Spermidine-preferential Uptake System in Escherichia coli

ATP HYDROLYSIS BY PotA PROTEIN AND ITS ASSOCIATION WITH MEMBRANES*

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Keiko Kashiwagi‡, Hiroko Endo‡, Hiroshi Kobayashi‡, Koji Takio§, and Kazuei Igarashi‡

From the ‡Faculty of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Inageku, Chiba 263 and the §Division of Biomolecular Characterization, The Institute of Physical and Chemical Research (RIKEN), 2-1 Hirosawa, Wako-shi, Saitama 350-01, Japan

PotA protein, one of the components of the spermidine-preferential uptake system in Escherichia coli, was purified to homogeneity, and some of its properties were examined. PotA protein showed Mg2+- and SH-dependent ATPase activity. The specific activity was approximately 400 nmol/min/mg of protein and the K_m value for ATP was 385 μM. The nature of the ATP binding site was explored by identification of the amino acid residue photoaffinity-labeled with 8-azido-ATP. It was found that 8-azido-ATP was attached to cysteine 26. In the spermidine transport-deficient mutant E. coli NH1596, valine 135 of PotA protein, which is located between two consensus amino acid sequences for nucleotide binding (50–57 and 168–173), was replaced by methionine (Kashiwagi, K., Miyamoto, S., Nukui, E., Kobayashi, H., and Igarashi, K. (1993) J. Biol. Chem. 268, 19358–19363). This mutant PotA protein could be labeled with 8-azido-ATP, but showed very low ATPase activity. To identify which cysteine is involved in the function of PotA protein, cysteines 26, 54, and 276 were replaced by alanine, threonine, and alanine, respectively. Among the three mutated PotA proteins, the mutated PotA protein C54T only lost both ATPase and spermidine uptake activities. The results taken together indicate that the adenine portion of ATP interacts with a domain close to the NH2-terminal end of PotA protein, and active centers of ATP hydrolysis are located both within and between the two consensus amino acid sequences for nucleotide binding. Association of PotA protein with membranes was strengthened by the existence of channel forming PotB and PotC proteins. ATPase of PotA protein was inhibited by spermidine, suggesting that uptake inhibition by spermidine may function during this process.

Polyamines (putrescine, spermidine, and spermine) are known to be necessary for cell growth (1, 2). It is thus important to understand the mechanism by which the cellular polyamine is regulated. Polyamine transport is one of the important determination factors of polyamine content in cells. In Escherichia coli, polyamine uptake is energy-dependent, and the putrescine transport system is different from the spermidine (spermine) transport system (3, 4). Furthermore, two transport systems for putrescine have been suggested in E. coli K12 grown in a low osmolarity medium (5). We recently obtained and characterized three clones of polyamine transport genes (pPT104, pPT79, and pPT71) in E. coli (6). The system encoded by pPT104 was a spermidine-preferential uptake system and that encoded by pPT79 a putrescine-specific uptake system. Furthermore, these two systems were periplasmic systems (7) consisting of four kinds of proteins: pPT104 clone encoded PotA, PotB, PotC, and PotD proteins and pPT79 clone encoded PotF, PotG, PotH, and PotI proteins, judging from the deduced amino acid sequences of the nucleotide sequences of these clones (8, 9). PotD and PotF proteins were periplasmic substrate binding proteins, and PotA and PotG proteins were membrane-associated proteins having the nucleotide-binding site. PotB and PotC proteins, and PotH and PotI proteins, were transmembrane proteins probably forming channels for spermidine and putrescine, respectively. In contrast, the putrescine transport system encoded by pPT71 consisted of one membrane protein (PotE protein) having 12 transmembrane segments (10) and was active in the excretion of putrescine from cells through putrescine-ornithine antiporter (11). We also found that spermidine uptake by membrane vesicles was strongly dependent on PotD protein, and the uptake by intact cells was completely dependent on ATP through its binding to PotA protein (12).

