Functions of PotA and PotD Proteins in Spermidine-preferential Uptake System in *Escherichia coli*

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The polyamine content in cells, which plays important roles in cell proliferation and differentiation (1, 2), is regulated by both polyamine biosynthesis and its transport. Although polyamine biosynthesis has been studied extensively, its transport has not. We recently obtained and characterized three clones of polyamine transport genes (pPT104, pPT79, and pPT71) in *Escherichia coli* (3). The system encoded by pPT104 was the spermidine-preferential uptake system and that encoded by pPT79 was the putrescine-specific uptake system. Furthermore, these two systems were periplasmic transport systems (4, 5) consisting of four kinds of proteins: pPT104 clone encoded potA, -B, -C, and -D proteins and pPT79 clone encoded potF, -G, -H, and -I proteins, judging from the deduced amino acid sequences of the nucleotide sequences of these clones (6, 7). PotD and -F proteins were periplasmic substrate-binding proteins, and potA and -G proteins were membrane-associated proteins having the nucleotide-binding site. PotB and -C proteins, and potH and -I proteins were transmembrane proteins probably forming channels for spermidine and putrescine, respectively. Their amino acid sequences in the corresponding proteins were similar to each other. In contrast, the putrescine transport system encoded by pPT71 consisted of one membrane protein (potE protein) having 12 transmembrane segments (8) and was active in the excretion of putrescine from cells through putrescine-ornithine antiporter activity (9).

In this study, we examined the functions of potA and -D proteins in the spermidine-preferential uptake system encoded by pPT104 clone in detail through a combined biochemical and genetic approach. Spermidine uptake by membrane vesicles was strongly dependent on potD protein, indicating that the spermidine-potD protein complex is a real substrate, and the uptake by intact cells was completely dependent on ATP through its binding to potA protein.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Culture Conditions**—A polyamine-requiring mutant, *E. coli* MA261 (10), provided by Dr. W. K. Maas, New York University School of Medicine, and its polyamine transport-deficient mutant NH1596 (3) were grown in medium A in the absence of polyamines as described previously (11). Another polyamine-requiring mutant, *E. coli* DR112 (12), provided by Dr. D. R. Morris, University of Washington, was grown as described previously (13). A proton-translocating ATPase mutant, *E. coli* DRK8 (14), provided by Dr. M. Futai, Osaka University, was grown in N-C medium (15) to deplete ATP. *E. coli* JM105 (16) was from Pharmacia LKB Biotechnology Inc.

*E. coli* MA261 potA::Km and MA261 potD::Km were prepared according to the method of Winans et al. (17). pPT72 (6) containing *potAB* genes was digested with Sall and BamHI (*BamHI* site was from vector pACYC184 (18)), and the 2.6 kb fragment was inserted into the *BamHI* and *SalI* sites of pUC118, digested with Sall and EcoRV, and the 2.6 kb fragment was inserted into the *BamHI* and *SalI* sites of pUC118 (ToKRa Biomedicals, Japan). The plasmid, termed pUCpotAB, was digested with *Csp*45I, and the termini made blunt-ended with a Klenow fragment (TOYOBO, Japan). Then, Km′ gene, a 1.3-kb HindII fragment of pUC4K (Pharmacia LKB Biotechnology), was inserted into the cut site. The linearized 3.7 kb fragment, digested with Sall and EcoRV, was introduced into a recD−Tn10 derivative of *E. coli* W3110, and Km′ transformant (*E. coli* W3110 recD::Tn10 potA::Km) was isolated. *E. coli* MA261 potA::Km was then derived from *E. coli* MA261 by transduction of a P1 phage-infected lysate of the above Km′ transformant according to the method of Lennox (19). Similarly, the 1.7-kb BglII−Smal fragment containing potD from pPT04 was inserted into the *BamHI−Smal* sites of pUC119 (ToKRa Biomedicals, Japan). The plasmid, termed pUCpotD, was digested with *BamHI*, and the Km′ gene, a 1.3-kb *BamHI* fragment of pUC4K, was inserted into the cut site.

