1,335 bp encoding 445 amino acids starting from an ATG codon (*5, 6*). During attempts to construct the MBP-ACP fusion gene, we found that the proposed initiation codon, ATG, was unable to produce active ACP in RM1 cells (data not shown) and hence the real initiation codon is a GTG codon 105 bases upstream from the ATG codon (Fig. 2). This was confirmed by peptide sequencing (see below). Thus the correct structural gene of ACP is 1,440 bp (480 amino acids).

Overexpression of MBP-ACP in E. coli—The results described above indicated that introduction of the *acp* and *malE* fusion genes into *E. coli* RM1 resulted in activation of alanine/glycine transport, but induction with a higher amount of the inducer, IPTG, had a severe deleterious effect on the host cells, similar to those reported in other gene expression experiments (*3, 14, 15*). It is generally accepted that the toxic effect of overexpression of foreign genes can sometimes be overcome by selecting a suitable host *E. coli* strain and appropriate culture conditions. Thus we examined a variety of *E. coli* strains and culture conditions, and selected an *E. coli* TB1 strain as the host and established culture conditions for overexpression of the MBP-ACP gene, as described under "MATERIALS AND METHODS." An important factor for successful overexpression was that IPTG was administered at the late-logarith-

TABLE I. **Competition of alanine transport in *E. coli* RM1(pAC6268).**

Competitor	Inhibition (%)	
	ACP [RM1(pAC5629)]	MBP-ACP [RM1(pAC6268)]*
D-Alanine	98	99
Glycine	99	71
L-Serine	53	99
D-Cycloserine	72	99
L-Glutamine	85	31
L-Asparagine	72	60
L-Threonine	27	42
L-Histidine	53	51
L-Arginine	0	0
L-Lysine	0	0
L-Leucine	0	0
L-Isoleucine	0	0
L-Phenylalanine	0	0
L-Methionine	0	0
L-Aspartic acid	0	0
L-Glutamic acid	0	0

The transport of [^{14}C]-L-alanine was measured in RM1(pAC6268) in the presence of a 25-fold molar excess (500 μM) of a competitor amino acid. **E. coli* RM1(pAC6268) cells were grown and induced with 5 μM IPTG for 2 h as described under "MATERIALS AND METHODS."

GAGGGAAGGATTTCAGAATTCGGATCCTCTAGAGTCGACCAGCTTGTAGAAACGGTGAACGGGTGGCTATGGAGTCCTTTTTTGATTGCATTC
E G R **I S E F G S S R V D Q L V E T V N G W L W S P F L** I A F

ATTGTTTGTTGCGGTTTGTATTTTAGCATTCGCACCCGTTTCTTGCAAATCCGCCATGTGAAAGAGATGATCCGGCTTGTGACGATGGGGAAA
I V C C G L Y F S I R T R F L Q I R H V K E M I R L V T M G K

Fig. 2. **The amino terminal sequence of ACP.** The amino acids in bold face are those detected on peptide sequencing. The junction sequence followed by the N-terminal sequence of ACP is shown. The double-underlined GTG codon is the initiation codon identified, and the previously proposed initiation ATG codon is single-underlined. An arrow indicates the cleavage site for factor Xa.

mic phase of growth. *E. coli* TB1 harboring pAC6268 [TB1(pAC6268)] was grown in LB medium, transferred to NZCY medium, and then allowed to grow to the late-log phase when IPTG (1 mM) was added. Under these conditions, there was no serious deleterious effect on the growth of TB1(pAC6268), in contrast to the case of RM1(pAC-6268) (data not shown). When cell lysates were subjected to SDS-PAGE, the 90 kDa MBP-ACP band was visualized by Coomassie Brilliant Blue staining immediately after the induction, and reached the maximum level after 3 h of incubation (Fig. 3). The amount of MBP-ACP protein reached more than 20% of the total membrane proteins, as determined by densitometric analysis (data not shown).

Subcellular Fractionation and Purification of MBP-ACP—After 3 h of IPTG induction, TB1(pAC6268) cells were harvested and treated with lysozyme-EDTA to obtain spheroplasts, followed by disruption with a French pressure cell disrupter. The resulting cell lysates were subjected to sucrose density gradient centrifugation as described under "MATERIALS AND METHODS." When fractionated samples were examined for either alanine or proline transport activity driven by D-lactate oxidation as a marker of the inner membrane, both were found exclusively in fractions 4 and 5 (Fig. 4a). On the other hand, the 90 kDa MBP-ACP protein was distributed rather broadly in fractions 1 to 6 (Fig. 4b), but was found mainly in fractions 2 to 4, which were slightly denser than those containing the cytoplasmic membranes. Since the host TB1 cells carry a native alanine transporter, the alanine transport activity in fractions 4 and 5 could not be distinguished from that of the expressed MBP-ACP. Thus we proceeded to purify MBP-ACP from the concentrated fractions.

