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Recombinant Polyamine-Binding Protein of *Synechocystis* sp. PCC 6803 Specifically Binds to and Is Induced by Polyamines

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Abstract—His-tagged *Synechocystis* sp. PCC 6803 PotD protein (rPotD) involved in polyamine transport was overexpressed in *Escherichia coli*. The purified rPotD showed saturable binding kinetics with radioactively labeled polyamines. The rPotD exhibited a similar binding characteristic for three polyamines, with putrescine having less preference. The K_d values for putrescine, spermine, and spermidine were 13.2, 8.3, and 7.8 μM, respectively. Binding of rPotD with polyamines was maximal at pH 8.0. Docking of these polyamines into the homology model of *Synechocystis* PotD showed that all three polyamines are able to interact with *Synechocystis* PotD. The binding modes of the docked putrescine and spermidine in *Synechocystis* are similar to those of PotF and PotD in *E. coli*, respectively. Competition experiments showed specific binding of rPotD with polyamines. The presence of putrescine and spermidine in the growth medium could induce an increase in PotD contents, suggesting the role of PotD in mediating the transport of polyamine in *Synechocystis* sp. PCC 6803.

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The polyamine-binding transport protein D (PotD) is produced and accumulated in the periplasm of several gram-negative bacterial species, where PotD interacts with high affinity with its substrates spermidine or putrescine before their uptake into the cell interior. These specialized high-affinity transport systems are classified as ABC (ATP-binding cassette) type transporters [1-5]. In *Escherichia coli*, the preferential active transport system for spermidine depends upon the PotD [6], two integral membrane-associated transport proteins (PotB and PotC) and ATP-hydrolyzing subunit (PotA) [7]. Another distinct four-gene operon coding for a putrescine-specific transporter consists of a periplasmic-binding protein (PotF), two transmembrane proteins (PotH and PotI), and an ATPase (PotG) [8]. The PotE protein, which is specific for putrescine, enabling both its uptake and release, has been described [9]. *Escherichia coli* PotD exhibits preferential binding to spermidine over putrescine [10], whereas *E. coli* PotF possesses a high binding affinity to only putrescine and does not bind other polyamines [11]. A similar model for polyamine transporter has been proposed for the *potABCD* operon of *Streptococcus pneumoniae*, showing a high similarity of amino acid sequence to the *E. coli potABCD* operon. Additionally, *Str. pneumoniae* PotD possesses characteristics of an extracellular protein that binds polyamines and possibly other structurally related molecules [4]. Furthermore, *in vitro* studies have shown that polyamines can bind to artificial membranes, and the stability of the complexes depends on the type and density of acidic phospholipids present in the vesicles [12].

The gram-negative, unicellular *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) contains a gene (*slr0401*) encoding a periplasmic binding protein D (PotD) of an ATP-binding cassette (ABC)-type transporter for polyamine [13]. Polyamine transport by *Synechocystis* has been recently demonstrated [14, 15]. The uptake activity is dependent on pH, electrochemical potential gradient, and substrate concentration. However, the mechanism of the interactions between polyamines

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and PotD protein at the molecular level is largely unknown.

In the present work, we report the capability of recombinant *Synechocystis* PotD to bind polyamines and evaluate its functionality under *in vitro* conditions. Polyamine docking experiments were also done to investigate the determinants for the polyamine binding specificity of *Synechocystis* PotD at the molecular level.

MATERIALS AND METHODS

Organism and culture conditions. *Synechocystis* was grown photoautotrophically at 30°C in BG11 medium [16] under continuous illumination provided by warm white fluorescent tubes (3 × 30W). The incident light intensity in the growth chamber was 50 μmol photons·m^{-2}·sec^{-1}. Growth of liquid cultures was monitored by measuring the optical density at 730 nm (OD_{730}) with a Spectronic Genesys 2 spectrophotometer.

Genomic organization and plasmid construction. The *slr0401* sequence encoding the putative periplasmic binding protein D of *Synechocystis* (PotD) [13] was amplified from the genomic DNA of *Synechocystis* using gene-specific primers. *Nde*I and *Bam*HI restriction sites (underlined) were added to *potD* forward primer (5′-CCA TAT GAA TTT ACC CTG CTA TTC CCG CCG-3′) and *potD* reverse primer after the stop codon (5′-CGG GAT CCC TAA GCA CTC CGC ATG GTT T-3′). The PCR products were fractionated on agarose gel, and the DNA fragments were recovered and cloned into pET19b (Promega, USA) to construct pET*potD*. *Escherichia coli* BL21 (DE3) was transformed with pET*potD* for protein expression.