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Polyamine Uptake by Intact Cells and by Right-side-out Membrane Vesicles—This was performed as described previously (25). The cell suspension was diluted with buffer A (25) so as to have a linear polyamine uptake for 5 min. Right-side-out membrane vesicles were prepared from *E. coli* DR112/pPT86 according to the procedure of Kaback (26), except that the concentration of lysozyme was decreased from 500 to 50 μg/ml. Protein content was determined by the method of Lowry et al. (27).

Purification of PotD Protein and Its Polyamine Binding Activity—PotD protein was purified from the shock fluid (28) of *E. coli* NH1596/pPT104 as described previously (6). The binding assay of polyanion to PotD protein was performed by gel filtration as described previously (29). The binding constant and the number of binding sites were calculated from a Scatchard plot (30) of the results.

Measurement of ATP Content, Δψ, and ΔpH—Assay of ATP was performed by the luciferase enzyme system (31). ATP was extracted with 0.2 M HClO₄ and measured after neutralization with 1 M KOH containing 50 mM KH₂PO₄. The rate of light emission was linearly dependent on the square of the ATP concentration. Δψ and ΔpH were measured in parallel experiments by determining the relative distribution of [³H]tetraphenylphosphonium bromide and [³⁵S]spermidine, respectively, across the membrane according to the method of Joshi et al. (32). Correction for the nonspecific binding of tetraphenylphosphonium bromide or benzoic acid was made by treating a sample with 10 μM CCCP and subtracting the value thus obtained from the experimental samples. The Nernst equation and the Henderson-Hasselbalch equations were used to calculate Δψ (millivolts, negative inside) and ΔpH (millivolts, alkaline inside) (33).

DNA Sequencing—The portion of the potA (1.5 kb) gene of spermidine transport-deficient mutant, *E. coli* NH1596, was sequenced. The 1.0-kb EcoRI fragment was inserted into the same restriction site of M13mp19 at both orientations (16). The DNA was sequenced by the dideoxy method of Sanger et al. (34), using the M13 phage (16). The nucleotide sequence of residual 0.5 kb of potA was directly determined using the polymerase chain reaction product.

Western Blot Analysis of PotA Protein—Immunoglobulin for potA protein was prepared as described previously (35), using the conjugate of the deduced carboxy-terminal 14 amino acid residues (VE-SWEYVLADDEHK) of potA protein and bovine thyroglobulin (36). Western blotting was performed according to the method of Nielsen et al. (37).

Photosaffinity Labeling of PotA Protein—*E. coli* JM105/pPKpotABC and JM105/pPKpotABC cells were cultured as described (13), and 0.5 mM isopropyl-β-D-thiogalactopyranoside was added as described above. Appropriate antibodies (30 μg/ml chloramphenicol, 100 μg/ml ampicillin, and 50 μg/ml kanamycin) were added during the culture of *E. coli*.

Plasmids—Plasmids pT7-BC and pT7-BCD containing the genes of potBC and potBCD were constructed from pT7-5 as described previously (6). pT7-5, a modified plasmid of pT7-1 (20) in which the 5.8-kb fragment was isolated. Then, the 1.1-kb Csp45I-DraIII fragment of pT7-5 described above was digested with DraIII and CiaI (Clal site was from vector pACYC184), and the termini made blunt-ended with T4 DNA polymerase (TOYOBO, Japan) and religated (pPTpT7a).