Fractions 2 to 4 obtained above were combined, solubilized with 1% Triton X-100, and then mixed with an amylose resin slurry. The MBP-ACP was specifically eluted from the resin with 10 mM maltose as described under "MATERIALS AND METHODS." A single 90 kDa band was detectable for the eluate (Fig. 4c). The recovery of MBP-ACP was estimated to be 5 mg protein from 50 mg protein of total membrane proteins. In order to evaluate the correct translation of the MBP-ACP gene in *E. coli* TB1 cells, the purified MBP-ACP was digested with lysyl endopeptidase, and the resulting peptides were separated by HPLC and then subjected to peptide sequencing. Among the various peptide fragments corresponding to the internal sequences of MBP and ACP, there was a peptide, sequenced as RWEEKK, which corresponded to the C-terminal region of ACP, indicating that the translation of the MBP-ACP fusion gene occurred correctly. In addition, treatment of MBP-ACP with protease factor Xa, which was expected to cleave at a specific site at the junction between MBP and ACP, resulted in the appearance of the peptide sequence of ISEFGSSRVDQLVETVNGWLWSPFL, which corresponded to the junction, followed by the correct N-terminal of ACP (see Fig. 2). Unfortunately, the factor Xa treatment

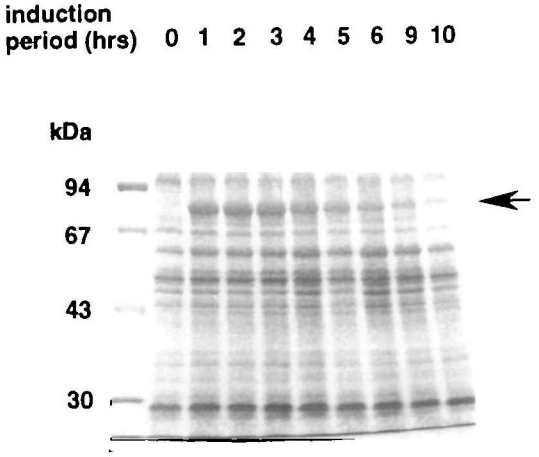

Fig. 3. **Expression of MBP-ACP in *E. coli* TB1(pAC6268).** *E. coli* TB1 harboring pAC6268 was grown in LB broth medium and then transferred to NZCY medium. When cell growth reached the late logarithmic phase, 1 mM IPTG was added. Cells were collected as indicated and disrupted with a sonifier, and then the resulting cell lysates were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue as described under "MATERIALS AND METHODS." An arrow indicates the 90 kDa MBP-ACP fusion protein.

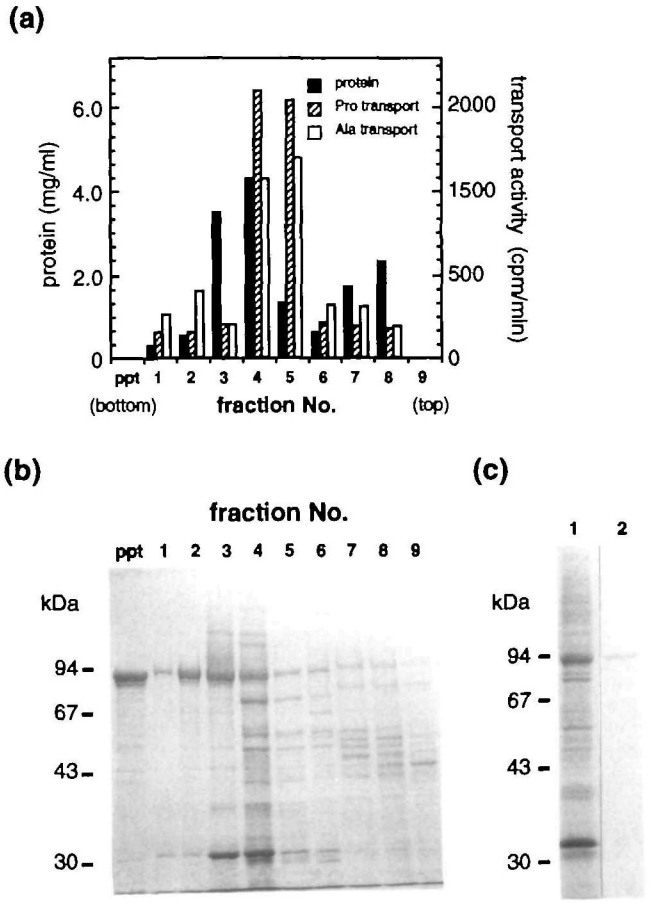

Fig. 4. **Subcellular fractionation of *E. coli* TB1(pAC6268) cell lysates and affinity-purified MBP-ACP.** *E. coli* TB1(pAC6268) was cultured, induced with IPTG, harvested, and then subjected to EDTA-lysozyme treatment to produce spheroplasts. (a) Cell lysates were subjected to sucrose density gradient centrifugation as described under "MATERIALS AND METHODS." Fractions were collected and the proline- or alanine-transport activity in each fraction was measured. (b) SDS-PAGE pattern of each fraction stained with Coomassie Brilliant Blue. (c) Fractions 2 to 4 were combined (lane 1), solubilized with Triton X-100, and then subjected to affinity separation using amylose resin (lane 2) as described under "MATERIALS AND METHODS."