Expression and purification of recombinant *Synechocystis* PotD. The recombinant *E. coli* BL21 (DE3) cells harboring pET*potD* were grown at 37°C in LB medium containing 100 μg/ml ampicillin until OD_{600} reached 0.6. Protein production was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Recombinant cells were harvested by centrifugation at 4000*g*, 4°C for 15 min. The pellets were washed twice with 50 mM Tris-HCl buffer, pH 8.0, resuspended in the same buffer, and disrupted by sonication with three short pulses of 30 sec. After centrifugation at 18,000*g*, 4°C for 60 min, soluble and insoluble fractions were analyzed by SDS-PAGE.

His-tagged *Synechocystis* PotD protein (rPotD) was found in the pellet fraction as inclusion bodies, the pellet was subjected to purification using a Ni^{2+}-affinity chromatography column according to the manufacturer's suggestions for insoluble proteins (His GraviTrap kit; GE Healthcare, England). The purification was done according to Schlicke and Brakmann [17]. Briefly, inclusion bodies were washed twice in 50 mM Tris-HCl, pH 8.0, and dissolved in buffer A (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 20 mM imidazole, 0.1% Triton X-100, 0.5 mM phenylmethanesulfonyl fluoride (PMSF), and 8 M urea). The insoluble proteins were removed by centrifugation. The clear solution was applied onto a Ni^{2+}-Sepharose column equilibrated with three volumes of buffer A. The rPotD was eluted by modified buffer A containing 500 mM instead of 20 mM imidazole.

Protein refolding. The rPotD was refolded by dialysis overnight at 4°C against 50 mM Tris-HCl buffer, pH 8.0, containing 0.1% Triton X-100 and 0.5 mM PMSF. The dialyzed protein sample was then centrifuged at 18,000*g* at 4°C for 20 min to remove unfolded or aggregated proteins and analyzed by 15% (w/v) SDS-PAGE. The concentration of rPotD was determined by Bradford's method [18].

Binding of polyamine to purified His-tagged *Synechocystis* rPotD. The reaction mixtures containing 100 μg of purified rPotD protein, 50 mM Hepes-KOH, pH 7.6, 30 mM KCl, and 50 μM each radioactively labeled polyamine (i.e. [^{14}C]putrescine, [^{14}C]spermidine, and [^{14}C]spermine) with a specific activity of 2 μCi/μmol were incubated at 37°C for 5 min. The reaction was stopped by rapid filtration through a filter. The filter was rinsed with 5 ml of cold buffer (50 mM Hepes-KOH, pH 7.6, 30 mM KCl). The radioactivity was counted with a liquid scintillation counter (Beckman Coulter Multipurpose Scintillation Counter LS6500, USA). Competition by substrate analogs was assayed by adding 20-fold excess of unlabeled analogs to the mixture. The apparent dissociation constant (K_d) and the maximum binding (B_{max}) parameters were calculated from Scatchard plots as described previously [10].

Docking of polyamines into homology model of *Synechocystis* PotD. Spermine, spermidine, and putrescine were docked into the homology model of *Synechocystis* PotD [19] using the GOLD 3.2 program [20, 21]. Prior to the docking experiments, spermidine was removed from the *Synechocystis* PotD model and hydrogens were added in Sybyl 8.0 (Tripos International, USA). In the putrescine docking experiment the conserved water molecule involved in putrescine binding in the *E. coli* PotF (PDB code 1A99) crystal structure was added to the *Synechocystis* PotD model prior to adding hydrogens. Ten independent genetic algorithm runs with the default docking parameters were made in GOLD. The binding site in the *Synechocystis* PotD model was restricted within 15 Å radius from the OD2 atom of Asp206 in the spermidine and spermine docking experiments, while the putrescine docking experiment was centered at the OD2 atom of Asp295. The docking was stopped if three docking solutions were within 1.5 Å rmsd. The docking results were visualized and examined with the Bodil modeling and visualization environment [22], and figures were prepared with PyMOL [23].

Induction of PotD. PotD induction was studied in cells grown for 5 days in BG11 medium containing

0.5 mM each of putrescine, spermidine, and spermine as well as 0.55 M NaCl, 0.3 M sorbitol, or the combination of either NaCl or sorbitol with each polyamine. Soluble proteins (50 μg) were separated by SDS-PAGE using 15% (w/v) acrylamide gels. After electrophoresis, proteins were electroblotted onto a polyvinylidene fluoride (PVDF) membrane (Millipore, UK) and subsequently probed with polyclonal anti-PotD. The detected bands were analyzed using Image Analysis Software.

