The ATPase activity and the Functional Domain of PotA, a Component of the Spermidine-preferential Uptake System in Escherichia coli*

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The ATPase activity of PotA, a component of the spermidine-preferential uptake system consisting of PotA, B, C, and D, was studied using purified PotA and a PotABC complex on inside-out membrane vesicles. It was found that PotA can form a dimer by disulfide cross-linking but that each PotA molecule functions independently. When PotA was associated with the membrane proteins PotB and PotC, the Km value for ATP increased and PotA became much more sensitive to inhibition by spermidine. It was also shown that spermidine uptake in cells was gradually inhibited in parallel with spermidine accumulation in cells. The results suggest that spermidine functions as a feedback inhibitor of spermidine transport. The function of PotA was analyzed using PotA mutants obtained by random mutagenesis. There are two domains in PotA. The NH2-terminal domain (residues 1–250) contains the ATP binding pocket formed in part by residues Cys36, Phe57, Phe46, Cys54, Leu66, and Leu76, the active center of ATPase that includes Val113 and Asp172, and amino acid residues necessary for the interaction with a second PotA subunit (Cys36) and with PotB (Cys54). The COOH-terminal domain (residues 251–378) of PotA contains a site that regulates ATPase activity and a site involved in the spermidine inhibition of ATPase activity.

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The cellular content of polyamines, which play important roles in cell proliferation and differentiation (1, 2), is regulated by biosynthesis, degradation, and transport. In Escherichia coli, the genes for three different polyamine transport systems have been cloned and characterized (3). Two uptake systems (spermidine-preferential, PotABCD, and putrescine-specific, PotFGHI) were ABC (ATP binding cassette) transporters, each consisting of a periplasmic substrate-binding protein (PotD or PotF), two transmembrane proteins (PotB and C or PotH and I), and a membrane-associated ATPase (PotA or PotG) (4). The third transport system, catalyzed by PotE, comprises of HisP (12) and MalK (13), membrane-associated ATPases of the histidine and maltose uptake systems, respectively, which have an extra COOH-terminal domain, similar to that in MalK. PotA (378 amino acid residues) is also expected to have an extra COOH-terminal domain, similar to that in MalK. The present work was studied by using purified PotA and a PotABC complex.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Culture Conditions—A polyamine-requiring mutant, E. coli MA261 (16), was generously provided by Dr. W. K. Maas, New York University School of Medicine. E. coli MA261 potA::Km was prepared from E. coli MA261 as described previously (17) and was grown in medium A in the absence of polyamines (18). A proton-translocating ATPase mutant, E. coli DK8 (19), was kindly supplied by Dr. M. Futai, Osaka University, and E. coli JM105 atpC was prepared by transduction of a P1 phage-infected lysate of E. coli DK8 (ΔatpB-atpC ilv-tk(A1001) and grown in an 18-amino acid-supplemented medium (20) containing 1% glucose. Plasmids pMWpotAB and pKK-potABC were prepared as described previously (17). Transformation of E. coli cells with plasmids was carried out as described by Maniatis et al. (21). Appropriate antibiotics (100 µg/ml ampicillin, 15 µg/ml tetracycline, and/or 50 µg/ml kanamycin) were added during the culture of E. coli having the above plasmids.

Random Mutagenesis and Selection of PotA Mutants—Random mutagenesis was carried out using a PCR-based strategy (22). To obtain 1.5 kilobase pairs of mutated potA genes, PCR was performed using 5'-TAAGGTCACCAAGGTTGGTTACCC-3' (P1, sequence for –61 to –38 of potA gene) and 5'-CGCGGCCCATGGTGGCAACATT-3' as

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The conserved amino acid residues are shown by black boxes with white lettering. Walker motif A and B, ABC signature, and switch region are indicated. PotA, *E. coli*; MalK, *Thermococcus litoralis*; HisP, *Salmonella typhimurium*. *", amino acid residues of PotA identified as functional amino acids in this and previous studies (11, 17).