In this study, we tried to identify the functional domain in PotA protein using the purified and several mutated PotA proteins. We found that 8-azido-ATP was attached to cysteine 26, and replacement of cysteine 54 and valine 135 by threonine and methionine, respectively, led to the loss of ATPase and spermidine uptake activities. The results also indicate that PotA protein is associated with membranes through the interaction with PotB and PotC proteins.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions—A polyamine-requiring mutant, E. coli MA261 (13), generously provided by Dr. W. K. Maas, New York University School of Medicine, was grown in medium A in the absence of polyamines as described previously (14). E. coli MA261 potA::Km was prepared from E. coli MA261 by P1kc transduction as described previously (12) according to the method of Lennox (15). A proton-translocating ATPase mutant, E. coli DK8 (16), was kindly provided by Dr. M. Futai, Osaka University, and E. coli J M105 (17) was purchased from Pharmacia Biotech Inc. E. coli J M105atpD was derived from E. coli J M105 by transduction of a P1 phage-infected lysate of E. coli DK8 (ΔatpB-atpC) Ilv::Tn10 and grown in a 17 amino acid-supplemented medium (18) containing 1% glucose. Appropriate antibiotics (30 μg/ml chloramphenicol, 100 μg/ml ampicillin, 50 μg/ml kanamycin, and 15 μg/ml tetracycline) were added during the culture of E. coli.

Plasmids—Plasmids pPT104, pPT79, pMWpCadB, pMWpCadB (V135M), pKKpotABC, and pKKpotABC (V135M) were prepared as described previously (8, 12). Plasmid pKKpotA was constructed from pKKpotABC by deleting the 1.8-kb DraII-PstI fragment (PstI site from the vector). Plasmids pMWpCadB and pMWpCadB (V135M) were

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† To whom correspondence should be addressed.

1 The abbreviations used are: kb, kilobase(s); PCR, polymerase chain reaction; HPLC, high performance liquid chromatography; NEM, N-ethylmaleimide.
prepared using the modified pMW119 (19), in which the EcoRI-recognized segment had disappeared (12). Site-directed mutagenesis by overlap extension using polymerase chain reaction (PCR) (20) was used for the preparation of mutated potA genes (potA2 (C26A), potA3 (C54T), and potA4 (C276A)). The primers used for mutagenesis were 5'-GGAGATTCCAAAGGCTTGTGATGTTA-3', 5'-GCCCT-TCTGTTACGGTTAAACCA-3', 5'-GCCGCGGCAACTATATTTACGTTA-3' and their complementary deoxynucleotides. To obtain 1.5-kb mutated potA genes, PCR was performed using 5'-TAAGAATCCACGGGTTTGCCTGTTA-3' and 5'-GGAGATTCCAAAGGCTTGTGATGTTA-3' as the 5'- and 3'-primers, respectively. PCR products of the 1.5-kb mutated potA2, potA3, and potA4 genes were purified by digestion with StuI, KpnI, and SspI, respectively. The PCR products were then digested with EcoRI, and 1.0-kb fragments were ligated with a 5.8-kb fragment obtained from pMW119AB. As a result, pMW119AB (C26A), pKK223−3 (C54T), and potA4 (C276A) were constructed.

To construct pKKpotA2BC, the 1.3-kb Styl fragment and the 5.1-kb StyldraII fragment were prepared from pKKpotA2 (C26A), and pKKpotA4BC, respectively. The 1.4-kb StyldraII fragment prepared from pMW119AB was then ligated together with the 1.3-kb Styl and 5.1-kb StyldraII fragments described above. Plasmid pKKpotA2BC was constructed by ligating the 0.8-kb SphI fragment of PCR product for the potA3 gene and the 7.0-kb SphI fragment of potA2BC. Plasmid pKKpotA4BC was constructed by ligating the 0.6-kb XbaI–StyldraII fragment of pMW119AB and the 7.2-kb XbaI–StyldraII fragment of pKKpotA2BC. Transformation of E. coli cells with various plasmids was carried out as described by Maniatis et al. (21).

