Identification and Functions of Amino Acid Residues in PotB and PotC Involved in Spermidine Uptake Activity

Received for publication, September 18, 2010. Published, JBC Papers in Press, October 11, 2010, DOI 10.1074/jbc.M110.186536

Kyohei Higashi†‡§, Yoshiharu Sakamaki†‡, Emiko Herai†, Risa Demizu†, Takeshi Uemura†, Sunil D. Saroj‡, Risa Zenda‡, Yusuke Terui‡, Kazuhiro Nishimura‡, Toshihiko Toida‡, Keiko Kashigawa‡, and Kazuei Igarashi†‡

From the †Graduate School of Pharmaceutical Sciences, Chiba University, 1-8-1 Inohana, Chu-ku, Chiba 260-8675, the ‡Amine Pharma Research Institute, Innovation Plaza at Chiba University, 1-8-15 Inohana, Chuo-ku, Chiba 260-0856, and the §Faculty of Pharmacy, Chiba Institute of Science, 15-8 Shiomi-cho, Choshi, Chiba 288-0025, Japan

Amino acid residues on PotB and PotC involved in spermidine uptake were identified by random and site-directed mutagenesis. It was found that Trp8, Tyr13, Trp100, Leu110, and Tyr261 in PotB and Trp96, Asp108, Glu169, Ser196, Asp198, and Asp199 in PotC were strongly involved in spermidine uptake and that Tyr160, Glu172, and Leu274 in PotB and Tyr19, Tyr88, Tyr149, Glu160, Leu195, and Tyr211 in PotC were moderately involved in spermidine uptake. Among 11 amino acid residues that were strongly involved in spermidine uptake, Trp8 in PotB was important for insertion of PotB and PotC into membranes. Tyr13, Trp100, and Leu110 in PotB and Trp96, Asp108, Ser196, and Asp198 in PotC were found to be involved in the interaction with PotD. Leu110 and Tyr261 in PotB and Asp108, Asp198, and Asp199 in PotC were involved in the recognition of spermidine, and Trp100 and Tyr261 in PotB and Asp108, Glu169, and Asp198 in PotC were involved in ATPase activity of PotA. Accordingly, Trp100 in PotB was involved in both PotD recognition and ATPase activity, Leu110 in PotB was involved in both PotD and spermidine recognition, and Tyr261 in PotB was involved in both spermidine recognition and ATPase activity. Asp108 and Asp198 in PotC were involved in PotD and spermidine recognition as well as ATPase activity. These results suggest that spermidine passage from PotD to the cytoplasm is coupled to the ATPase activity of PotA through a structural change of PotA by its ATPase activity.

Polyamines (putrescine, spermidine, and spermine) play important roles in cell proliferation and differentiation (1–3), and cellular polyamine content is regulated by biosynthesis, degradation, and transport (4, 5). With regard to transport, we characterized three polyamine transport systems in Escherichia coli (5–7). Those were the spermidine-preferential and putrescine-specific uptake systems, which belong to the family of ATP-binding cassette transporters (8), and a protein, PotE, involved in the excretion of putrescine by a putrescine-ornithine antiporter activity. We also examined the properties of CadB, which is a cadaverine-lysine antiporter, and found that both PotE and CadB play important roles for cell growth at acidic pH (9). It has been also reported that a new putrescine uptake protein, PuuP, functions when putrescine is used as an energy source (10). Two uptake systems (spermidine-preferential, PotABCD, and putrescine-specific, PotFGHI) consist 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) (5). Crystal structures of the two substrate-binding proteins (PotD and PotF) have been determined (11, 12). Each consists of two domains with an alternating β-α-β topology, similar to other periplasmic binding proteins (13, 14). The polyamine binding site lies in a cleft between the two domains as determined by crystallography and site-directed mutagenesis (11, 12, 15). We also purified a membrane-associated ATPase (PotA) of the spermidine-preferential uptake system, and some properties of PotA, including the existence of an ATP-binding site in the NH2 terminus, have been determined (16). The crystal structures of HisP (17) and MalK (18), membrane-associated ATPases of the histidine and maltose uptake systems, have been determined. The structure of HisP was very similar to that of the NH2-terminal domain of MalK (17, 18), but HisP (258 amino acid residues) was smaller than MalK (372 amino acid residues), and MalK had an extra domain in the COOH terminus. It has been reported that the COOH terminus of MalK was critical for negative regulation of the mal operon (19). Furthermore, it was found that a mutant (E306K) of the COOH terminus of MalK affects its ATPase activity, suggesting a role for this region in the ATPase activity (20). PotA (378 amino acid residues) was also expected to have an extra COOH-terminal domain, similar to that in MalK. We found that the NH2-terminal domain (residues 1–250) was involved in the recognition of ATP and in the interactions of PotA with a second PotA subunit and with PotB and PotC. The COOH-terminal domain (residues 251–378) of PotA contained a site that regulates ATPase activity and a site involved in the spermidine inhibition of ATPase activity (7). In this study, we tried to identify the amino acids of PotB and PotC involved in spermidine transport by random and site-directed mutagenesis and determined the functions of individual amino acid involved in spermidine transport.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Culture Conditions—A polyamine-requiring mutant, E. coli MA261 (speB speC serA

