Spermidine-preferential Uptake System in Escherichia coli
IDENTIFICATION OF AMINO ACIDS INVOLVED IN POLYAMINE BINDING IN PotD PROTEIN∗

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Spermidine-binding sites on PotD protein, a substrate-binding protein in periplasm, in the spermidine-preferential uptake system in Escherichia coli were studied by measuring polyamine transport activities of right-side-out membrane vesicles with mutated PotD proteins prepared by site-directed mutagenesis of the potD gene and by measuring polyamine binding activities of these mutated PotD proteins. Polyamine transport activities of the mutated PotD proteins paralleled their polyamine binding activities. It was found that Trp-34, Thr-35, Glu-36, Tyr-37, Ser-83, Tyr-85, Asp-168, Gln-257, Tyr-293, Asp-257, Tyr-85, Asp-168, Glu-171, Trp-229, Trp-255, Asp-257, Tyr-293, and Gln-327 of PotD protein were involved in the binding to spermidine. When spermidine uptake activities were measured in intact cells expressing the mutated PotD proteins, it was found that Gln-171, Trp-255, and Asp-257 were more strongly involved in the binding of spermidine to PotD protein than the other amino acids listed above. The dissociation constants of spermidine for the mutated PotD proteins at Glu-171, Trp-255, and Asp-257 increased greatly in comparison with those for the other mutated PotD proteins. Since these three amino acids clearly interact with the diaminopropane moiety of spermidine, the results are in accordance with the finding that PotD protein has a higher affinity for spermidine than for putrescine. Putrescine was found to bind at the position of the diaminobutane moiety of spermidine.

The polyamine content in cells, which plays important roles in cell proliferation and differentiation (1, 2), is regulated by polyamine biosynthesis, degradation, and transport. As for the latter, we obtained and characterized three clones of polyamine transport genes (pPT104, pPT79, and pPT71) in Escherichia coli (3). The system encoded by pPT104, PotA, PotB, and PotC proteins and PotD and PotG proteins are membrane-associated proteins with a nucleotide-binding site. PotB and PotC proteins and PotH and PotI proteins are transmembrane proteins that probably form channels for spermidine and putrescine, respectively. Their amino acid sequences in the corresponding proteins are similar to each other. In contrast, the putrescine transport system encoded by pPT71 consists of one membrane protein (PotE protein) with 12 transmembrane segments (8) and is active in the excretion of putrescine from cells through putrescine-ornithine antiporter activity (9). We also found that spermidine uptake by membrane vesicles is strongly dependent on PotD protein, and the uptake by intact cells is completely dependent on ATP through its binding to PotA protein (10). Furthermore, PotA protein was shown to have ATPase activity, and its association with membranes is strengthened by the existence of channel-forming PotB and PotC proteins (11).

Recently, we determined the crystal structure of PotD protein in a complex with spermidine at 2.5-Å resolution (12). It was revealed that four acidic and five aromatic amino acid residues in PotD protein interact with spermidine (13). In this study, we tried to identify the amino acid residues in PotD protein that are involved in the binding of spermidine by using mutated PotD protein produced by site-directed mutagenesis of the potD gene. We found that Gln-171, Trp-255, and Asp-257, among 13 amino acids involved in the interaction with spermidine, are particularly important in the recognition.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Culture Conditions—A polyamine-requiring mutant, E. coli DR112 (14), provided by Dr. D. R. Morris (University of Washington), was grown as described previously (15). E. coli TG1 (∆lac-pro), supE, thi, hsdS5FtraD36, proAB, 21, lacI, lacZAM15), which has a higher transformation efficiency, was grown in 2YT medium (16). A spermidine transport- and polyamine biosynthesis-deficient mutant, MA261 potD::Km (10), was grown in medium A in the absence of polyamines as described previously (17). Plasmids pPT86 containing the potABC gene and pUCpotsD were prepared as described previously (10). Plasmid pMMVpotD was prepared by inserting the 1.7-kilobase EcoRI-HindIII fragment of pMMVpotD into the same restriction site of pMW119 (18). Transformation of E. coli cells with plasmids was carried out as described by Maniatis et al. (19). Appropriate antibiotics (30 μg/ml chloramphenicol, 100 μg/ml ampicillin, and 50 μg/ml kanamycin) were added during the culture of E. coli cells.

Mutagenesis of potD Gene—To prepare potD mutants, a 1.7-kilobase EcoRI-HindIII fragment of pMMVpotD was inserted into the same site of M13mp9 (20). Site-directed mutagenesis was carried out by the method of Sayers et al. (21) with a Sculptor™ in vitro mutagenesis system (Amersham Corp.), using the oligonucleotides shown in Table I. The mutated DNA fragments were isolated from the replicative form of M13 and religated into the same site of pUCpotsD. Mutations were confirmed by DNA sequencing (22) using the M13 phage system (20) with commercial and synthesized primers.

