Identification of the Putrescine Recognition Site on Polyamine Transport Protein PotE*

Received for publication, July 11, 2000, and in revised form, August 28, 2000
Published, JBC Papers in Press, August 29, 2000, DOI 10.1074/jbc.M006083200

Polyamines play important roles in cell proliferation and differentiation (1, 2), and cellular polyamine content is regulated by polyamine biosynthesis, degradation, and transport. We obtained three clones of polyamine transport genes (pPT104, pPT79, and pPT71) in *Escherichia coli* (3). The pPT104 clone encodes PotA, PotB, PotC, and PotD proteins and preferentially catalyzes the uptake of spermidine (4). The pPT79 clone encodes PotF, PotG, PotH, and PotI proteins and can catalyze the specific uptake of putrescine (5). These two transport systems are ATP-binding cassette transporters (6, 7). We have characterized these systems, especially the structure of substrate binding proteins, PotD and PotF, by x-ray analysis and site-directed mutagenesis (8–11).

The PotE protein can catalyze both uptake and excretion of putrescine. The *Km* values of putrescine for uptake and excretion are 1.8 and 73 μM, respectively. Uptake of putrescine is dependent on the membrane potential, whereas excretion involves putrescine-ornithine antiporter activity. Amino acids involved in both activities were identified using mutated PotE proteins. It was found that Cys62, Trp201, Trp292, and Tyr425 were strongly involved in both activities, and that Tyr290, Cys210, Cys285, and Cys286 were moderately involved in the activities. Mutations of Tyr290, Trp301, and Trp322 mainly affected uptake activity, and the *Km* values for putrescine uptake by these PotE mutants increased greatly, indicating that these amino acids are involved in the high affinity uptake of putrescine by PotE. Mutations of Lys201 and Tyr308 mainly affected excretion activity (putrescine-ornithine antiporter activity), and excretion by these mutants was not stimulated by ornithine, indicating that these amino acids are involved in the recognition of ornithine. It was found that the putrescine and ornithine recognition site on PotE is located at the cytoplasmic surface and the vestibule of the pore consisting of 12 transmembrane segments. Based on the results of competition experiments with various putrescine analogues and the disulfide cross-linking of PotE between cytoplasmic loops and the COOH terminus, a model of the putrescine recognition site on PotE consisting of the identified amino acids is presented.

Another putrescine transport system, encoded by pPT71, consists of one membrane protein (PotE) (12) and is active in both excretion and uptake of putrescine (13, 14). Excretion is based on a putrescine-ornithine antiporter activity (13), and uptake is dependent on the membrane potential (14). The topology of PotE involves 12 transmembrane segments with the NH2 and COOH termini being located in the cytoplasm (14). Furthermore, three glutamic acid residues (Glu77, Glu207, and Glu133) located on the cytoplasmic side of PotE have been found to be important for both uptake and excretion of putrescine (14). In the polyamine binding proteins PotD and PotF, not only acidic amino acids but also aromatic amino acids, especially tryptophan and tyrosine residues, are important for the recognition of putrescine and spermidine (8–11). Recently, Hu and King (15) reported that a cysteine residue is important for the activity of the γ-aminobutyric acid transporter in *E. coli*, which also has 12 transmembrane segments. Thus, we tried to identify additional residues that are involved in the activities of PotE and focused on Trp, Tyr, and Cys residues. The activities of a number of PotE mutants containing mutations at some basic amino acid residues were also examined to look for the site that recognizes the carboxyl group of ornithine. We found several amino acid residues that are involved in the uptake and/or excretion of putrescine. Disulfide cross-linking experiments were performed to determine the proximity of cytoplasmic loops and the COOH terminus of PotE that contain amino acid residues involved in the uptake and excretion activities. Based on the results of the disulfide cross-linking of PotE and the competition experiments with various putrescine analogues, a model of the putrescine recognition site on PotE consisting of the identified amino acids is presented.

### EXPERIMENTAL PROCEDURES

#### Bacterial Strains, Plasmids, and Culture Conditions—A polyamine-requiring mutant, *E. coli* MA261 (speB speC thr leu ser thi; Ref. 16), provided by Dr. W. K. Maas (New York University School of Medicine), and its polyamine uptake-deficient mutant KK313potF::Km (5) were grown in medium A in the absence of polyamines as described previously (17). *E. coli* JM105 (supE endA sbcB15 hsdR4 rpsL thi Δlac-proAB/F’ (trdD36 proAB+ lacIq lacZΔM15); Ref. 18) was cultured in a 19-amino acid supplemented medium containing 1% glycerol (19). Plasmid pMWpotE was prepared as described previously (14).