‡ This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.
1 Both authors contributed equally to this work.
2 To whom correspondence should be addressed: Graduate School of Pharmaceutical Sciences, Chiba University, 1-8-1 Inohana, Chu-ku, Chiba 260-8675, Japan. Tel.: 81-43-224-7500; Fax: 81-43-379-1050; E-mail: iga16077@faculty.chiba-u.jp.
PotABCD Spermidine Uptake System in E. coli

thr leu thi) (21), was generously provided by W. K. Maas, New York University School of Medicine. E. coli MA261 potB::Km was prepared from E. coli MA261 as described previously (22) and was grown in medium A in the absence of polyamines (23). Another polyamine-requiring mutant, E. coli DR112 (speA speB thi) (24), generously provided by D. R. Morris, University of Washington, was grown as described previously (25). An ornithine deficiency in DR112 was achieved by the addition of 1 mg of arginine/ml to the medium. A proton-translocating ATPase mutant, JM105atp−, was prepared as described previously (7) and grown in an 18-aminos acid-supplemented medium containing 1% glucose (26). Protease OmpT- and Lon-deficient mutant BL21(DE3) (27) and a membrane-bound protease FtsH-deficient mutant AR3291 (28), kindly supplied by T. Ogura, Kumamoto University School of Medicine, were also grown in an 18-amino acid-supplemented medium containing 1% glucose (26). A low copy number plasmid pMW119 (Nippon Gene) (29) was digested with BamHI and HindIII. The termini were made blunt-ended with a Klenow fragment and religated, and as a result the BamHI- and HindIII-recognized sequence disappeared from the plasmid. Then, the plasmid was digested with KpnI and Cfr9I, and the 5.5-kbp KpnI and Cfr9I fragment from pPT104 (23) was inserted (pMWpotABCD, 9.7 kbp). The plasmid was further digested with KpnI and Bpu110I, treated with T4 DNA polymerase, and religated. The pMWpotABCD thus obtained was 8.5 kbp and used for the experiments in this study. Transformation of E. coli cells with pMWpotABCD was carried out as described by Maniatis et al. (30). Appropriate antibiotics (100 μg/ml ampicillin and 50 μg/ml kanamycin) were added during the culture of E. coli-containing pMWpotABCD.

Random and Site-directed Mutagenesis and Selection of PotB and PotC Mutants—Random mutagenesis was carried out using a PCR-based strategy (31). To obtain 1.2 kbp of mutated potB and potC genes, PCR was performed using 5′-GAGCGTACAAAACGTCGCCGAAAAC-3′ and 3′-GAGCAAGAGGGCACGCCGCG-3′ as primers for potB and 5′-GTGATGCTGCC-3′ and 3′-TCACGGGCATTGTCGGT-CAAC-3′ as the 5′- and 3′-primers for potC. The first cycle was carried out in the presence of a 200 μM concentration of each of three dNTPs and a 0.5 μM concentration of the fourth dNTP, with a 200 μM concentration 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 XbaI and Sall for potB gene and with Sall and BamHI for potC gene. The digested fragments were inserted to the same restriction sites of pMWpotABCD. The mutated potABCD was transformed to E. coli MA261 potB::Km, and mutants were selected judging from low activity of spermidine uptake. Site-directed mutagenesis of Asp to Asn, Glu to Gln, and Trp and Tyr to Leu on potB and potC genes was carried out with the QuikChange Site-directed mutagenesis kit (Stratagene). A list of oligonucleotide primers for mutagenesis has not been included but is available from the authors upon request. Mutations were confirmed by DNA sequencing using a CEQ8000 DNA genetic analysis system (Beckman Coulter).