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FIG. 1. Alignment of the amino acid sequence of membrane-associated ATPase of some ABC transporters. The conserved amino acid residues are shown by black boxes with white lettering. Walker motif A and B, ABC signature, and switch region are indicated. PotA, *E. coli*; MalK, *Thermococcus litoralis*; HisP, *Salmonella typhimurium*. *", amino acid residues of PotA identified as functional amino acids in this and previous studies (11, 17).

the 5'- and 3'-primers, respectively. The first cycle was carried out in the presence of 200 μM of each of three dNTPs and 0.5 μM of the fourth dNTP, with 200 μM of the fourth dNTP added in the subsequent 24 cycles. Four separate reaction mixtures (with dA, dC, dG, and dT, each at low concentrations in the first cycle) were combined, purified, and digested with EcoRI. The digested fragments, which encode 24–345 amino acid residues of PotA, were ligated with a 5.8-kilobase pair fragment obtained with pMWpotAB. *E. coli* MA261 potA::Km was transformed with the mutated pMWpotAB, and cells were grown on 1.5% agar plates containing medium A and 30 μg/ml spermidine. Colonies thus obtained were used for the assay of spermidine transport described below.

**Construction of pMWpotAC1 and pMWpotAC2**—To construct pKppotAC1, PCR was performed using pMWpotAB as template, P1 as described above, and 5'-CCCCCTGCAGTTAAACACGTAAGTCTT-3' as primers. The PCR product thus obtained was digested with Csp45I and inserted into the same restriction site of pMWpotABC. Site-directed mutagenesis by overlap extension using PCR (23) was performed to prepare pKppotAC2. The template used for the first PCR was pMWpotAB. Primers used for first PCR were P1 and 5'-AACAAA-CAGGTTTAAACGCTGCTGCTG-3' (complementary sequence for 732-707 except underlined base), and 5'-AGGGTTAACGTAGATATTACAT-3' (P2, complementary sequence for 845–825). A second PCR was performed using initial PCR products as templates and P1 and P2 as primers. PCR product thus obtained was digested with Csp45I and XbaI and inserted into the same restriction sites of pKppotAC1 (11). pMWpotAC1 was constructed by inserting the StyI-XbaI fragment of pKppotAC2 into the same restriction sites of pMWpotAB. Similarly, pMWpotAC2 was constructed by inserting the

StyI-XbaI fragment of pKppotAC1 into the same restriction sites of pMWpotAB.

**Preparation of pMWpotE297Q** and pE297D—To construct these mutants, site-directed mutagenesis was carried out by using the QuikChange™ site-directed mutagenesis kit (Stratagene).

**Construction of pKppotABC Mutants**—To examine the ATPase activity of the mutants whose spermidine uptake activity was decreased, pKppotABC mutants were constructed. As for PotA F27L, F45L, L60F, F27L, F45L, L60F, L76P, D172N, and C2, pKppotABC mutants were constructed by inserting the MunI-XbaI fragment of pMWpotAB mutants into the same restriction sites of pKppotABC. As for PotA E297K, E297Q, E297D, and E297Q, site-directed mutagenesis was carried out by using the QuikChange™ site-directed mutagenesis kit (Stratagene).

**Construction of pKppotABC Mutants**—To examine the ATPase activity of the mutants whose spermidine uptake activity was decreased, pKppotABC mutants were constructed. As for PotA F27L, F45L, L60F, L76P, D172N, and C2, pKppotABC mutants were constructed by inserting the MunI-XbaI fragment of pMWpotAB mutants into the same restriction sites of pKppotABC. As for PotA E297K, E297Q, E297D, and C1, pKppotABC mutants were constructed by inserting the XbaI-DraI fragment of pMWpotAB mutants into the same restriction sites of pKppotABC. Nucleotide sequences of all mutants described above were determined by DNA Sequencer DQ-1000 (Shimazu) or Seq 4 personal sequencing system (Amersham Biosciences). pKK potA1BC(V135M), potA2BC(C26A), potA3BC(C54T), and potA4BC(C343E) were prepared as described previously (11, 17).

**Assays for Spermidine Uptake and ATPase**—Spermidine uptake by intact cells (*E. coli* MA261 potA::Km/pMWpotAB) was performed as described previously (24) using 10 μM [14C]spermidine as substrate.