A low copy number of plasmid pMW119 (Nippon Gene, Japan) (21) was digested with EcoRI. The termini were made blunt-ended with a Klenow fragment and religated, and as a result the EcoRI recognized sequence disappeared from the plasmid. The plasmid was then digested with BamHI and SalI and the 2.6-kb SalI-BamHI fragment was ligated with a 5.8-kb fragment obtained from the pMWpotAB (wild) digested with SmaI and treated with alkaline phosphatase (Boehringer Mannheim). Then, the portion of potA (1.5 kb) of the spermidine transport-deficient mutant, *E. coli* NH1596, was amplified by polymerase chain reaction by the method of Saiki et al. (22). The polymerase chain reaction product was digested with EcoRI, and the 1.0-kb fragment was ligated with a 5.8-kb fragment obtained from the pMWpotAB (wild) digested with EcoRI (pMWpotAIB (V135M)). Plasmid pKK223-5 (Pharmacia LKB Biotechnology) for the overexpression of protein (23) was digested with SmaI and treated with alkaline phosphatase (Boehringer Mannheim). Then, the 3.2-kb StyI-BanHI fragment was isolated. Then, the 1.1-kb Csp45I-DraIII fragment of pT7-5 was made blunt-ended with a Klenow fragment and religated, and as a result the EcoRI recognized sequence disappeared from the plasmid. The plasmid was then digested with BamHI and SalI and the 2.6-kb SalI-BamHI fragment was ligated with a 5.8-kb fragment obtained from the pMWpotAB (wild) digested with EcoRI (pMWpotAIB (V135M)). Plasmid pKKpotABC was digested with Csp45I and DraIII, treated with alkaline phosphatase, and the 6.7-kb Csp45I-DraIII fragment was isolated. Then, the 1.1-kb Csp45I-DraIII fragment of pMWpotAIB was inserted into the cut site of the 6.7-kb Csp45I-DraIII fragment of pKKpotABC (pKKpotAIBC). Transformation of *E. coli* cells with various plasmids was carried out as described by Maniatis et al. (24).

The strains and plasmids used in this study are listed in Table I.

| Strain or plasmid | Relevant Characteristics | Comments, source, or Refs. |
|-------------------|--------------------------|---------------------------|
| **E. coli strains** |                          |                           |
| W3110 recD::Tn10  | W3110 recD               | Laboratory stock          |
| JM106             | supE endA sbcB15 hsdR4 repL thi Δlac-proAB/ΔF' [traD36 proAB+ lacI* lacZD15M] | Commercial source (16) |
| MA261             | speB speC thr leu ser thi | Polymamine-requiring mutant; W. K. Maas (10) |
| MA261             | pKKpotABC                | Spermidine and putrescine transport-deficient mutant of MA261 (3) |
| NH1596            | supE endA sbcB15 hsdR4 repL thi Δlac-proAB/ΔF' [traD36 proAB+ lacI* lacZD15M] | Spontaneous mutant (12) |
| DR112             | speB speC thr leu ser thi | Polymamine-requiring mutant; D. R. Morris (12) |
| DK8               | ΔapdB-aptC               | Spermidine transport-deficient mutant of MA261; this study |
| MA261 potD::Km    | MA261 potA               | Spermidine transport-deficient mutant of MA261; this study |
| MA261 potD::Km    | MA261 potD               | Spermidine transport-deficient mutant of MA261; this study |
| **Plasmids**      |                          |                           |
| pACYC184          | Vector                   | S. N. Cohen (18)          |
| pPTT104           | pKAT* Pp* PotC* PotD*    | Spermidine-preferential transport protein gene (6) |
| pPTT86            | pKAT* Pp* PotC*          | Ref. 6                   |
| pPpT7a            | pKAT* Pp* PotC*          | Insertion of potA into pACYC184; this study |
| pUPgotABC         | pKAT* PotC*             | Insertion of potAB into pUC118; this study |
| pUCpOTD           | pKAT* PotC*             | Insertion of potD into pUC119; this study |
| pT7T-BC           | pKAT* PotC*             | Insertion of potBC into pT7-5 (6) |
| pT7-BCD           | pKAT* PotC*             | Insertion of potBCD into pT7-5 (6) |
| pMWpotABC         | pKAT* PotC*             | Insertion of potBC into pMW119; this study |
| pPKpotABC         | pKAT* PotC*             | Insertion of potABC into pPK223-3; this study |
| pKKpotABC         | pKAT* PotC*             | Insertion of potABC into pKK223-3; this study |

*Valine at the 135th residue from the amino-terminal of potA protein was replaced by methionine.