Western Blot Analysis of PotA, B, C, and D Proteins on Rightside-out Membrane Vesicles—Antibodies against PotA and PotD were prepared as described previously (32). Antibodies against PotB and PotC were prepared according to the method of Posnett et al. (33) using the multiple antigenic peptides LYYWRASRLNNKVK, which corresponds to the PotB COOH-terminal peptide, and LIARDKTKGNTGVDVK, which corresponds to the PotC COOH-terminal peptide. Rightside-out membrane vesicles were prepared from E. coli MA261 potB::Km/pMWpotABCD according to the procedure of Kaback (34), except that the concentration of lysozyme was decreased from 500 to 50 μg/ml. For Western blot analysis of PotA, PotB, PotC, and PotD proteins, rightside-out membrane vesicles (10 μg of protein for PotA and PotD, and 30 μg of protein for PotB and PotC) were separated by SDS-PAGE (35) on a 12% acrylamide gel and transferred to a polyvinylidine fluoride membrane (Immobilon P; Millipore). The levels of four spermidine uptake proteins were detected with ECL Western blotting reagents (GE Healthcare), except that 0.2% Triton X-100 was used instead of 0.05% Tween 20 (36) and quantified by a LAS-1000 plus luminescent image analyzer (Fuji Film).

Assays for Spermidine Uptake and ATPase—Spermidine uptake by intact cells (E. coli MA261 potB::Km/pMWpotABCD) was measured as described previously (37) using 50 μg of protein of intact cells and 10 μM [14C]spermidine as substrate. Incubation was carried out for 10 min at 30 °C. Rightside-out membrane vesicles from E. coli DR112/pACYCpotABC (22) were prepared as described above. PotD protein was purified as described previously (11). Spermidine uptake by rightside-out membrane vesicles was measured as described above using 100 μg of protein of membrane vesicles and various amounts of PotD protein as shown in the Fig. Inside-out membrane vesicles were prepared from E. coli JM105 atp−/pMWpotABCD 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. (38). ATPase activity was measured by the method of Lill et al. (39), 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 [γ-32P]ATP (specific activity, 20–50 cpm/pmol), and 10 μg of protein of inside-out membrane vesicles. Protein content was determined by the method of Lowry et al. (40).

Assay for PotD Binding to Rightside-out Membrane Vesicles—The reaction mixture (95 μl), containing buffer 1 (50 mM potassium phosphate buffer, pH 6.6, 50 mM Hepes–KOH, pH 7.6, 10 mM MgSO4, 20 mM ascorbic acid, and 10 μM phenazine methosulfate), 50 μg of protein of rightside-out membrane vesicles prepared from E. coli DR112/pACYCpotABC (22), and 1 μg of PotD, was preincubated at 30 °C for 5 min. The reaction was started by the addition of 5 μl of 200 μM spermidine. After incubation at 30 °C for 5 min, the reaction mixture was placed on the top of 20% sucrose in buffer 1 containing 10 μM spermidine, and centrifuged at 12,000 X g for 15 min. The level of PotD in the precipitate containing right-
side-out membrane vesicles was detected by Western blotting after SDS-PAGE (35) as described above.

In Vitro Translation of PotB, PotC, and PotD Proteins—DNA-dependent transcription-translation reactions (41) were carried out as described previously (42) with some modifications using an *E. coli* T7 S30 Extract System for Circular DNA (Promega). A reaction mixture (0.02 ml) contained 0.8 μg of pT7-BCD or pT7-BC (22), 240 kBq of [35S]methionine (27 TBq/mmol), 0.1 mM each 19 amino acids without methionine, 6 μl of T7 S30 extract, and 8 μl of the attached reaction mixture. After incubation at 37 °C for 60 min, 5 μl of 20 μm methionine and 1 ml of ice-cold 5% trichloroacetic acid were added to the reaction mixture. After removal of supernatant, the pellet was dissolved with 25 μl of sample buffer for SDS-PAGE (35) and boiled for 2 min. A 20-μl aliquot was used for 15% SDS-PAGE, and fluorography was performed according to the method of Laskey and Mills (43). Radioactivity of labeled protein was quantified using a BAS-2000II imaging analyzer (Fuji Film).