The strains and plasmids used in this study are listed in Table I. Purification of PotA Protein—E. coli J M105/pKKpotA2 was grown in 20 liters of LB medium at 37°C. When cell growth reached A600nm = 0.3, 0.5 mM isopropl-β-D-thiogalactopyranoside was added to the medium, and the culture was continued for 3 hours. 100,000 g supernatant (3.9 g of protein) was prepared as described previously (22), using Buffer A containing 0.1 M potassium phosphate buffer, pH 7.5, 10 mM EDTA, and 20 µM FUT-175 (6-amino-2-naphthyl-4-quinodino-benzoate dihydrochloride), a protease inhibitor (23). The proteins (2.5 g) precipitated with 50% saturation of (NH4)2SO4 were dissolved in Solution A (1 M diethylthiolretol, 10 µM FUT-175, and 10% glycylglycin) containing 0.15 M potassium phosphate buffer, pH 7.5, and were applied to a DEAE-Sephadex A-50 column (4.6 cm × 13 cm) previously equilibrated with Solution A containing 0.15 M potassium phosphate, pH 7.5. The column was washed with a linear gradient of 0.08–0.4 M potassium phosphate, pH 7.5, in Solution A (600 ml). The PotA protein was eluted at 0.4 M potassium phosphate, and the protein fraction containing PotA protein (110 mg) was concentrated by ultrafiltration. After the concentration of potassium phosphate was adjusted to 80 mM, the protein was applied to a Bio-Rad Econo-Pac Q Cartridge (5 ml) previously equilibrated with Solution A containing 80 mM potassium phosphate buffer, pH 7.5. This column was washed with a linear gradient of 0.08–0.4 M potassium phosphate, pH 7.5, in Solution A (200 ml). The fraction containing PotA protein (23.4 mg) was concentrated and chromatography with a Bio-Rad Econo-Pac Q Cartridge was repeated. Finally, 10.2 mg of PotA protein (95% purity) was obtained (Fig. 1). PotA protein was identified by Western blotting using antibody for PotA protein as described below. Mutated PotA protein (V135M) (12) was purified from E. coli JM105/pKKpotABC (C26A).

Assays for ATPase and Spermidine Uptake—Inside-out membrane vesicles were prepared by French press treatment of E. coli cells suspended in 0.1 M potassium phosphate buffer, pH 6.6, and 10 mM EDTA according to the method of Houn et al. (24). ATPase activity was measured by the method of Lill et al. (25), except that the reaction mixture (0.025 ml) contained 50 mM Hepes-KOH, pH 7.5, 50 mM KCl, 10 mM magnesium acetate, 1 mM [γ-32P]ATP (specific activity, 10–20 cpn/pmol), and purified PotA protein or inside-out membrane vesicles. Spermidine uptake by intact cells was performed as described previously (4) using 10 µM [14C]spermidine as substrate.

Photoaffinity Labeling of PotA Protein with 8-Azido ATP—Inside-out membrane vesicles (100 µg of protein) were added to a buffer containing 10 mM Tris-HCl, pH 7.4, 5 mM CaCl2, and 4 µM 8-azido-[32P]ATP (111 kBq) in a final volume of 0.1 ml and placed in a well on ice. The reaction mixture was irradiated for 3 min with UV light (12 watts) at 365 nm at a distance of 2 cm (26). The samples were then centrifuged for 20 min at 500,000 × g, resuspended without boiling in Laemmli sample buffer (27) with the omission of 2-mercaptoethanol, and subjected to electrophoresis on a sodium dodecyl sulfate-12% polyacrylamide gel. Autoradiography of dried gel was performed at −80°C using an intensifying screen.