*Inside-out membrane vesicles were prepared from *E. coli* JM105 Δatp-D-1-thiogalactopyranoside for 2 h at 37°C by French press treatment of the *E. coli* cells suspended in 0.1 M potassium phosphate buffer, pH 6.6, and 10 mM EDTA according to the method of Houng et al. (25). ATPase

The mutated PotA protein E297Q contains glutamine instead of glutamic acid at position 297.

**Fig. 1. Alignment of the amino acid sequence of membrane-associated ATPase of some ABC transporters.** The conserved amino acid residues are shown by black boxes with white lettering. Walker motif A and B, ABC signature, and switch region are indicated. PotA, *E. coli*; MalK, *Thermococcus litoralis*; HisP, *Salmonella typhimurium*. *", amino acid residues of PotA identified as functional amino acids in this and previous studies (11, 17).

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activity was measured by the method of Lill et al. (26), except that the reaction mixture (0.1 ml) contained 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM MgCl₂, 0.5 mM \( [\text{H}^32\text{P}]\text{ATP} \) (specific activity, 20–50 cpm/pmol), and 10 µg of protein of inside-out membrane vesicles. PotA was purified as described previously (11). ATPase activity of purified PotA was measured using 4 µg of protein instead of 10 µg of protein of inside-out membrane vesicles.

**Photoaffinity Labeling of PotA Protein with 8-Azido-ATP**

Inside-out membrane vesicles (50 µg of protein) were added to a buffer containing 10 mM Tris-HCl, pH 7.4, 5 mM CaCl₂, and 4 µM 8-azido-[\( \gamma ^{32}\text{P} \)]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 (122 watts) at 365 nm (27). The samples were then centrifuged for 20 min at 150,000 \( \times \) g, resuspended without boiling in 2-mercaptoethanol-free SDS sample buffer (28), and subjected to 12% SDS-polyacrylamide gel electrophoresis. Binding strength of 8-azido-[\( \gamma ^{32}\text{P} \)]ATP to PotA was estimated with a Fujix Bas 2000II imaging analyzer using the dried gel.

**Western Blot Analysis of PotA Protein**

Antibodies for PotA protein were prepared as described previously (29) using the conjugate of the deduced NH₂-terminal 18 amino acids (GQSKKLNKQPSSLSPLVQ) or the deduced COOH-terminal 14 amino acid residues (VESWEVVLAEDEEHK) of PotA protein together with bovine thyroglobulin. Western blotting was performed according to the method of Nielsen et al. (30).

**Disulfide Cross-linking**

Disulfide cross-linking was performed by the method of Kubo et al. (31) with some modifications. The reaction mixture (50 µl) containing 10 mM Tris-HCl, pH 8.0, 28 mM KCl, 2% glycerol, 0.4 mM 2-mercaptoethanol, and 50 µg of inside-out membrane vesicle protein was incubated with 1,6-bis-maleimidohexane (BMH; spacer arm, 16 Å) (32) at a final concentration of 0.5 mM at 25 °C for 30 min. After the samples were mixed with 2-mercaptoethanol-free SDS sample buffer (28), samples containing 10 µg of protein were subjected to 12% SDS-polyacrylamide gel electrophoresis, and subsequent Western blotting was performed as described above. pKK potAB(C69S)C, potAB(C201S)C, potAB(C69S/C201S)C, potABC(C139S), potABC(A74C), potABC(S192C), potA(E297K)BC(C139S), and potA(E297K)B-
Polyamine levels in E. coli—PotABC Complex

The ATPase activity of purified PotA and of a PotABC complex on inside-out membrane vesicles was measured as described under "Experimental Procedures." Spermidine uptake and ATPase activity of native PotA were 1.21 nmol/min/mg of protein and 454 nmol/min/mg of protein, respectively. ATPase activity (51 nmol/min/mg of protein) by inside-out membrane vesicles of JM105amp/pKK223–3 (vector) was subtracted from the data. The values are expressed as the mean ± S.D. for triplicate determinations. B, positions of amino acid residues identified by random mutagenesis as being involved in spermidine uptake and ATPase activity in PotA are shown by boxes.