RESULTS

Identification of Amino Acid Residues in PotB and PotC Involved in Spermidine Uptake—To determine which amino acids in PotB and PotC are involved in spermidine uptake, PotB and PotC mutants that influence spermidine uptake were isolated by random mutagenesis. In some mutants, it was found that there were two mutations. In that case, individual mutants were constructed, and spermidine uptake was measured. If the activity of the mutant was reduced to <30% or 30–60% of the control, the mutated amino acids were defined as having strong and moderate involvement, respectively, in the activity. *E. coli* MA261 potB::Km was transformed with the mutant potABCD, and spermidine uptake was measured. As shown in Fig. 1, Leu<sup>110</sup> in PotB and Ser<sup>196</sup> and Asp<sup>198</sup> in PotC were strongly involved in spermidine uptake, and Leu<sup>274</sup> in PotB and Leu<sup>195</sup> and Tyr<sup>211</sup> in PotC were moderately involved. Furthermore, Asp<sup>349</sup> and Asp<sup>374</sup> in PotA were strongly involved, and Val<sup>38</sup> in PotD was moderately involved in spermidine uptake.

It is known that polyamines are recognized by proteins through interactions with acidic and aromatic amino acid residues (11, 12, 15). There are 9 aspartic acid, 6 glutamic acid, 6 tryptophan, and 9 tyrosine residues in PotB, and 8 aspartic acid, 4 glutamic acid, 4 tryptophan, and 9 tyrosine residues in PotC. These residues were individually mutated to asparagine, glutamine, and leucine, respectively, and spermidine uptake was measured. As shown in Fig. 2, Trp<sup>8</sup>, Tyr<sup>43</sup>, and Trp<sup>198</sup> in PotB and Trp<sup>46</sup>, Asp<sup>108</sup>, Glu<sup>160</sup>, Asp<sup>198</sup>, and Asp<sup>199</sup> in PotC were strongly involved in spermidine uptake, and Tyr<sup>160</sup> and Glu<sup>172</sup> in PotB and Tyr<sup>19</sup>, Tyr<sup>88</sup>, Tyr<sup>148</sup>, Glu<sup>160</sup>, and Tyr<sup>211</sup> in PotC were moderately involved. In contrast, mutation of Tyr<sup>261</sup> to Leu in PotB enhanced spermidine uptake activity by 2.4-fold.

When the hydropathy profile of the proteins was evaluated, it became apparent that PotB and PotC proteins contained six putative transmembrane-spanning segments linked by hydrophilic segments of variable length (32). Most of the key amino acids identified by mutagenesis were located either on the cytoplasmic or the periplasmic sides, but not on the transmembrane segments (Fig. 3). The results suggest that interactions between PotA or PotD and the transmembrane proteins PotB and PotC occur on both surfaces of the membrane.

Characteristics of Functional Amino Acid Residues in PotB and PotC Involved in Spermidine Uptake—We next examined the functions of 11 amino acids in PotB and PotC that are strongly involved in spermidine uptake. The association of PotA, PotB, PotC, and PotD in the membrane was studied by Western blot analysis using rightside-out membrane vesicles. As shown in Fig. 4, association of the four proteins was only
disrupted by the Trp8 mutant in PotB. With the Trp8 mutant, the level of all four proteins, but especially PotB and PotC, on the membrane was low. The results suggest that Trp8 in PotB is important for the insertion of PotB and PotC into the membrane, so that association of PotA and PotD to the membrane was inhibited in the Trp8 mutant. To show the importance of Trp8 for the insertion of PotB and PotC to membrane, Trp8 was mutated to 10 other amino acids, consisting of hydrophilic, hydrophobic, acidic, and basic amino acids. In all mutants, association of PotA and PotD to membrane was strongly inhibited because the insertion of PotB and PotC to membrane was inhibited (Fig. 5A).

To confirm that Trp8 in PotB is important for the insertion of PotB and PotC into the membrane rather than increasing or accelerating degradation of PotB, possible degradation of the Trp8 mutant by the OmpT, Lon, and FtsH proteases was studied using mutants deficient in these proteases. The levels of PotA, B, C, and D on membranes in these mutants did not increase significantly, suggesting that the OmpT, Lon, and FtsH proteases do not influence the membrane expression of PotA, B, C, and D (Fig. 5B). It was determined whether synthesis of PotB and PotC is influenced by mutation of Trp8 in PotB in a cell-free protein synthetic system. As shown in Fig. 5C, synthesis of PotB and PotC was nearly equal in both wild type and Trp8-mutated PotB, and synthesis of PotB and PotC was very low compared with synthesis of PotD in both cell-free systems synthesizing normal and Trp8-mutated PotB. These results suggest that synthesis of PotB and PotC may be partially coupled with the insertion of PotB and PotC, and the insertion of PotB and PotC to membrane is inhibited in the Trp8-mutated PotB.