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added when A600 reached 0.3. The culture was then continued for 2 h. The cells were collected and inside-out membrane vesicles were prepared by French press treatment according to the method of Houn et al. (38). The membrane samples (100 μg of protein) were added to a buffer containing 10 mM Tris-HCl, pH 7.4, 5 mM CaCl2, and 2 mM 8-azido-[α-32P]ATP (N[32P]-ATP) (55.5 kBq) in a final volume of 0.1 ml and placed in a well on ice. The reaction mixture was irradiated for 3 min with UV light (12 watts) from a lamp emitting at 365 nm at a distance of 2 cm (39). The samples were then centrifuged for 20 min at 150,000 × g, resuspended without boiling in Laemmli sample buffer (40) with the omission of 2-mercaptoethanol, and subjected to electrophoresis on a sodium dodecyl sulfate-12% polyacrylamide gel. Radioactivity on dried gel was quantified by a Fujix Imaging Analyzer BAS 2000 (Fuji Photo Film Co. Ltd., Tokyo).

Proline Uptake by Intact Cells—This was performed as described previously (41) using 10 μM [3H]proline as substrate.

RESULTS

Dependence of Spermidine Uptake on PotA and -D Proteins—In order to clarify whether both potA and -D proteins are absolutely necessary for spermidine uptake, the genes for potA and potD in E. coli were disrupted by inserting the gene for kanamycin resistance into potA and potD genes. As shown in Table II, spermidine uptake was not observed in E. coli MA261 potA::Km and MA261 potD::Km, in which the genes for potA and potD were disrupted. The transformation of plasmids containing the genes for potA and potD into these cells recovered spermidine uptake activity. The results indicate that both potA and -D proteins are essential for spermidine uptake, and the spermidine uptake system encoded by the potABCD operon is the only system for spermidine uptake expressed under these experimental conditions.

Function of PotD Protein—Polyamine dissociation constants of potD protein were measured by gel filtration under the condition of 1 mM Mg2+ and 100 mM K+ at pH 7.5. As shown in Fig. 1, the dissociation constants of spermidine and putrescine for potD protein were 3.2 and 100 μM, respectively. These values reflect the uptake affinity of spermidine and putrescine through the spermidine-preferential uptake system in intact cells (3). The binding site for polyamines on potD protein is the only site for spermidine uptake expressed under these experimental conditions.

Function of PotA Protein—ATP dependence for spermidine uptake was examined using right-side-out membrane vesicles. When membrane vesicles were prepared from the cells containing the plasmid for potABCD genes, they had significant uptake activity, and the addition of potD protein did not stimulate spermidine uptake strongly (data not shown), probably due to the association of potD protein with membrane vesicles. This finding confirmed the previous results (25). When membrane vesicles were prepared from the cells containing the plasmid for potABC genes, spermidine uptake with membrane vesicles became potD protein-dependent (Fig. 2). The optimal concentration of potD protein was 5 μM when the assay was carried out using 10 μM spermidine as substrate.

Function of potD protein on spermidine uptake was then examined using the proton-translocating ATPase mutant E. coli DK8, transformed with the potABCD genes. When cells were grown in N-C medium (15), ATP

![Fig. 1. Scatchard plot of polyamine binding to potD protein.](image)

**TABLE II**

PotA and potD protein-dependent spermidine uptake

| Strain                      | Spermidine uptake |
|----------------------------|-------------------|
|                            | nmoles/min/mg protein % |
| MA261                     | 1.72 ± 0.22 ± 0.02 |
| MA261 potA::Km             | 0.049 ± 0.009 ± 0.009 |
| MA261 potA::Km/pPTS6       | 1.55 ± 0.21 ± 0.02 |
| MA261 potD::Km             | 0.029 ± 0.003 ± 0.003 |
| MA261 potD::Km/pUCpotD     | 0.712 ± 0.055 ± 0.055 |

*Mean ± S.D.

content in the cells became very low, but the membrane potential generated was nearly normal (Table III). When cells were energized with succinate by incubation at 37 °C for 10 min, both membrane potential and ATP increased slightly. The addition of glucose greatly increased ATP content but not membrane potential through 10 min of incubation. The change in pH was small (less than 0.1) under these experimental conditions.