#### Mutagenesis of potE Gene—Site-directed mutagenesis was carried out by the method of Sayers et al. (20) with a Sculptor™ in vitro mutagenesis system (Amersham Pharmacia Biotech) or by using QuikChange™ site-directed mutagenesis kit (Stratagene). Mutations were confirmed by DNA sequencing using Seq 4 × 4 personal sequencing system (Amersham Pharmacia Biotech). A list of oligonucleotide primers used for mutagenesis has not been included but is available from the authors upon request.

#### Construction of the Genes Encoding PotE Dimers, Wild/Wild, Wild/ *E207Q*, *E207Q/Wild*, and *E207Q/E207Q*—For the construction of genes encoding the NH2-terminal half of PotE, polymerase chain reac-
ation was performed using primers 5'-TAGTGTCAGCCGATTCCGCCAGTGGAAC-3' and 5'-GGTTGTCAGACCGTTTATTTCGAGTTC-3' and the pMWpOE or pMWpotE E207Q as template. For the construction of genes encoding the COOH-terminal half of PotE, polymerase chain reaction was performed using primers 5'-GGAGTGACAGTGAGTCAGGCTAAATCGCA-3' and 5'-GGTTGTCAGCTACACCIGTTGTA-3' (40 universal primer; Amersham Pharmacia Biotech) and pMWpOE or pMWpotE E207Q as template. The polymerase chain reaction product of the former was digested with SphI and SacI, and that of the latter was digested with SalI and BamHI. The fragments thus obtained were ligated with pMW119 previously digested with SphI and BamHI. PotE Uptake by Intact Cells—E. coli: KK31poptF::Kmp/pMWpotE cells grown in medium A containing 0.5 mM isopropyl-1-thiogalactopyranoside and 0.5% glucose were suspended in buffer 1 containing 0.4% glucose, 62 mM potassium phosphate, pH 7.0, 1.7 mM sodium citrate, 7.6 mM MgSO4 to yield a protein concentration of 0.1 mg/ml. The cell suspension (0.48 ml) was preincubated at 30 °C for 5 min, and the reaction was started with the addition of 0.02 ml of 0.25 mM [14C]putrescine (370 MBq/mmol). After incubation at 30 °C for 30 s to 3 min, the cells were collected on membrane filters (cellulose acetate, 0.45 μm; Advantec Toyo), and the filters were washed three times with a total of 12 ml of buffer 1. The radioactivity on the filters was assayed with a liquid scintillation spectrometer. Protein content was determined by the method of Lowry et al. (21).

Polyamine Transport in E. coli

**RESULTS**

**Identification of Amino Acid Residues Involved in Uptake and Excretion of Putrescine**—There are 10 tryptophan residues in PotE. These residues were individually mutated to leucine using site-directed mutagenesis of the potE gene. Putrescine uptake activity was measured using the E. coli mutant KK31poptF::Kmp, which is deficient in polyamine uptake, transformed with pMW encoding wild type or mutant potE. PotE activity was induced by isopropyl-β-D-thiogalactopyranoside. As shown in Fig. 1, both uptake and excretion were greatly decreased with the PotE mutants W201L and W292L, whereas uptake of putrescine decreased more than excretion with W422L.

There are 14 tyrosine residues in PotE. These residues were also replaced by leucine. Both uptake and excretion of putrescine decreased greatly with the PotE mutant Y425L and moderately with Y92L, and uptake decreased greater than excretion with Y78L and Y60L. In W292L mutants, putrescine excretion decreased more than uptake with Y308L, suggesting that this residue may be involved in the recognition of ornithine in the case of putrescine-ornithine antiporter activity (Fig. 1).
putrescine were seen with the PotE mutants C62A, W201L, W292L, and Y425A (Fig. 1). To confirm the importance of these amino acid residues, each was replaced by several amino acids, and the activities were measured (Fig. 2). Replacement of Trp201 and Trp292 by Phe and Tyr did not restore the activities of PotE, indicating that these tryptophan residues are critical for activity. In contrast, replacement of Tyr425 by Trp restored the activities almost completely, and replacement by Phe partially restored activity. Replacement of Cys62 by Ser restored the activities partially, but that by Thr did not restore the activities significantly. Expression of mutated PotE proteins was examined by Western blot analysis of inside-out membrane vesicles. Comparable amounts of mutated PotE were expressed on the membranes (Fig. 2). The results strongly suggest that Cys62, Trp201, Trp292, and Tyr425 are important for both uptake and excretion of putrescine together with Glu77, Glu207, and Glu433 (14).