The $K_m$ and $V_{max}$ values were then determined in the 10 mutants of PotB and PotC. As shown in Table 1, the $K_m$ value was greatly increased in PotB Leu110 and Tyr261 and PotC Asp108, Asp199, and Asp199 mutants, and $V_{max}$ was greatly decreased without significant change of the $K_m$ value in PotC Trp46, Glu169, and Ser190 mutants, suggesting that the former is involved in recognition of spermidine and the latter in recognition of the PotD or PotA protein.

The ATPase activity of PotABC protein complex was determined in the 10 mutants using inside-out membrane vesicles. As shown in Fig. 6A, ATPase activity was greatly decreased in mutants of PotB Trp100 and PotC Asp108, Glu169, and Asp198. A decrease in both the affinity for spermidine and the ATPase activity was observed in mutants of PotC Asp108 and Asp198, suggesting that spermidine movement from PotD to cytoplasm and the structural change of PotB and PotC by PotA ATPase were coupled. At the Tyr261 PotB mutant, ATPase activity was greatly enhanced (Fig. 6B), although the $K_m$ value for spermidine increased (see Table 1). Enhancement of spermidine uptake by the PotB Tyr261 mutant is probably due to an increase in ATPase activity.

Among 11 PotB and PotC mutants, the function of PotB Tyr43 and PotC Trp46 and Ser190 was not yet identified. These amino acid residues are located on the periplasmic side, and association of PotD with the membrane seems to be weak in these mutants compared with wild type PotB and PotC (see Fig. 4). Thus, association of PotD with the 10 mutants was examined by measuring spermidine uptake activity using rightside-out membrane vesicles with various amounts of PotD. As shown in Fig. 7, association of PotD protein with PotB Y43L and W100L and PotC W46L and S196P was weak compared with association with wild type PotB and PotC. Apparent $K_d$ values of PotD binding to the PotABC complex were 0.7 μM (wild type), 3.4 μM (PotB Y43L), 3.7 μM (PotB W100L), 4.1 μM (PotC W46L), and 5.6 μM (PotC S196P). However, spermidine uptake activity of six other mutants was
not recovered even if 10 μM PotD protein was added to the reaction mixture (Fig. 7).

We next measured the direct binding of PotD to membrane vesicles, i.e. PotB and PotC proteins. As shown in Fig. 8A, PotD binding to membrane vesicles was dependent on the presence of both spermidine and ATP. It was confirmed that spermidine and ATP-dependent PotD binding to the membrane vesicles was weakened in the vesicles containing PotB Y43L and W100L and PotC W46L and S196P mutants. Furthermore, PotD binding was weakened in the presence of spermidine and ATP in vesicles containing PotC D108N mutant, but it was not significantly altered in vesicles containing PotC E169Q and D199N mutants. In the case of PotB L110P and PotC D198N, PotD binding in the absence of spermidine and ATP was greater than that to normal PotB and PotC. These results suggests that PotD may directly bind vesicles containing PotB L110P and PotC D198N mutants in the absence of spermidine and ATP, and release of PotD from these vesicles may be disturbed.

The characteristics of the key amino acids are illustrated on a model of the PotABCD spermidine transporter (Fig. 9) following the model of maltose transport system (44). Amino acid residues (PotB Tyr43 and Leu110 and PotC Trp46, Ser196, and PotC E169Q and D199N mutants...
Asp<sup>198</sup>, and Asp<sup>199</sup>) involved in PotD binding and spermidine recognition during spermidine passage from PotD to cytoplasm are located mainly at the periplasmic side, and those involved in the insertion of PotB and PotC proteins and PotA ATPase (PotB Trp<sup>8</sup> and Tyr<sup>261</sup> and PotC Asp<sup>108</sup> and Glu<sup>169</sup>) are located mainly at the cytoplasmic side. Amino acid residues involved in both PotD and spermidine recognition as well as PotA ATPase are located in both periplasmic (PotC Asp<sup>198</sup>) and cytoplasmic (PotC Asp<sup>108</sup>) sides. The results suggest that spermidine passage from PotD to cytoplasm and PotA ATPase are coupled each other.