Data analysis. The data are presented as means ± SD. Comparisons were made using Student's *t*-test. Significance was accepted at $P < 0.05$, $n = 3$.

RESULTS

Expression and purification of the His-tagged *Synechocystis* rPotD. The *potD* gene was inserted into a pET19b vector to construct pET*potD* (Fig. 1a) and subsequently expressed in *E. coli* BL21 (DE3) cells. Whole cells and a crude extract from sonicated cells were analyzed by SDS-PAGE. The induction of an approximate 43 kDa recombinant protein occurred after 1 h of induction by IPTG and gradually increased over the 5 h induction (Fig. 1b). The rPotD content induced for 5 h was 10-fold higher than that of the non-induced. The rPotD was found in the pellet fraction as inclusion bodies (data not shown). By Ni^{2+}-affinity chromatography purification, rPotD was detected in the fraction eluted with 500 mM imidazole. The recombinant PotD was purified to homogeneity as determined by SDS-PAGE (Fig. 1c). Analysis by immunoblotting with anti-His monoclonal antibody and polyclonal PotD antiserum indicated that the rPotD had apparent molecular mass of 43 kDa, corresponding to the size of the predicted rPotD (Fig. 1c).

Table 1. Parameters for rPotD binding to different polyamines

Polyamine	K_d, μM	B_{max}, mol/mol
Putrescine	13.2 ± 0.1	0.74 ± 0.05
Spermidine	7.8 ± 0.06	1.42 ± 0.41
Spermine	8.3 ± 0.05	0.13 ± 0.03

Affinities for polyamine binding. The binding of putrescine, spermidine, or spermine to rPotD was saturable (Fig. 2a). Scatchard plots showed linear transformation of the binding of all three polyamines to rPotD (Fig. 2b). The values of the dissociation constant (K_d), calculated from the reciprocal of the slope for putrescine, spermidine, and spermine *in vitro* were 13.2, 7.8, and 8.3 μM, respectively (Table 1). The maximum binding (B_{max}) values, the x-intercepts of the Scatchard plot, of putrescine, spermidine, and spermine were 0.74, 1.42, and 0.13 mol/mol rPotD, respectively (Table 1). The data suggest that the three polyamines bind to rPotD with a 1 : 1 binding stoichiometry based on the molecular mass for rPotD of 43 kDa. The seemingly low values of less than 1 for putrescine and spermine might be due to dissociation of the complex occurring during filtration/washing of the membrane. Another possibility is that a portion of improperly refolded rPotD was present in the final preparation that was incompetent to bind polyamines.

Changes in polyamine binding capacity as a function of external pH were observed (Fig. 2c). The specific binding of each of the polyamines to rPotD displayed an optimum at pH 8.0. It is noted that rPotD had the high-