Mutants at PotE Y78L, Y90L, and W422L had a greater effect on putrescine uptake than on excretion (Fig. 1). The $K_m$ value for putrescine uptake by wild type PotE is 1.8 $\mu$M, and that for putrescine excretion is 73 $\mu$M. Therefore, the $K_m$ values for putrescine and $V_{max}$ values of putrescine uptake of these PotE mutants were determined by Lineweaver-Burk plots. As shown in Fig. 5, the $K_m$ values for putrescine increased greatly with these three PotE mutants, and the change in the $V_{max}$ value was small compared with the change in the $K_m$ value. The results indicate that these Trp and Tyr residues are involved in the high affinity uptake of putrescine by PotE.

Putrescine excretion (via a putrescine/ornithine antiporter) decreased more than uptake with the PotE mutant Y308L (Fig. 1). If other residues are involved in the recognition of ornithine, we might expect basic amino acids to recognize the carboxyl group of ornithine. Because Tyr308 is located in the hydrophilic loop region on the cytoplasmic side of PotE, arginine and lysine residues, located on the cytoplasmic side and structurally close to Tyr308, were mutated to alanine. As shown in Fig. 4, both uptake and excretion were reduced slightly with the PotE mutants K68A and K82A, whereas putrescine excretion was reduced more than uptake with K301A. The activities did not change significantly with other mutated PotE proteins. To ensure that Lys301 and Tyr308 are involved in the recognition of ornithine, inside-out membrane vesicles were prepared in the presence and absence of ornithine, and putrescine uptake by the inside-out membrane vesicles was compared. Putrescine uptake activity with wild type PotE was stimulated by ornithine existing in the vesicles, whereas the activity with mutated PotE K301A and Y308L was not stimulated by ornithine (Fig. 5). The results support the idea that Lys301 and Tyr308 are involved in the recognition of ornithine.

Location of Amino Acid Residues Involved in the Activities—Amino acid residues involved in both uptake and excretion of putrescine are shown in Fig. 6. The important residues are located in the transmembrane helices III, VI, VIII, and XII or in the cytoplasmic region between transmembrane helices II and III (C1), VI and VII (C4), VIII and IX (C5), and in the COOH terminus (C7) of PotE. Amino acid residues in the transmembrane helices are also located in the cytoplasmic side rather than the periplasmic side. It is expected that these amino acid residues are located close to each other in the tertiary structure of PotE so that putrescine (or ornithine) can be recognized.

Putrescine Uptake and Excretion Activities of PotE Dimer—To identify the substrate recognition site on PotE, we first tested whether PotE functions as a monomer or dimer. It has been reported that the functional unit of lactose permease is a monomer by measuring the activity of permease dimer, a fusion protein containing two lactose permease molecules (26). If the protein functions as a dimer, it would be expected that the nonfunctional half inhibits the activity dominantly negatively. Thus, we also constructed dimer PotE, a fusion protein containing two PotE molecules of Wild/Wild, Wild/E207Q, E207Q/Wild, and E207Q/E207Q, and examined the putrescine uptake and excretion activities. As shown in Fig. 7A, the activities of Wild/Wild PotE dimer were about twice of the of monomer, Wild/E207Q dimer, and E207Q/Wild dimer. Essentially no significant activities were observed with E207Q/E207Q dimer. The amount of PotE dimer in cells was nearly equal in Wild/Wild, Wild/E207Q, E207Q/Wild, and E207Q/E207Q dimer (Fig. 7B). The results indicate that the functional unit of PotE is a monomer.

Effect of Putrescine Analogues on Putrescine Uptake by PotE and PotF, G, H, and I—The putrescine recognition site on PotF, a substrate-binding protein of the putrescine uptake system consisting of PotF, G, H, and I (3), was identified by the crystal structure of PotF as well as the mutational analysis of PotF (11). To compare the putrescine recognition site on PotE and
brane segments, because rather than the surface of the pore consisting of 12 transmembrane segments has been stabilized by PotE. We have previously determined the putrescine and spermine excretion activities were measured by the putrescine uptake of the inside-out membrane vesicles prepared in the presence or absence of 2.5 mM ornithine as described under “Experimental Procedures.” Each point represents the mean of duplicate measurements.