RESULTS

Comparison of the Amino Acid Sequences of PotA, MalK, and HisP—The amino acid sequence of PotA was first compared with the sequences of MalK and HisP, whose structures have already been determined by x-ray crystallography (12, 13). MalK has a longer COOH-terminal domain compared with HisP. As shown in Fig. 1, the NH2-terminal domain (amino acid residues 1–250) of PotA was similar to that of MalK and to the sequence of HisP. The COOH-terminal domain of PotA (amino acid residues 251–378) had only limited identity to that of MalK, in which the regulatory site of mal regulon (14) and ATPase activity (15) exists. We have previously reported that the NH2-terminal domain of PotA has the active site of ATPase (11, 17). We hypothesized that the COOH-terminal domain of PotA has a unique function related to the spermidine uptake system of which PotA is a component.

Comparison of ATPase Activities of Purified PotA and a PotABC Complex—The ATPase activity of purified PotA and of a PotABC complex on inside-out membrane vesicles was measured in the presence of 50 mM K+ and 10 mM Mg2+. As shown in Fig. 2A, the Km value for ATP with purified PotA was 390 μM with a Hill coefficient of 0.98. The Km value for ATP increased about 3-fold for the PotABC complex (1.49 mM) compared with PotA, and the Hill coefficient was 1.02 (Fig. 2B). The results indicate that the affinity for ATP decreases when PotA makes a complex with PotB and PotC and that there is no cooperativity for ATP during ATP hydrolysis even in the PotABC complex.

When PotA was complexed with PotB and PotC, sensitivity to stimulation by Mg2+ and to inhibition by spermidine are both greatly increased (Fig. 3). The Ks value for spermidine was ~10 μM at the PotABC complex, and spermidine uncontrollably inhibited PotA activity, suggesting that spermidine binds at a site on PotA different from the ATP recognition site. When cells were incubated for 40 min in the presence of 100 μM spermidine, spermidine gradually accumulated in cells, and spermidine uptake activity was reduced in parallel with spermidine accumulation (Fig. 4). Under these conditions, the level of PotA in cells did not change significantly as determined by Western blotting of PotA (data not shown). These results suggest that spermidine functions as a feedback inhibitor of spermidine uptake through inhibition of the ATPase activity of PotA.

Characteristics of PotA Mutants That Influence Spermidine Uptake—To determine which regions are involved in the function and regulation of PotA, PotA mutants that influence spermidine uptake were isolated by random mutagenesis. E. coli MA261 potA::Km was transformed with the mutant pMWpotA, colonies were isolated, and the spermidine uptake of the colonies were measured. As shown in Fig. 5A, a number of PotA mutants that reduced spermidine uptake were isolated. These were PotA F27L, F45L, L60F, L76P, D172N, and E297K. Another mutant, PotA V135M, was an ATPase-deficient mutant that has been previously isolated (17). All mutants except PotA E297K were located in the NH2-terminal domain in which the active center of ATPase is located (Fig. 5B). To clarify the role of the COOH-terminal domain in ATPase activity, PotA mu-
Levels of native PotA, PotA C1, and PotA C2 were measured of ATP using 8-azido-ATP binding to Cys26 (11) with inside-out mutants, suggesting that Asp172 and Val 135 are part of the binding was observed at both the D172L and V135M (11), although ATPase activity was abolished, similar to effects seen with a V135M mutant (11), although ATP binding was reduced due to a decrease in \( V_{\text{max}} \). Although the \( K_m \) value for ATP was decreased with PotA L76P, the strength of ATP binding was also reduced. Interaction of 8-azido-ATP with Cys26 on PotA L76P may be reduced due to the structural change of PotA L76P.