Identification of 8-Azido-ATP Photoaffinity-labeled Amino Acid in PotA Protein—Photoaffinity labeling of PotA protein was carried out as described above except that the reaction mixture (0.1 ml) contained 40 µg of purified PotA protein and 50 µM 8-azido-ATP. Digestion of 8-azido-ATP-labeled PotA protein with 1-teramino-2-phenylethyl chloromethyl ketone-treated trypsin was performed according to the method of Mimura et al. (28) using 0.8 µg of the enzyme. Peptides thus obtained were separated by HPLC as described previously (29). The peptide peaks absorbing ultraviolet rays at 260 nm were analyzed by automated Edman degradation on a protein sequencer (Applied Biosystems model 477A) equipped with a phenylthiohydantoin analyzer (model 120A).

Binding of N-[14C]ethylmaleimide to PotA Protein—The reaction mixture (1.0 ml) containing 50 µM Hepes-KOH, pH 7.5, 50 mM KCl, 0.1 M magnesium acetate, 0.16 mM N-[14C]ethylmaleimide (37 kBq) and 50 µg of protein of inside-out membrane vesicles was incubated at 37°C for 30 min. The reaction was terminated by the addition of dithiothreitol at the final concentration of 1 mM. The proteins of membrane vesicles were subjected to gel electrophoresis (27) followed by fluorography (30). Radioactivity on dried gel was quantified by Fuji imaging analyzer Fuji BAS 2000 (Fuji Photo Film Co. Ltd).
from the nucleotide sequence of to be 45 kDa, close to the deduced molecular mass (43 kDa). The molecular mass of the band was estimated during this process. The specific activity was approxi-

mately 400 nmol/min/mg of protein (Table II), and the

already accumulated spermidine, the inhibition may be in op-

FIG. 2. Effect of Mg$^{2+}$ (A) and spermidine (B) on ATPase activity of PotA protein. Assays were performed under standard condi-

tions except that Mg$^{2+}$ in the reaction mixture was changed and sper-

midine was added to the reaction mixture as shown in the figure. Each value is the average of three determinations. The standard deviation was within ±10% for each data point.

FIG. 3. Effect of N-ethylmaleimide (A) and p-chloromercuri-

benzoic acid (B) on ATPase activity of PotA protein. Assays

were performed with (●) or without (○) 2 mM dithiothreitol. Each value is the average of three determinations. The standard deviation was within ±10% for each data point.

FIG. 4. 8-azido-[α-$^{32}$P]ATP labeling of PotA1 (V135M) protein in inside-out membrane vesicles. Numbers on the left represent molecu-

lar mass in Da. A. Coomassie Blue staining of protein; lane 1, the vesicles prepared from E. coli JM105, lane 2, the vesicles prepared from E. coli JM105/pKKpotABC, B. 8-azido-[α-$^{32}$P]ATP labeling of proteins; lanes 1 and 2, labeling was performed using the vesicles prepared from E. coli JM105 in the absence and presence of 1 mM ATP, respectively; lanes 3 and 4, labeling was performed using the vesicles prepared from E. coli JM105/pKKpotABC in the absence and presence of 1 mM ATP, respectively.

| Vesicles          | ATPase activity$^*$ (nmol/min/mg protein) |
|-------------------|------------------------------------------|
| No plasmid        | 20.2 ± 1.2                               |
| pKKpotABC         | 198 ± 15                                  |
| pKKpotA1BC        | 25.8 ± 1.9                                |
| PotA              | 396 ± 23                                  |
| PotA1 (V135M)     | <5                                       |

$^*$ Mean ± S.D.
membrane vesicles did not have any ATPase activity (Table II). The inability of PotA1 protein to hydrolyze ATP was confirmed with purified PotA1 protein. These results indicate that PotA1 protein is a mutant of ATPase activity.