PotF, the effect of putrescine analogues on putrescine uptake activity was examined. The putrescine analogues added to the reaction mixture of PotE and PotF, G, H, and I were 200 and 50 \( \mu \text{M} \), respectively, because the \( K_m \) value for putrescine in PotE and PotF, G, H, and I is 1.8 and 0.5 \( \mu \text{M} \), respectively (14, 27). The putrescine analogue, trans-1,4-diamino-2-butene, inhibited the putrescine uptake activity by PotE and PotF, G, H, and I (Fig. 8, number 6). N-Ethylputrescine and 1,3-diaminopropane inhibited the uptake activity by PotF, G, H, and I but not by PotE (Fig. 8, numbers 1 and 5). Because putrescine binds to the cleft between the \( \text{NH}_2 \)- and the COOH-terminal domains of PotF (11), one side of the substrate recognition site of PotF is open. Thus, N-ethylputrescine, which has extra ethyl group, could be recognized by PotF. Furthermore, 1,3-diaminopropane was also recognized by PotF. PotE could recognize trans-1,4-diamino-2-butene only, indicating that putrescine recognition site of PotE is more rigid than that of PotF. The results also suggest that putrescine may be recognized at the vestibule rather than the surface of the pore consisting of 12 transmembrane segments, because N-ethylputrescine could not be recognized by PotE.

**Disulfide Cross-linking of Cytoplasmic Loops and the COOH Terminus of PotE**—Proximity of periplasmic loops of transport protein consisting of 12 transmembrane segments has been recently studied by the disulfide cross-linking method using Cu\(^{2+} /\)o-phenanthroline (23, 28). The method was applied to determine the proximity of the cytoplasmic loops and the COOH terminus of PotE, which contain amino acid residues involved in the uptake and excretion activities. Because the mobility of mutated PotE (C62S/C210A) during gel electrophoresis did not change with or without N-ethylmaleimide treatment (Fig. 9A, number 10), it was judged that either a disulfide bond was not formed or a structural change seen by the shift of mobility during gel electrophoresis did not occur in four cysteines (Cys\(^{359}\), Cys\(^{165}\), Cys\(^{285}\), and Cys\(^{296}\)) located in the transmembrane segments. Judging from the shift of the mobility of PotE in the absence of N-ethylmaleimide, disulfide cross-linking was only observed between the C2 and C4 loops as indicated by the asterisk (Fig. 9A). The shift of the mobility was not observed with PotE having cysteine residue in either the C2 or the C4 loop. The results suggest that the relative positions of C2 and C4 are close. Furthermore, when a cysteine residue is present in the COOH terminus (C7) of PotE, a dimer was formed as indicated by the double asterisk (cf. Fig. 7 as for the position of the dimer). When a cysteine residue is present in the C2, C4, or C5 loop, formation of the PotE dimer was not apparent. The results suggest that the COOH terminus is located at the surface of the pore consisting of 12 transmembrane segments. Because the shift of the mobility of PotE having two cysteines in the C2 and C4 loops was small, disulfide cross-linking was performed using \( N,N' -\)o-phenylenediamine (6 A) and \( N,N' -p\)-phenylenediamine (10 A) instead of Cu\(^{2+} /\)o-phenanthroline (24). The results obtained above were confirmed with \( N,N' -o\)-phenylenediamine and \( N,N' -p\)-phenylenediamine (Fig. 9B).

From the data of Fig. 9 (A and B) and the putrescine recognition model of PotF (11), a model of the putrescine recognition site on PotE is proposed in Fig. 9C. One amino group of putrescine is recognized by Glu\(^{77}\) and Glu\(^{207}\), and the other amino group is recognized by Glu\(^{439}\). The position of Glu\(^{439}\) is closer to cytoplasm than Glu\(^{77}\) and Glu\(^{207}\). Two Trp residues (201 and 292) and Tyr\(^{425}\) are important for the recognition of butane moiety of putrescine. Tyr\(^{78}\), Tyr\(^{90}\), and Trp\(^{422}\) are important for the recognition of putrescine from periplasmic side. Cys\(^{359}\) may stabilize the structure of putrescine recognition site through disulfide bond formation with Cys\(^{210}\). Although Cys\(^{285}\) and Cys\(^{296}\) were not shown in the model, these two cysteine residues may also be involved in the maintenance of active structure of PotE through disulfide bond formation. We could not identify the relative position of Lys\(^{921}\) and Tyr\(^{1068}\), which recognize the carboxyl group of ornithine.

**DISCUSSION**

From the present and previous studies (14), we have identified acidic, basic, and aromatic amino acids and cysteine residues that are involved in both uptake and excretion by PotE. These residues are located in the loop and transport passage region on the cytoplasmic side of PotE. It has also been shown that the main functional amino acids of the lactose/H\(^+\) symporter (29, 30) and metal tetracycline/H\(^+\) antipporter (31) are located on the cytoplasmic side of the protein. If functional amino acids exist on the cytoplasmic side, they may be able to respond quickly to any metabolic change in the substrate concentration in the cytoplasm.