**DISCUSSION**

With regard to ATP-binding cassette transporters, the crystal structures of the maltose (44) and molybdenum (45) transporters in complex with their binding proteins have been resolved. The structure of these two transporters is similar, and it is thought that the structural change for substrate influx is caused by ATPase activity (44–46).

The spermidine uptake system comprising PotABCD is one of the ATP-binding cassette transporters. The structure of
PotD, the periplasmic substrate (spermidine)-binding protein, and some properties of PotA, the membrane-bound ATPase have been clarified (7, 11, 15). Thus, the structure-function relationships of the membrane proteins PotB and PotC were studied using mutant PotB and PotC proteins. It was found that Trp\(^8\) located in the NH\(_2\) terminus of PotB protein is essential for insertion of PotB and PotC into the membrane. Trp cannot be replaced by any other amino acid. Because insertion of PotC was also inhibited by the PotB Trp\(^8\) mutant, association between PotB and PotC is probably important for insertion of both proteins. It has been reported that a Trp residue was necessary for correct trafficking of proteolipid protein 1 (PLP1) from the endoplasmic reticulum to plasma membrane in COS-7 cells (47). These results indicate that Trp on membrane protein plays important roles for correct arrangement of membrane proteins.

Spermidine uptake by PotABCD may be analogous to maltose uptake by the maltose transport system (8, 44, 46). It has been reported that EAA loops present in the cytoplasmic loops of MalF and MalG, membrane proteins of the maltose transporter, are important for interaction with ATPase subunit, MalK (48, 49). An EAA motif also exists in the cytoplasmic loop of PotB and PotC (see Fig. 3). When Glu\(^{172}\) in PotB and Glu\(^{169}\) in PotC were mutated to Gln, the spermidine transport activity was decreased by about 50%. Thus, the EAA motif in PotB and PotC is important for interaction with PotA. It is thought that PotA dimers interact with PotB and PotC with different angles (see Fig. 9). Thus, the existence of Glu\(^{169}\) in PotC next to the EAA motif is probably important for the interaction of PotA with PotC. We also found that Tyr\(^{43}\) and Leu\(^{110}\) in PotB and Trp\(^{46}\) and Ser\(^{196}\) in PotC, which exist in the periplasmic loop, are important for the interaction with spermidine-binding protein, PotD. It was also found that Val\(^{38}\) and Phe\(^{46}\) in PotD are involved in spermidine uptake (see Fig. 1). The sequence motif at residues 46–54 (FTKETGIKV) of PotD (11) is highly conserved with residues 53–61 (FEKDTGIKV) of the maltose-binding protein. The results suggest that this region, located at the loop between α1 and βB in PotD, may be important for the interaction with PotB and PotC.

Amino acid residues involved in recognition of spermidine were defined as those that influence the \(K_m\) value for spermidine. They were located either at the periplasmic side (Leu\(^{110}\) in PotB and Asp\(^{198}\) and Asp\(^{199}\) in PotC) or at the cytoplasmic side (Tyr\(^{261}\) in PotB and Asp\(^{199}\) in PotC). Amino acid residues at the periplasmic side may be involved in the release of spermidine from PotD, and those at the cytoplasmic side may be involved in the release of spermidine to the cytoplasm together with ADP (see Fig. 9). With a Tyr\(^{261}\) mutation to Leu in PotB, both spermidine uptake activity and ATPase activity of PotA increased by 2.4-fold, and the \(K_m\) value for spermidine increased by 3.9-fold. These data support an idea that
Tyr\textsuperscript{261} in PotB is involved in the release of spermidine to the cytoplasm by PotA ATPase.

Amino acid residues affecting ATPase activity are also located at both the periplasmic side (Asp\textsuperscript{198} in PotC) and at cytoplasmic side (Tyr\textsuperscript{261} in PotB and Asp\textsuperscript{108} and Glu\textsuperscript{169} in PotC). A plausible model is one in which the structures of the transmembrane proteins (PotB and PotC) change as a consequence of ATP binding to PotA (see Fig. 9), analogous to the mechanism reported for the maltose uptake system (44, 46, 50, 51). Asp\textsuperscript{108} and Asp\textsuperscript{198} in PotC were involved in both PotD and spermidine recognition as well as PotA ATPase, strongly suggesting that spermidine influx and ATPase are coupled. The results also indicate that these two amino acid residues are key amino acid residues for spermidine uptake by PotABCD because they are involved in all three functions.