As shown in Fig. 3A, spermidine uptake was only observed with cells energized by glucose but not by succinate. When

![Fig. 2. Effect of potD protein on spermidine uptake by membrane vesicles of DR112/pPT66. Various concentrations of potD protein were added to the reaction mixture as shown in the figure.](image)

**TABLE III**

ATP content and membrane potential for the atp mutant DK8/pPT104 cells energized by different energy sources

| Addition                  | ATP content | Δψ |
|---------------------------|-------------|----|
|                           | nmoles/mg protein % | mV |
| 0.4% glucose              | 3.70 ± 0.43 ± 0.30 | 100 | -116 |
| 0.4% glucose + 40 μM CCCP | 3.72 ± 0.45 ± 0.32 | 101 | -214 |
| 0.5% succinate            | 0.99 ± 0.11 ± 0.09 | 27  | -124 |
| None                      | 0.37 ± 0.05 ± 0.05 | 10  | -112 |

*Mean ± S.D.
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A. SPD uptake

B. Pro uptake

FIG. 3. Energy-dependent spermidine (A) and proline (B) uptake by the atp mutant DK8/pPT104 cells. After being grown as described (13) until $A_{600} = 0.5$, the cells were incubated while shaking in N-C- medium (15) for 18 h to deplete ATP (31). Then, protein (23) was used as a vector.

Proline uptake was observed in the ATP-depleted cells and the cells energized by glucose and succinate. The results indicate that ATP is also involved in the uptake. Proline uptake, which is known to be membrane potential dependent (42), was measured as a control (Fig. 3B). Proline uptake was observed in the ATP-depleted cells and the cells energized by glucose and succinate. However, the uptake was completely inhibited by CCCP.

Then, we examined whether potA protein could react with the photoaffinity labeling reagent 8-azido-ATP, since consensus nucleotide-binding sequences were found in potA protein as observed in the sequences of $\alpha$ and $\beta$ subunits of E. coli ATPase (43), hisP protein (44), and malK protein (45). For this purpose, the plasmid pKK223-3 for overproduction of potA protein (43), hisP protein (44), and malK protein (45). For this purpose, the plasmid pKK223-3 for overproduction of potA, -B, and -C proteins was transformed into E. coli JM105/pKKpotABC instead of pKKpotABC, the photolabeling of potA protein, but not the 29-kDa protein, disappeared (data not shown). These results strongly suggest that potA is involved in the energy-coupling step in the spermidine-preferential uptake system.

The nucleotide specificity of binding to potA protein was examined by adding competing nucleotides during the photoaffinity reaction. The degree of inhibition of 8-azido-$[\alpha-3^2P]$ATP labeling of potA protein was in the order ATP > GTP = ADP > CTP = UTP (Table IV). The nucleotide specificity was almost the same as that in histidine and iron citrate transport systems (39, 46).

Identification of the Site of Mutation in the Spermidine Transport-deficient Mutant E. coli NH1596—Recovery of spermidine uptake activity in E. coli NH1596 was examined by transforming the genes for potA, potBC, or potBCD into the mutant cells. As shown in Table V, spermidine uptake activity was observed only when potA, but not potBC or potBCD, was transformed into E. coli NH1596. Thus, the nucleotide sequence of potA gene in E. coli NH1596 was determined, and the deduced amino acid sequence of mutated potA protein was compared with that of normal potA protein. As shown in Fig. 5, valine 135 (GTG codon) of potA protein, which is located between two consensus amino acid sequences for nucleotide binding, was replaced by methionine (ATG codon) in the mutated potA protein. Among the nucleotide-binding proteins in periplasmic transport systems, there are four other conserved sequences (sites C to F) in addition to two consensus sequences for nucleotide binding (sites A and B) according to the analysis by Ames (4). The mutated position in potA protein corresponds to site C.