The COOH-terminal mutants E297K, E297Q, and E297D had ATP binding affinities and an intact ATP binding site similar to native PotA, judging from the \( K_m \) values and binding of 8-azido-ATP to Cys26 (Table I). However, the ATPase activity of these mutants was greatly reduced, indicating that the COOH-terminal domain is involved in regulation of ATPase activity. Similar results were obtained with the COOH-terminal-truncated mutants C1 and C2, but the apparent decrease in ATP binding at these mutants (0.64 and 0.70 in Table I) may be due to a decrease in the affinity of the C1 and C2 mutants for PotB and PotC. To test this idea, the total amount of native and mutant PotA in cells and the amount in the cell membranes were measured by Western blotting. Although comparable amounts of native PotA and C1 and C2 mutants were produced in cells (Fig. 6A), the amount of C1 and C2 associated with membrane was less than that of the native PotA (Fig. 6B). These results are consistent with the idea that the COOH-terminal domain stimulates the association between PotA and the membrane-bound PotB and PotC subunits.

The COOH-terminal domain of PotA is involved in the inhibition of ATPase activity by spermidine. As shown in Fig. 7A, mutants in the NH\(_2\)-terminal domain did not affect inhibition by spermidine, whereas mutants in the COOH-terminal domain greatly reduced inhibition by spermidine (Fig. 7B). This suggests that the COOH-terminal domain of PotA has a spermidine binding site involved in the regulation of its ATPase activity.

Interaction of PotA with a Second PotA Subunit and with PotB and PotC—To examine how PotA interacts with a second PotA subunit and with PotB and PotC, cross-linking of cysteine residues between subunits was studied using BMH (spacer arm, 16 Å). In PotA, there are three cysteine residues (Cys206, Cys274, and Cys375, see Fig. 5B). There are two cysteine residues in PotB (Cys65 on the second transmembrane segment and Cys201 on the fifth transmembrane segment) and one cysteine residue in PotC (Cys139 on the fourth transmembrane segment) (29).

When native PotA was used for the cross-linking experiment, a PotA dimer as well as a PotA-PotB complex were formed (Fig. 8A, lane 2). Similar results were obtained with PotA V135M, F27L, F45L, L60F, L172N, E297Q, and E297D (Fig. 8A). However, no PotA-PotB complex was obtained with PotA L76P (Fig. 8A, lane 7), and a PotA-PotC complex was obtained instead of

### Table I

| Domain                  | Mutant | \( K_m \) \( \mu \text{M} \) | \( V_{\text{max}} \) \( \text{nnoles/mg protein} \) | Relative binding strength |
|-------------------------|--------|-----------------------------|-------------------------------------|--------------------------|
| NH\(_2\)-terminal domain|        |                             |                                     |                          |
| Wild                    | 1290   | 833                         | 1.00                                |                          |
| F27L                    | 7000   | 2000                        | 0.55                                |                          |
| F45L                    | 2010   | 1830                        | 0.61                                |                          |
| L60F                    | 2630   | 1110                        | 0.42                                |                          |
| L76P                    | 318    | 282                         | 0.65                                |                          |
| D172N                   | ND     | ND                          | 0.50                                |                          |
| E297K                   | 633    | 158                         | 1.12                                |                          |
| E297Q                   | 481    | 50                          | 0.84                                |                          |
| E297D                   | 855    | 208                         | 0.92                                |                          |
| C1                      | 769    | 77                          | 0.64                                |                          |
| C2                      | 925    | 83                          | 0.70                                |                          |
| COOH-terminal domain    |        |                             |                                     |                          |

**Fig. 6. Levels of C1 and C2 PotA mutants in whole cells (A) and on inside-out membrane vesicles (B).** MA261(potA::kan) carrying pKKpotA, pKKpotAC1, or pKKpotAC2 was cultured in medium A until \( A_{540} = 0.3 \), and cell lysate and inside-out membrane vesicles were prepared. Levels of native PotA, PotA C1, and PotA C2 were measured by Western blot analysis using antibody against the NH\(_2\)-terminal region of PotA.
PotA-PotB complex with PotA E297K (Fig. 8A, lane 9).