Unlike maltose binding on the equivalent proteins in the maltose transport system (44), a spermidine recognition site on the transmembrane segments of PotB and PotC could not be identified. Thus, our experimental results support the idea that the cytoplasmic side of PotB and PotC is open for binding to the PotD-spermidine complex in the presence of ATP, and the periplasmic side of PotB and PotC is open for the release of spermidine together with hydrolysis of ATP.

It has been reported that the NH\textsubscript{2}-terminal domain (residues 1–250) of PotA contains an active ATPase center and that the COOH-terminal domain (residues 251–378) contains a regulatory site for ATPase activity (7). We found that Asp\textsuperscript{349} and Asp\textsuperscript{374} in the COOH-terminal domain are involved in spermidine uptake activity (see Fig. 1), indicating that these two aspartic acid residues are involved in regulation of ATPase activity of PotA.

Acknowledgments—We thank Dr. K. Williams for help in preparing the manuscript and Drs. W. K. Maas, D. R. Morris, and T. Ogura for providing E. coli strains.

REFERENCES

1. Cohen, S. S. (1998) A Guide to Polyamines, Oxford University Press, New York
2. Wallace, H. M., Fraser, A. V., and Hughes, A. (2003) Biochem. J. 376, 1–14
3. Igarashi, K., and Kashiwagi, K. (2010) Int. J. Biochem. Cell Biol. 42, 39–51
4. Pegg, A. E. (1988) Cancer Res. 48, 759–774
5. Igarashi, K., and Kashiwagi, K. (2010) Plant Physiol. Biochem. 48, 506–512
6. Kashiwagi, K., Kuraishi, A., Tomitori, H., Igarashi, A., Nishimura, K., Shirahata, A., and Igarashi, K. (2000) J. Biol. Chem. 275, 36007–36012
7. Kashiwagi, K., Innami, A., Zenda, R., Tomitori, H., and Igarashi, K. (2002) J. Biol. Chem. 277, 24212–24219
8. Eitinger, T., Rodionov, D. A., Grote, M., and Schneider, E. (2010) FEMS
PotABCD Spermidine Uptake System in E. coli