To confirm that the mutated potA protein is inactive, the membrane potential was extinguished by CCCP, the uptake activity was decreased by 60% even if ATP existed and the change in pH was small. The results indicate that ATP is essential for spermidine uptake and that membrane potential is also involved in the uptake. Proline uptake, which is known to be membrane potential dependent (42), was measured as a control (Fig. 3B). Proline uptake was observed in the ATP-depleted cells and the cells energized by glucose and succinate. However, the uptake was completely inhibited by CCCP.

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![Diagram.png](attachment:Diagram.png)

FIG. 4. 8-azido-$[\alpha-3^2P]$ATP labeling of the potA protein in the inside-out membrane vesicles. SDS-gel electrophoresis was performed using 10 µg of proteins of the inside-out membrane vesicles and 12% acrylamide slabs gels. Numbers on the left represent M, A, Coomassie Blue staining of protein; lane 1, the vesicles prepared from E. coli JM105; lane 2, the vesicles prepared from JM105/pKkopABC, 3, 8-azido-$[\alpha-3^2P]$ATP labeling of proteins; lanes 1 and 2, labeling was performed using the vesicles prepared from E. coli JM105 in the absence and presence of 1 mM ATP, respectively; lanes 3 and 4, labeling was performed using the vesicles prepared from E. coli JM105/pKKpotABC in the absence and presence of 1 mM ATP, respectively.

| Nucleotide | Conc. (mM) | % labeling |
|------------|------------|------------|
| None       | 0.1        | 100        |
| ATP        | 0.1        | 42         |
| ADP        | 1.0        | 12         |
| GTP        | 0.1        | 62         |
| CTP        | 1.0        | 35         |
| UTP        | 0.1        | 78         |
|            | 1.0        | 77         |

| Strain      | Spermidine uptake | % |
|-------------|--------------------|---|
| MA261       | 2.02 ± 0.21        | 100|
| NH1596      | 0.034 ± 0.004      | 1.7 |
| NH1596/pTpotA | 0.549 ± 0.066     | 27.2|
| NH1596/pGP1-2 + pT7-BC | 0.069 ± 0.008 | 3.4 |
| NH1596/pGP1-2 + pT7-BCD | 0.133 ± 0.015 | 6.6 |

* Mean ± S.D.
Fig. 5. Deduced amino acid sequence of mutated potA protein. Boxed residues are two separate blocks (A and B) which comprise the consensus nucleotide-binding fold. The mutated position is shown by the arrow. Valine 135 was replaced by methionine in the mutated potA protein. The numbers on the right refer to the deduced amino acid sequence of potA protein.

Fig. 6. Expression of normal and mutated potA protein. Western blot analysis of potA protein was performed using 30 μg of total cellular protein. Numbers on the left represent Mᵋ. Lane 1, proteins from E. coli MA261; lane 2, proteins from NH1596; lane 3, proteins from MA261 potA::Km; lane 4, proteins from MA261 potA::Km/pMWpotAB; lane 5, proteins from MA261 potA::Km/pMWpotA1B (V135M). potA1 is a gene for mutated potA protein.

TABLE VI

| Strain          | Spermidine uptake by normal and mutated potA protein |
|-----------------|------------------------------------------------------|
|                 | Spermidine uptake (nmol/min/mg protein) %             |
| MA261           | 2.05 ± 0.21*                                         |
| MA261 potA::Km  | 0.066 ± 0.008                                        |
| MA261 potA::Km/pMWpotAB (wild) | 2.19 ± 0.23                                         |
| MA261 potA::Km/pMWpotA1B (V135M) | 0.083 ± 0.010                                        |

* Mean ± S.D.

gene for normal and mutated potA was transformed into E. coli MA261 potA::Km, in which the potA gene was disrupted by the Km' gene. In this experiment, pMW119, a low copy number of plasmid, was used as a vector. The expression of normal and mutated potA protein was confirmed by Western blot analysis using the antibody for carboxyl-terminal 14 amino acids of potA protein. As shown in Fig. 6, the expression of normal and mutated potA protein was observed in E. coli MA261 and NH1596, but not in E. coli MA261 potA::Km. Greater amounts of these proteins were produced in the transformed cells by pMWpotAB or pMWpotA1B (plasmid containing the gene for mutated potA protein).