Identification of 8-Azido-ATP Photoaffinity-labeled Amino Acid—Purified PotA protein was photoaffinity-labeled with 8-azido-ATP in the presence of 5 mM Ca^{2+}, in which the ATPase activity of PotA protein was inhibited. Then, the PotA protein was digested with trypsin and subjected to HPLC to separate the hydrolyzed products. Fig. 5 shows the peptide elution profile obtained by HPLC. There were two peaks showing an absorbance at 260 nm: one (peak A) was eluted at 11.3 min and the other (peak B) at 24.5 min. Since peptide B also showed absorbance at 275 nm and $A_{275}$ was higher than $A_{260}$, it was expected that peptide B contained tryptophan. The sequence of peptide B was MAINWVESWVLADIEHK, corresponding to the carboxyl-terminal tryptic peptide (360–378) of PotA protein (Fig. 6A). However, peptide A did not show strong absorbance at 275 nm, indicating that it contained the 8-azido-ATP photoaffinity-labeled amino acid. The sequence of peptide A was XFDGK (Fig. 5). The first amino acid was not identified and the others corresponded to the 27–30 amino acids of PotA protein (Fig. 6A). Thus, it was concluded that cysteine 26 was photoaffinity-labeled with 8-azido-ATP.

Determination of Cysteine Residue Involved in ATPase Activity of PotA Protein—PotA protein contains three cysteine residues (Cys-26, Cys-54, and Cys-276). To identify which cysteine is involved in its ATPase activity, they were converted to alanine, threonine, and alanine, respectively, using site-directed mutagenesis on the potA gene. ATPase activity was measured using inside-out membrane vesicles prepared from E. coli JM105 atp/pKK potABC, in which the potA gene was modified by site-directed mutagenesis. As shown in Table III, only PotA3 (C54T) protein did not have significant ATPase activity. The other proteins A2 (C26A) and A4 (C276A) showed the ATPase activity. Since the amount of PotA4 protein on the vesicles was small (Figs. 7, C and D), the ATPase activity of the vesicles with PotA4 protein was lower than with PotA2 protein. When another PotA3* (C54A) protein was used, the vesicles containing the protein did not show any ATPase activity, in spite of its small amount (rapidly degraded) (data not shown). Spermidine uptake activity was measured using E. coli MA261 potA::Km/pMWpotAB, in which potA gene was modified by site-directed mutagenesis. The uptake activity paralleled the activity of ATPase. E. coli MA261 potA::Km/pMWpotA3B showed very low spermidine uptake activity (Table III).

PotA2, PotA3, and PotA4 proteins were photoaffinity-labeled with 8-azido-ATP (Fig. 7A). In a foregoing paragraph, we identified cysteine 26 as photoaffinity-labeled amino acid. Thus, threonine 39 may be photoaffinity-labeled with 8-azido-ATP.
ATPase activity of normal and mutated PotA proteins was measured using inside-out membrane vesicles prepared from E. coli JM105/pKK carrying pKKpotABC, pKKpotA2BC, pKKpotA3BC, or pKKpotA4BC, and spermidine uptake activity was measured using E. coli MA261/pKK carrying pMWpotA4B, pMWpotA2B, pMWpotA3B, or pMWpotA4B, as described under “Experimental Procedures.” Each value is the average of three determinations.

### Table III

| PotA Protein | ATPase Activity -NEM | ATPase Activity +2 mM NEM | Spermidine Uptake Activity -NEM | Spermidine Uptake Activity +2 mM NEM |
|--------------|----------------------|--------------------------|-------------------------------|-----------------------------------|
|              | nmol/min/mg protein  | nmol/min/mg protein      | nmol/min/mg protein            | nmol/min/mg protein               |
| Normal       | 267 ± 19 (100)       | 13                       | 1.08 ± 0.14 (100)             | 0.05                              |
| A2 (C26A)    | 256 ± 18 (96)        | 11                       | 0.92 ± 0.09 (85)              | 0.06                              |
| A3 (C54T)    | 18 ± 19 (6.7)        | 6.2                      | 0.08 ± 0.02 (7.4)             | 0.03                              |
| A4 (C276A)   | 169 ± 13 (63)        | 10                       | 0.70 ± 0.08 (65)              | 0.04                              |

*Mean ± S.D. Values in parentheses represent percent activity.