9. Soskawatmaekhin, W., Kuraishi, A., Sakata, K., Kashiwagi, K., and Igarashi, K. (2004) Mol. Microbiol. 51, 1401–1412
10. Kuribara, S., Oda, S., Kato, K., Kim, H. G., Koyanagi, T., Kumagai, H., and Suzuki, H. (2005) J. Biol. Chem. 280, 4602–4608
11. Sugiyama, S., Vassylev, D. G., Matsushima, M., Kashiwagi, K., Igarashi, K., and Morikawa, K. (1996) J. Biol. Chem. 271, 9519–9525
12. Vassylev, D. G., Tomitori, H., Kashiwagi, K., Morikawa, K., and Igarashi, K. (1998) J. Biol. Chem. 273, 17604–17609
13. Vyas, N. K., Vyas, M. N., and Quiocho, F. A. (1988) Science 242, 1290–1295
14. Kang, C. H., Shin, W. C., Yamagata, Y., Gokcen, S., Ames, G. F., and Kim, S. H. (1998) Nature 396, 703–707
15. Kashiwagi, K., Pistocchi, R., Shibuya, S., Sugiyama, S., Morikawa, K., and Igarashi, K. (1996) J. Biol. Chem. 271, 12205–12208
16. Kashiwagi, K., Endo, H., Kobayashi, H., Takio, K., and Igarashi, K. (1995) J. Biol. Chem. 270, 25377–25382
17. Hung, L. W., Wang, I. X., Nikaido, K., Liu, P. Q., Ames, G. F., and Kim, S. H. (1998) Nature 396, 703–707
18. Diederichs, K., Diez, J., Greller, G., Müller, C., Breed, J., Schnell, C., Vonrhein, C., Boos, W., and Welte, W. (2000) EMBO J. 19, 5951–5961
19. Kühnau, S., Reyes, M., Sievertsen, A., Shuman, H. A., and Boos, W. (1991) J. Bacteriol. 173, 2180–2186
20. Hunke, S., Landmesser, H., and Schneider, E. (2000) J. Bacteriol. 182, 1432–1436
21. Cunningham-Rundles, S., and Maas, W. K. (1975) J. Bacteriol. 124, 791–799
22. Kashiwagi, K., Miyamoto, S., Nukui, E., Kobayashi, H., and Igarashi, K. (1993) J. Biol. Chem. 268, 19358–19363
23. Kashiwagi, K., Hosokawa, N., Furuchi, T., Kobayashi, H., Sasakawa, C., Yoshikawa, M., and Igarashi, K. (1990) J. Biol. Chem. 265, 20893–20897
24. Linderoth, N., and Morris, D. R. (1983) Biochem. Biophys. Res. Commun. 117, 616–622
25. Igarashi, K., Kashiwagi, K., Hamasaki, H., Miura, A., Kakegawa, T., Hirose, S., and Matsuizaki, S. (1986) J. Bacteriol. 166, 128–134
26. Kashiwagi, K., and Igarashi, K. (1988) J. Bacteriol. 170, 3131–3135
27. Gottesman, S. (1996) Annu. Rev. Genet. 30, 465–506
28. Ogura, T., Inoue, K., Tatsuta, T., Suzuki, T., Karata, K., Young, K., Su, L. H., Fierke, C. A., Jackman, J. E., Raetz, C. R., Coleman, J., Tomoyas, T., and Matsuizaki, H. (1999) Mol. Microbiol. 31, 833–844
29. Yamaguchi, K., and Masamune, Y. (1985) Mol. Gen. Genet. 200, 362–367
30. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, pp. 250–251, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
31. Leng, X. H., Manolson, M. F., and Forgac, M. (1998) J. Biol. Chem. 273, 6717–6723
32. Furuchi, T., Kashiwagi, K., Kobayashi, H., and Igarashi, K. (1991) J. Biol. Chem. 266, 20928–20932
33. Posnett, D. N., McGrath, H., and Tam, J. P. (1988) J. Biol. Chem. 263, 1719–1725
34. Kaback, H. R. (1971) Methods Enzymol. 22, 99–120
35. Laemmli, U. K. (1970) Nature 227, 680–685
36. Nielsen, P. J., Manchester, K. L., Towbin, H., Gordon, J., and Thomas, G. (1982) J. Biol. Chem. 257, 12316–12321
37. Kashiwagi, K., Kobayashi, H., and Igarashi, K. (1986) J. Bacteriol. 165, 972–977
38. Houng, H. S., Lynn, A. R., and Rosen, B. P. (1986) J. Bacteriol. 168, 1040–1044
39. Lill, R., Cunningham, K., Brundage, L. A., Ito, K., Oliver, D., and Wickner, W. (1989) EMBO J. 8, 961–966
40. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
41. Zubyk, G. (1973) Annu. Rev. Genet. 7, 267–287
42. Higashi, K., Kashiwagi, K., Taniguchi, S., Terui, Y., Yamamoto, K., Ishihama, A., and Igarashi, K. (2006) J. Biol. Chem. 281, 9527–9537
43. Laskey, R. A., and Mills, A. D. (1975) Eur. J. Biochem. 56, 335–341
44. Oldham, M. L., Khare, D., Quiocho, F. A., Davidson, A. L., and Chen, J. (2007) Nature 450, 515–521
45. Holenstein, K., Frei, D. C., and Locher, K. P. (2007) Nature 446, 213–216
46. Oldham, M. L., Davidson, A. L., and Chen, J. (2008) Curr. Opin. Struct. Biol. 18, 726–733
47. Koizume, S., Takizawa, S., Fujita, K., Aida, N., Yamashita, S., Miyagi, Y., and Osako, H. (2006) Neuroscience 141, 1861–1869
48. Mourez, M., Hofnung, M., and Dassa, E. (1997) EMBO J. 16, 3066–3077
49. Daus, M. L., Grote, M., Müller, P., Doebber, M., Herrmann, A., Steinhoff, H. J., Dassa, E., and Schneider, E. (2007) J. Biol. Chem. 282, 22387–22396
50. Chen, J., Sharma, S., Quiocho, F. A., and Davidson, A. L. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 1525–1530
51. Grote, M., Polyzah, Y., Jescke, G., Steinhoff, H. J., Schneider, E., and Bordignon, E. (2009) J. Biol. Chem. 284, 17521–17526