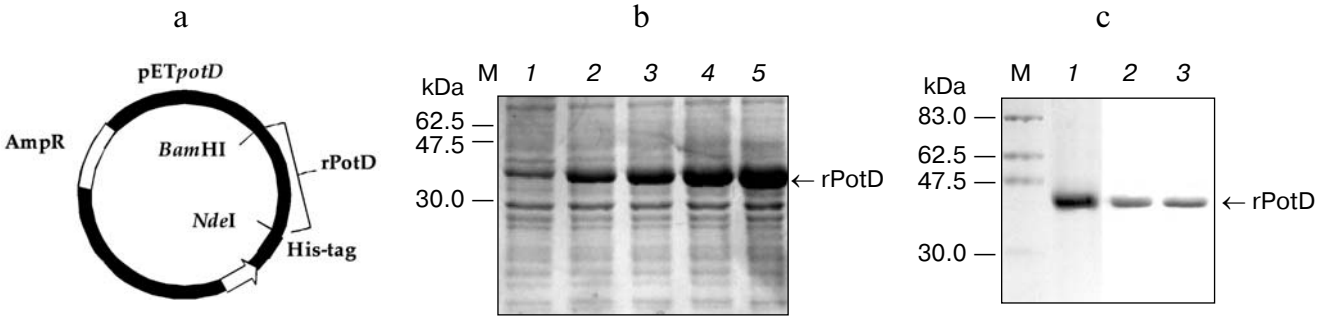

Fig. 1. Expression of recombinant *Synechocystis* PotD in *E. coli* BL21 (DE3). a) Schematic view of the recombinant plasmid. pET*potD* is a derivative of pET19b (Novagen) harboring *Synechocystis potD* gene. The vector carries an ampicillin resistance gene (*AmpR*), and the *potD* (*slr0401*) gene is inserted between *Nde*I and *Bam*HI restriction sites. b) Coomassie-stained gel for analysis of expression of His-tagged *Synechocystis* PotD (rPotD) in *E. coli* from the vector pET19b. Whole-cell extracts were loaded on 15% SDS-PAGE. Lanes: M) molecular weight markers (in kDa); *1*) non-induced cultures after overnight growth; *2-5*) cultures kept for 1, 2, 3, or 5 h, respectively, after IPTG induction in LB medium at 37°C. c) 15% SDS-PAGE analysis of eluted fraction from Ni^{2+}-chromatography purification of rPotD from *E. coli* and immunoblotting analysis. Lanes: M) molecular weight markers (in kDa); *1*) fraction eluted with buffer containing 500 mM imidazole; *2*) Ni^{2+}-purified rPotD probed with monoclonal anti-His antibody; *3*) Ni^{2+}-purified rPotD probed with polyclonal rabbit immune serum against purified rPotD.

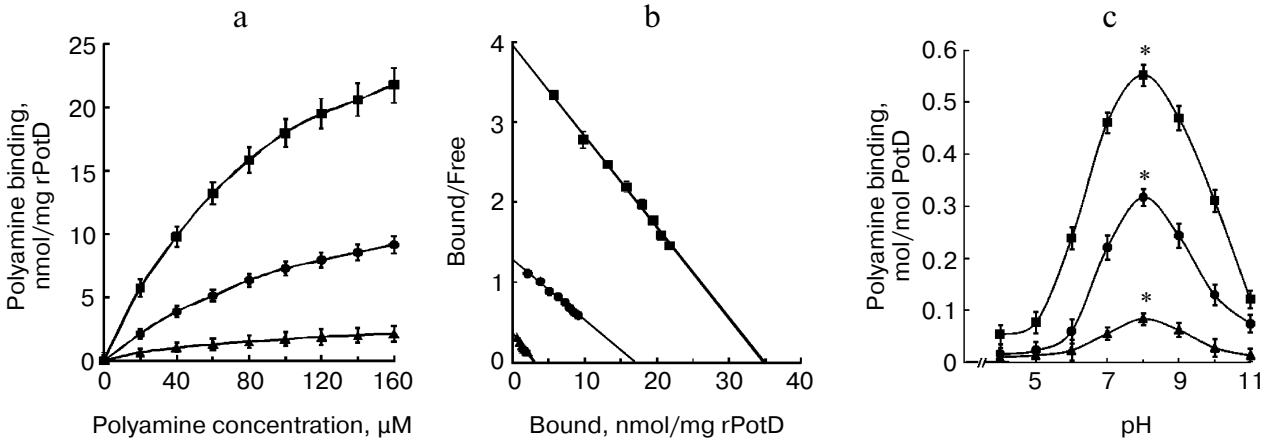

Fig. 2. Putrescine (circles), spermidine (squares), and spermine (triangles) binding. a) Different amounts of each polyamine were added to give different external polyamine concentrations. The data represent three independent biological replicates. b) Scatchard analysis of the data. The line drawn is derived from regression analysis of the data. K_d and B_{max} were obtained from the reciprocal of the slope and the intercept on the x-axis, respectively. c) Effect of external pH on polyamine binding. The binding assays were performed at different pH values. Asterisks indicate statistically significant differences.

est binding capacity for spermidine at all pH values tested.

Specificity of polyamine binding. Experiments were carried out to determine if the interactions between the rPotD and polyamines are specific. As shown in Table 2, the binding of each radioactively labeled polyamine to rPotD was not inhibited by compounds structurally related to polyamines, including L-arginine, L-asparagine, L-glutamic acid, L-lysine, and L-ornithine. Binding of [^{14}C]spermidine to rPotD was inhibited by non-labeled spermine and putrescine by 35 and 42%, respectively. Binding of [^{14}C]spermine to rPotD was inhibited by non-labeled putrescine and spermidine by 39 and 58%, respectively. Similarly, binding of [^{14}C]putrescine to rPotD was inhibited by non-labeled spermine and spermidine by 38 and 58%, respectively. These results prove that rPotD is able to bind specifically to the three polyamines – putrescine, spermidine, and spermine.