resulted in non-specific digestion of the ACP and hence made it impossible to isolate ACP from the fusion protein.

The MBP-ACP obtained was reconstituted into proteoliposomes and the alanine transport in response to an artificial membrane potential was estimated to be 0.4 nmol/min/mg protein, which was far less than that observed with the native ACP purified from PS3 membranes (40 nmol/min/mg protein) (*4*). The reason for this low activity is not known at present.

DISCUSSION

The present work demonstrated that the transformation of *E. coli* RM1 cells defective in alanine/glycine transport with a plasmid harboring the *malE-acp* fusion gene resulted in the triggering of transport activity. As we reported previously, there is an insertion sequence (IS)-like element in the 5′-upstream region of the *acp* gene in thermophilic bacterium PS3 chromosomal DNA. This IS-like sequence, including its putative 5′ promotor region, is a prerequisite for the expression of the *acp* gene in *E. coli* cells (*6*). Furthermore, during attempts to achieve the overexpression of *acp* in *E. coli*, we found that various plasmids in which the *acp* gene was cloned downstream of a strong inducible promotor, such as the *lac*-, *tac*-, or T7-promotor, failed to induce transport activity in *E. coli* RM1 (data not shown). Thus, we decided to construct another form of expression system, a *malE-acp* fusion gene, as described in this report. It is intriguing that the MBP-ACP fusion protein expressed in *E. coli* RM1 catalyzes alanine/glycine transport without removal of the MBP region and retains similar characteristics to ACP, as revealed by kinetic and competition studies. Induction of the fusion gene with low concentrations of IPTG (5 μM) resulted in slight activation of the transport activity together with the appearance of a 90 kDa band which cross-reacted with both anti-MBP and anti-ACP antibodies. However, induction with IPTG at high concentrations (1 mM) resulted in instantaneous cessation of cell growth. In a control experiment involving cells transformed with a plasmid harboring *malE* alone, IPTG induction had no effect on growth (data not shown), indicating that the expression of a high level of the fusion protein is toxic to the cells.

It is generally observed that many membrane proteins, even when expressed at low levels, are toxic to *E. coli* cells (*16-18*). Hence laborious searches for appropriate vectors, suitable host cells and optimal culture conditions are generally required to achieve overproduction of membrane proteins. In this study, we found that overexpression of the *malE-acp* fusion gene was best achieved in *E. coli* TB1 cells when IPTG induction was performed in the late logarithmic phase of growth. Three hours after induction, the expression of the 90 kDa MBP-ACP reached the maximum level and subcellular fractionation experiments revealed that it was located in fractions with a slightly heavier density than that of cytoplasmic membranes. It is also known that overproduced proteins tend to form insoluble aggregates or inclusion bodies which are easily precipitated on low speed centrifugation. Since the sucrose density gradient centrifugation was carried out with the supernatant fraction obtained on low speed centrifugation, the possibility that the MBP-ACP had formed inclusion bodies was excluded.

The amount of MBP-ACP produced reached more than 20% of the total membrane proteins from which the recombinant protein was solubilized with a detergent followed by one-step purification involving affinity separation with amylose resin. Unfortunately, the purified MBP-ACP exhibited very low transport activity when reconstituted into proteoliposomes compared with the native ACP, and attempts to remove the MBP region with the protease, factor Xa, unexpectedly resulted in the digestion of ACP.

During the course of our experiments we determined the real N-terminal sequence of ACP, which was not described in the previous reports (*5, 6*). When the *acp* gene, starting from ATG, which was previously proposed to be an initiation codon, was fused downstream of the *malE* gene and transformed into *E. coli* RM1, the cells were unable to transport alanine/glycine (data not shown). In contrast, plasmid pAC6268, which contained a GTG codon found 105 bases upstream from the ATG codon, induced transport activity effectively, as described. Peptide sequencing of the MBP-ACP fusion protein revealed that ACP was translated from the GTG codon. Thus the correct structural gene of ACP consists of 1,440 bp (480 amino acids) and the number of putative membrane spanning domains is calculated to be 10-12 instead of 8-10, as proposed previously (*5*).

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