Finally, spermidine uptake activity was measured (Table VI). Although the activity was observed in both E. coli MA261 and MA261 potA::Km containing the plasmid pMWpotAB, no significant uptake activity was observed in MA261 potA::Km and MA261 potA::Km containing the plasmid pMWpotA1B. Since mutated potA protein was associated with membranes and labeled with 8-azido-[α-32P]ATP (data not shown), the mutation probably influences the late stage of the energy-coupling step in the transport system.

DISCUSSION

The spermidine-preferential uptake system in E. coli consists of four kinds of proteins: a periplasmic protein (potD), a membrane-associated protein having the nucleotide-binding site (potA), and two other membrane proteins (potB and -C) consisting of six membrane-spanning segments linked by hydrophilic segments of variable length (6). In the present study, we examined the functions of potA and -D proteins through a combined biochemical and genetic approach. We could clearly show that spermidine uptake cannot take place without both potA and -D proteins.

When 10 μM spermidine was used as substrate in the assay with membrane vesicles, the optimal concentration of potD protein was 5 μM (Fig. 2). Since the dissociation constant of spermidine for potD protein was 3.2 μM, almost all potD protein (5 μM) can associate with spermidine under these conditions. This confirms that the spermidine-potD protein complex is the real substrate in this transport reaction. Free potD protein may compete with the spermidine-potD protein complex in terms of the binding to transmembrane proteins (potB and -C proteins) on membrane because the addition of 20 μM potD protein was observed to slightly inhibit the spermidine uptake (Fig. 2). This finding is in accordance with the previous results of Prossnitz et al. (47) that excess amounts of histidine-binding protein inhibited histidine transport on membrane vesicles.

It has been reported that the apparent Kₘ value for spermidine transport in intact cells is 0.1 μM (3). There is a great difference between that value and the dissociation constant of spermidine for potD protein (3.2 μM). PotD protein in the periplasm has been estimated as roughly 100 μM. If the spermidine concentration would be 0.1 μM (the Kₘ value), the concentration of spermidine-potD protein in the periplasm would be approximately 3.1 μM. Although free potD protein slightly inhibits spermidine uptake, spermidine could be effectively transported into cells since 3.1 μM of the real substrate, which is close to the dissociation constant of spermidine for potD protein, would exist in the periplasm.

We showed that ATP is absolutely necessary for spermidine uptake using a proton-translocating ATPase mutant of E. coli. This is in accordance with the results from a study of the histidine transport system (32). ATP could bind to potA protein but not to potB and -C proteins. We also showed that mutated potA protein, in which the mutation occurred at the position between two consensus amino acid sequences for nucleotide binding, could not support spermidine uptake. The mutated position corresponded to site C, which is one of the other four conserved amino acid sequences (4). This position may be important for the late stage of energy coupling such
as the hydrolysis of ATP or the dissociation of ADP, since the mutated potA protein was associated with membrane and labeled by 8-azido-[32P]ATP. The energy-coupling mechanism will be clarified in detail with a reconstituted system from purified proteins.

In contrast to the histidine transport system [32], spermidine uptake was inhibited significantly by the protonophore CCCP. This suggests that the membrane potential is also involved in the uptake. In the in vivo translocation of secretory proteins catalyzed by secE, -Y, and -A proteins, the membrane potential is also involved in the uptake. The energy-coupling mechanism will be clarified in detail with a reconstituted system from purified proteins.

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