Polyamine Uptake by Right-side-out Membrane Vesicles—Right-side-out membrane vesicles were prepared from E. coli DR112/pPT86 as described previously (23). Periplasmic proteins were obtained from E. coli DR112/pPT86 by making a break in the cells (11). Measurement of Polyamine Uptake Activities—Uptake activities were measured in intact cells expressing the mutated PotD proteins prepared by site-directed mutagenesis of the potD gene. The uptake activities were measured in right-side-out membrane vesicles prepared from E. coli DR112/pPT86 as described previously (23).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M64519.

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col. TG1 containing pUCpOD- or pUC-mutated potD according to the method of Oliver and Beckwith (24). PotD protein occupied ~60% of the total periplasmic proteins, and they were used for the PotD protein source. The reaction mixture (0.1 ml; containing 20 mK potassium phosphate buffer (pH 6.6), 50 mHEPES/KOH (pH 7.6), 5 mM MgSO₄, 20 mM 3-lactate (lithium salt), 100 μg of membrane vesicle protein, 70 μg of PotD or mutated PotD protein, and 20 μM [¹⁴C]putrescine (4.07 GBq/mmol) or 4 μM [¹⁴C]spermidine (4.07 GBq/mmol) was incubated at 37°C for 10 min. Membrane vesicles were collected on membrane filters (cellulose acetate, 0.45 μm; Advantec Toyo), and the radioactivity was counted with a liquid scintillation spectrometer. Protein content was measured by the method of Lowry et al. (25).

Assay for Polyamine Binding to PotD Protein—The reaction mixture (0.1 ml; containing 10 mM Tris-HCl (pH 7.5), 30 mM KCl, and a combination of 10 μg of PotD protein and 4 μM [¹⁴C]spermidine (2.03 GBq/mmol) or 100 μg of PotD protein and 35 μM [¹⁴C]putrescine (4.07 GBq/mmol)) was incubated at 30°C for 5 min. PotD protein was collected on membrane filters (cellulose nitrate, 0.45 μm; Advantec Toyo), and the radioactivity was counted with a liquid scintillation spectrometer. The dissociation constant (Kd) and the number of binding sites (Bmax) of spermidine for PotD protein were calculated from a Scatchard plot (26) by changing the substrate concentrations from 1 to 50 μM.

Spermidine uptake by intact cells—E. coli MA61 potD::Km containing pMWpotD- or pMW-mutated potD was grown in medium A until A600nm reached 0.3. The assay for spermidine uptake was performed as described previously (23), except that 1 μM (4.07 GBq/mmol) or 10 μM (370 MBq/mmol) [¹⁴C]spermidine was used as substrate.

RESULTS

Spermidine and Putrescine Uptake Activities of Right-side-out Membrane Vesicles and Mutated PotD Protein—Recently, we determined the tertiary structure of PotD protein in a complex with spermidine by x-ray crystallographic analysis (13). Fig. 1A shows 13 amino acids (Trp-34, Thr-35, Glu-36, Tyr-37, Ser-83, Tyr-85, Asp-168, Glu-171, Trp-229, Trp-255, Asp-257, Tyr-293, and Gln-327) that may be involved in the spermidine binding. In addition, it has been reported that Tyr-86 and Arg-170 might also participate in the spermidine binding from the prediction of the structural similarity between PotD protein and maltose-binding protein (27). Therefore, we prepared PotD proteins mutated at the 15 amino acids listed above by site-directed mutagenesis of the potD gene (Table I) and analyzed the activities of the mutated PotD proteins by three different assay methods.

First, spermidine and putrescine uptake activities were measured using right-side-out membrane vesicles and PotD or mutated PotD protein. Right-side-out membrane vesicles were prepared from E. coli DR112/pP786, in which relatively large amounts of P0A, P0B, and P0C proteins are synthesized (6). Periplasmic protein prepared from E. coli TG1/pUCpOD was used as the source of PotD and mutated PotD proteins. Spermidine uptake activities decreased greatly with mutated PotD proteins (W255L and D257N) in which a close located amino acid interacting with the second amine of spermidine is modified (Fig. 1B). The activity also decreased with all mutated PotD proteins (E36Q, Y37A, Y293A, W34L, E171Q, S83A, D168N, and Q327A) whose amino acids were strongly suggested to be involved in the binding of spermidine by x-ray analysis. Spermidine uptake activities with other mutated PotD proteins (Y86A and R170L) did not change significantly (data not shown).

Putrescine uptake activity greatly decreased with certain mutated PotD proteins (E36Q, Y37A, Y293A, W34L, and D257N) in which an amino acid interacting with the diaminobutane moiety of spermidine is modified (Fig. 1B). The activity did not significantly decrease with other mutated PotD proteins (E171Q, Y85A, and D168N) in which an amino acid interacting with the aminopropyl moiety of spermidine is modified. The mutated PotD protein W255L slightly decreased the activity. The results indicate that putrescine occupies the position corresponding to the binding site of the diaminobutane moiety of spermidine in PotD protein. It should also be noted that the substitution of Thr-35, which was involved in the binding of spermidine, was not connected with the interaction with putrescine.