### DISCUSSION

The spermidine-preferential uptake system belongs to periplasmic active transport systems (permeases), which consist of one periplasmic substrate-binding protein and three membrane-bound components (7). One of the membrane components is a nucleotide-binding protein involved in energy supply. A model for the structure of the nucleotide-binding protein (HisP) in the histidine transport system was proposed by analogy to the adenylate kinase structure (33). It was shown that site A (Gly-X-X-Gly-X-Gly-Lys) of consensus amino acid sequences for nucleotide binding is important for ATP hydrolysis. Furthermore, MalK protein, the nucleotide-binding protein in the maltose transport system, has been purified to homogeneity, and it was reported that the specific activity of MalK protein as ATPase was approximately 130 nmol/min/mg of protein (34). The specific activity of PotA protein as ATPase was 396 nmol/min/mg of protein, higher than that of MalK protein, and the activity was inhibited by NEM. We found that cysteine 54, located in site A (OPSGCGTK), is involved in ATP hydrolysis. This supports the idea that site A is important for ATP hydrolysis (33). We found that valine 135 is also located in the active center of ATPase in PotA protein. Replacement of proline 172 by threonine in site B (Pro-X-Val(Leu)-Leu-X-Leu-X-Asp-Glu) of the consensus amino acid sequences for nucleotide binding in HisP protein-stimulated ATPase activity (35, 36). These results indicate that the active sites of ATP hydrolysis of the nucleotide-binding protein in periplasmic active transport systems are located both within and between the two consensus amino acid sequences for nucleotide binding.

In MalK protein, cysteine is located at the equivalent position of cysteine 54 of PotA protein (37). When this cysteine was replaced by glycine, the transport activity did not change significantly (38). This suggests that cysteine may not necessarily be essential, but still be important for the ATPase and transport activities. When ATPase activities of HisP and MalK were measured using proteoliposomes equivalent to right-side-out membrane vesicles, the existence of substrate and substrate-binding protein in proteoliposomes was essential for the ATPase activity (39, 40). Structure of the nucleotide-binding protein in inside-out membrane vesicles or in free form may be different from that in proteoliposomes.

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**A. 8-Azido-[α-32P]ATP labeling**

![Image of ATP labeling](image)

**B. [14C]NEM labeling**

![Image of NEM labeling](image)

**C. Western blotting**

![Image of Western blotting](image)

**D. Coomassie blue staining**

![Image of Coomassie staining](image)
We also found that cysteine 26 was photoaffinity-labeled by 8-azido-ATP. Cysteine 26 is the 24th amino acid from site A of the NH2-terminal end of the nucleotide-binding protein in periplasmic active transport systems. When cysteine 26 of PotA protein was replaced by alanine, the mutated PotA protein was still photoaffinity-labeled by 8-azido-ATP. Threonine 39 in the mutated PotA protein might be photoaffinity-labeled by 8-azido-ATP instead of cysteine 26, since it has been reported that serine 41 in HisP protein was also photoaffinity-labeled in addition to histidine 19 in the presence of 5 mM Ca2+ and 10 mM Mg2+ (28). Thus, the pocket for ATP on the nucleotide-binding protein in periplasmic active transport systems may be wide. The results taken together indicate that the center of ATPase activity is located in the NH2-terminal portion in PotA protein. In fact, the NH2-terminal peptide consisting of 239 amino acids of PotA protein showed ATPase activity.2

It has been reported that the interaction of HisP protein with membranes was enhanced by HisQ and HisM membrane proteins (41). We also found that the association of PotA protein with membranes was strengthened by the existence of PotB and PotC channel-forming proteins. When the secondary structures of PotB and PotC proteins were compared, common amino acid sequences, LEAAR(K)DLGAS, were observed in the hydrophilic region (8). These sequences may be involved in the interaction with PotA protein. It has been reported that a similar amino acid sequence has been found in a hydrophilic loop of channel-forming membrane proteins in periplasmic active transport systems (42).

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