We have previously determined the putrescine and spermidine binding sites on PotD and PotF (8–11). According to the model of the lactose/H\(^+\) symporter (32, 33), six transmembrane segments among the 12 segments may form the transport passage for the substrate. Considering this model, the amino acid residues that are involved in putrescine recognition on PotE are arranged in a manner similar to the residues necessary for substrate recognition in PotD and PotF (Fig. 9C). One amino group of putrescine is recognized by Glu\(^{77}\) and Glu\(^{207}\), and the other amino group is recognized by Glu\(^{439}\). The butane moiety of putrescine is stacked by Tyr\(^{201}\), Trp\(^{292}\), and Tyr\(^{425}\).
The results suggest that putrescine is strongly recognized by PotE through the interaction of the N2 amino-terminal half of putrescine (Fig. 9C). Most important amino acid residues are Glu207 (14) and Trp201 and Trp292 (Fig. 1). When putrescine is recognized from the periplasmic side, the affinity of PotE for putrescine increases through its interaction with Tyr78, Tyr90, and Trp422 in addition to the above mentioned amino acid residues. When ornithine is recognized from the periplasmic side, as is the case when PotE functions as a putrescine-ornithine antiporter, the tertiary structure of PotE may be different from that which recognizes putrescine from the periplasmic side. Unfortunately, we could not identify the relative positions of Lys301 and Tyr308, which recognize the carboxyl group of ornithine. Even when \( N_2N_9-o\)-phenylenedimaleimide (rigid 6 Å), \( N_2N_9-p\)-phenylenedimaleimide (rigid 10 Å), or 1,6-bismaleimidohexane (flexible 16 Å) (data not shown) was used as a cross-linker instead of Cu\(^{2+}\)/o-phenanthroline (24), we could not determine the relative positions of Lys301 and Tyr308. In TPO1, which is a polyamine transporter of Saccharomyces cerevisiae (34), there are three glutamate residues in a similar position to the three glutamate residues in PotE. All three glutamate residues were involved in the transport activity. Thus, the
It is also of interest to know how PotE, which controls uptake and excretion of putrescine, is regulated. There are two ATP-dependent putrescine uptake systems: the spermidine-preferential and putrescine-specific uptake systems (3–5). Furthermore, the expression of the speF-potE operon including the genes for inducible ornithine decarboxylase and potE is weak at neutral pH (Figs. 1 and 4 and Ref. 35). Thus, the expression of the operon is probably induced when putrescine is accumulated in cells, and the main function of PotE in E. coli may be the excretion of putrescine. Induction may also occur when the two ATP-dependent uptake systems are absent or nonfunctional. In Rickettsia prowazekii, which has only one fourth the chromosome size of E. coli, only a PotE-like protein exists, not the ATP-dependent polyamine uptake systems (36).

Acknowledgments—We thank Drs. A. J. Michael and K. Williams for kind suggestions and help in preparing the manuscript.

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**Fig. 9.** Disulfide cross-linking of PotE catalyzed by Cu⁺/o-phenanthroline (A) and N,N-o-phenylelenedimaleimide and N,N'-o-phenylelenedimaleimide (B) and model of putrescine recognition site on PotE (C). A, disulfide cross-linking was performed with inside-out membrane vesicles prepared from E. coli JM105 containing the following pMWpotE. Lanes 1, pMW119 (vector); lanes 2, pMWpotE wild (Cys321 and Cys434); lanes 3, pMWpotE C120A (Cys321); lanes 4, pMWpotE C210A/L434C (Cys321 and Cys434); lanes 5, pMWpotE C210A/L434C (Cys321 and Cys434); lanes 6, pMWpotE C625/L434C (Cys321 and Cys434); lanes 7, pMWpotE C210A/L434C (Cys321 and Cys434); lanes 8, pMWpotE C625/L434C (Cys321 and Cys434); lanes 9, pMWpotE C625/L434C (Cys321 and Cys434); lanes 10, pMWpotE C210A/L434C (no cysteine residues in cytoplasmic loops and NH₂ and COOH termini) Cysteines with number in parentheses indicate the position of cysteine residues in the cytoplasmic loops and the COOH terminus of mutated PotE. Similar results were obtained with Cys321 and Cys434 in case of C5. B, disulfide cross-linking experiments were performed with N,N'-o-phenylelenedimaleimide and N,N'-o-phenylelenedimaleimide. C, model of putrescine recognition site of PotE was constructed according to the results obtained in the present and previous studies (14). Amino acid residues shown in rectangles are involved in both uptake and excretion of putrescine, and amino acid residues in ellipses are in the high affinity uptake of putrescine.