Spermidine and Putrescine Binding to Mutated PotD Protein—Polyamine binding to PotD protein was measured using 4 μM spermidine or 35 μM putrescine as substrate. As shown in Fig. 1C, the amounts of spermidine bound to the mutated PotD proteins almost paralleled the spermidine uptake activities of the proteins except for the E36Q mutated protein. The release of spermidine from this mutated protein may be slower than proteins (E171Q, Y85A, and D168N) in which an amino acid interacting with the aminopropyl moiety of spermidine is modified. The mutated PotD protein W255L slightly decreased the activity. The results indicate that putrescine occupies the position corresponding to the binding site of the diaminobutane moiety of spermidine in PotD protein. It should also be noted that the substitution of Thr-35, which was involved in the binding of spermidine, was not connected with the interaction with putrescine.

Spermidine and Putrescine Binding to Mutated PotD Protein—Polyamine binding to PotD protein was measured using 4 μM spermidine or 35 μM putrescine as substrate. As shown in Fig. 1C, the amounts of spermidine bound to the mutated PotD proteins almost paralleled the spermidine uptake activities of the proteins except for the E36Q mutated protein. The release of spermidine from this mutated protein may be slower than
that from the others. Putrescine binding was measured using a 10 times greater amount of mutated PotD protein since the affinity for putrescine (K_D = 100 μM) is much lower than that for spermidine (K_D = 3.2 μM) (7). The amounts of putrescine bound to the mutated PotD proteins also almost paralleled the putrescine uptake activities of the proteins. Since the W255L mutated protein significantly decreased both the putrescine uptake and binding activities, Trp-255 may also be involved in the interaction with the primary amine of putrescine, which corresponds to the secondary amine of spermidine.

Spermidine Uptake Activities of Intact Cells Containing Mutated PotD Proteins—Spermidine uptake activities were measured with E. coli MA261 potD::Km containing pMW-mutated potD, in which the expression of PotD or mutated PotD protein was about four times greater than that in wild-type MA261 cells. The activity with intact cells was measured with 1 or 10 μM spermidine, as the K_m value for spermidine is 0.1 μM (3); it was observed to be much greater than that with right-side-out membrane vesicles and PotD protein. One reason for this was that the possibility of PotD protein interaction with the channel-forming PotB and PotC proteins was increased by the existence of the outer membrane. When the activity was measured in intact cells, the difference among the activities of the mutated PotD proteins became more pronounced. As shown in Table II, Asp-257 was the most important amino acid for recognition of spermidine, with Trp-255 and Glu-171 also being strongly involved. Other mutated PotD proteins slightly decreased the spermidine uptake activity, but the mutated PotD protein S83A did not. Replacement of Asp-257 by Glu resulted in uptake activity at 70% of normal PotD protein, indicating that a negative charge at position 257 is important (data not shown).

The dissociation constants (K_D) and the number of binding sites (B_max) of spermidine for the mutated PotD proteins were then measured. Since the B_max value (1 mol/PotD protein) did not change significantly, the change in binding affinity was mainly due to the change in the K_D values. As shown in Table II, the K_D value of spermidine for normal PotD protein was estimated to be 3.7 μM. However, the K_D values of spermidine for the three mutated proteins D257N, W255L, and E171Q increased greatly, paralleling the decrease in spermidine uptake activities of intact cells. We previously reported that the concentration of spermidine-PotD protein in the periplasm would be ~3.1 μM if the spermidine concentration was 0.1 μM under standard conditions (10).

The amino acids involved in the recognition of spermidine are summarized in Fig. 2. There were four acidic amino acids,
tact cells with mutated PotD proteins as well as spermidine binding activities of mutated PotD proteins. We found that amino acids, especially Glu-171, Trp-255, and Asp-257, involved in the interaction with the diaminopropionate moiety of spermidine are more crucial in the binding of spermidine to PotD protein than those involved in the interaction with the aminobutyl moiety of spermidine. Putrescine was found to bind at the position of the diaminobutane moiety of spermidine. These results explain why spermidine has a higher affinity for PotD protein than putrescine. Among the above three amino acids, Asp-257, which interacts with the secondary amine of spermidine, are more crucial in the binding of spermidine to PotD protein than those involved in the interaction with spermidine.

In this connection, it should be noted that the secondary amine of spermidine or spermine most effectively contributes to the differential interaction with spermidine. Identical amino acids are indicated with asterisks (4).

Fig. 3. Comparison of amino acid sequences of PotD and PotF proteins. Asterisks indicate the amino acids involved in the differential interaction with spermidine. Identical amino acids are indicated with black shading, and equivalent or shuffled amino acids by shaded boxes.

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