PotA Cys26 was involved in the formation of PotA dimers because there was no dimer formation with PotA C26A (Fig. 8B, lane 2), and PotA Cys54 was involved in the formation of a PotA-PotB complex because this complex was not formed using PotA C54T (Fig. 8B, lane 3). The formation of a PotA-PotB

FIG. 7. Effect of spermidine on ATPase activity of PotA mutants. ATPase activity of mutated PotA in the NH2-terminal region (A) and in the COOH-terminal region (B) was measured in the presence of 2 mM Mg2+ and various concentrations of spermidine as described under “Experimental Procedures.” ATPase activity of PotA mutants in the absence of spermidine is shown in Fig. 5A. Each point is the average of duplicate determinations.

FIG. 8. Disulfide cross-linking between PotABC complex by BMH. Disulfide cross-linking between PotABC complex by BMH was performed with inside-out membrane vesicles in which mutant PotA, PotB, and/or PotC were included, and it was estimated by Western blot analysis using antibody against the COOH-terminal region of PotA. The positions of PotA dimer, PotA-PotB, PotA-PotC, and PotA are shown by arrows.
complex was confirmed by the finding that its formation was reduced when a cysteine-mutated version of PotB (C69S and C201S) was used (Fig. 8B, lanes 5 and 6). The PotB C69S mutant had a larger effect on formation of the PotA-PotB complex than did the PotB C201S mutant. The PotA-PotB complex was not observed with PotB C69S/C201S double mutant (data not shown). The formation of a PotA E297K-PotC complex was confirmed by the finding that a PotA E297K-PotB complex (rather than PotA E297K-PotC) was formed when Cys139 in PotC was mutated to serine (Fig. 8B, lane 9). Furthermore, no cross-linking between PotA E297K and PotC (or PotB) was obtained when Cys69 and Cys201 in PotB and Cys139 in PotC were mutated (Fig. 8B, lane 10). These results suggest that marked structural changes occurred in the PotA L76P and E297K mutants.

Finally, cysteines were inserted in PotC at positions similar to those where Cys is found in PotB (Cys74 on the second transmembrane segment and Cys192 on the fifth transmembrane segment), and cross-linking of this mutated PotC with PotA was studied. As shown in Fig. 8B, lanes 11 and 12, there was no PotA-PotC complex formed, only a PotA-PotB complex. The results suggest that PotA preferentially and predominantly interacts with PotB rather than PotC.

**DISCUSSION**

In this study, we have investigated the ATPase activity of PotA, a component of the spermidine-preferential uptake system in *E. coli*. Most ABC transporters are thought to utilize two ATPase subunits in the transport process (36), and positive cooperativity for ATP during ATP hydrolysis was reported in HisP (Hill coefficient = 2) (37) and MalK (Hill coefficient = 1.3) (38). However, it has been reported recently that there is no cooperativity for ATP using purified HisP (39) and that one intact HisP in the heterodimers between the wild type and mutant HisP can catalyze ATP hydrolysis and histidine transport (40). For PotA, no cooperativity for ATP was observed (Hill coefficient = 1.0). We have also observed that purified PotA exists predominantly as a monomer as determined by gel filtration (data not shown). Our results indicate that each PotA subunit functions independently, although PotA is able to form a dimer by disulfide cross-linking.

In the spermidine uptake system, ATPase activity is always greater than spermidine uptake activity. For example, when both activities were measured with *E. coli* JM105 Δatp AΔ maltA1 potABC, the ratio of ATPase and spermidine uptake activities was estimated to be 3−10. We reported that spermidine strongly inhibits ATPase activity through its interaction with the COOH-terminal domain of PotA. Spermidine not only functions as a feedback inhibitor of spermidine uptake through the COOH-terminal domain, which is the active center of ATPase. Thus, the RPECL may regulate the ATPase activity through its interaction with the Walker motif B. We expect that the RPECL motif in PotA has a similar function. If this is the case, spermidine may interact with Glu297 in the RPECL motif and inhibit ATPase activity. When PotA makes a complex with PotB and PotC, the cleft between the NH2- and COOH-terminal domains may become a suitable structure to interact with spermidine. Experiments are now in progress to clarify how spermidine and the COOH-terminal domain affect ATPase activity.

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**Spermidine-preferential Uptake System**
The ATPase Activity and the Functional Domain of PotA, a Component of the Spermidine-preferential Uptake System in Escherichia coli

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