Table 2. Effect of polyamine analogs on specific binding activity of *Synechocystis* rPotD

Analog	Polyamine binding, %		
	[^{14}C]putrescine	[^{14}C]spermidine	[^{14}C]spermine
Control	100 ± 2	100 ± 2	100 ± 2
Putrescine	–	58 ± 2*	61 ± 3*
Spermidine	42 ± 6*	–	42 ± 4*
Spermine	62 ± 3*	65 ± 5*	–
L-Arginine	94 ± 3	92 ± 3	93 ± 2
L-Asparagine	96 ± 6	95 ± 3	96 ± 5
L-Glutamic acid	96 ± 5	98 ± 3	97 ± 3
L-Lysine	90 ± 5	94 ± 6	92 ± 4
L-Ornithine	91 ± 4	91 ± 5	93 ± 5

Note: Data shown are means of three independent experiments representing percent binding activity relative to control (100%) for putrescine, spermidine, and spermine, which were 0.33 ± 0.03, 0.55 ± 0.18, and 0.05 ± 0.01 mol/mol, respectively.

* Statistically significant differences (Student's *t*-test, $P < 0.05$, $n = 3$) with respect to control.

Docking of polyamines into homology model of *Synechocystis* PotD. Putrescine, spermidine, and spermine were docked into the *Synechocystis* PotD model to examine differences in the binding of the three polyamines. The binding modes of spermidine and putrescine were also compared with their binding to the preferred periplasmic substrate-binding proteins in *E. coli*, PotD and PotF, respectively [24]. The binding mode of the docked spermidine in *Synechocystis* PotD was very similar to the binding mode of spermidine in the X-ray structure of *E. coli* PotD as well as to the binding mode of spermidine in the previously modeled complex of *Synechocystis* PotD and spermidine. *Escherichia coli* and *Synechocystis* PotD share conserved residues (Asp206, Glu209, Trp267, Trp293, and Asp295; *Synechocystis* numbering), which are involved in the binding of spermidine (Fig. 3a; see color insert). Additionally, Leu40 binds spermidine through hydrophobic interactions, while Asn269 and Gln270 bind the N1 nitrogen of spermidine. The volume of the active site cavity in *Synechocystis* PotD is large enough to accommodate spermine, which is largest of the docked polyamines. Based on the docking results, spermine binds similarly to spermidine (Fig. 3b; see color insert). An additional residue, Gln71, was found to interact with the N14 nitrogen of spermine. A conserved water molecule in *E. coli* PotF (water number 471

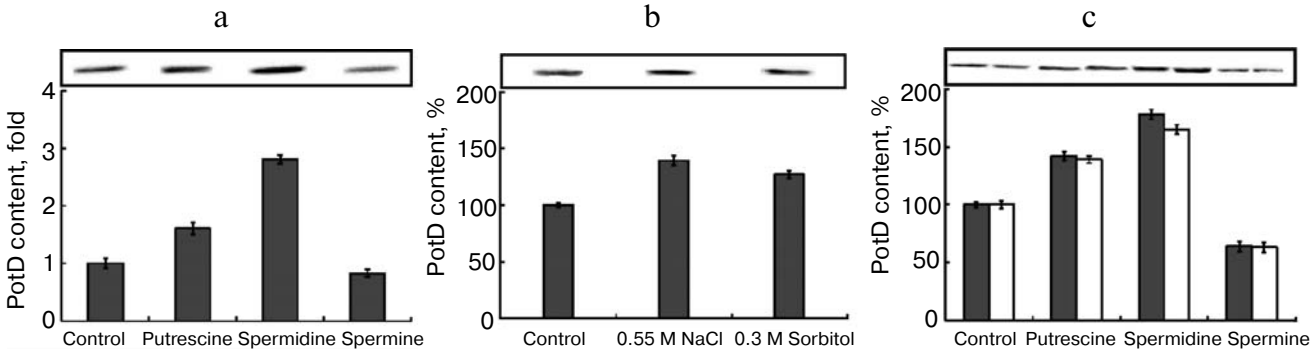

Fig. 4. Western blot analysis of PotD induction by 0.5 mM polyamine (a), osmotic stress of either 0.55 M NaCl or 0.3 M sorbitol (b), and the combination of polyamine and osmotic stress imposed by 0.55 M NaCl (black columns) or 0.3 M sorbitol (white columns) (c). *Synechocystis* cells grown for 5 days under various conditions were analyzed. The mean values (±SE) are calculated from the results representing three independent biological replicates. The top panel of each figure shows immunoblotting analysis probed with polyclonal rabbit immune serum against purified rPotD.

in PDB structure 1A99) was shown to be important for putrescine binding [11] and, thus, it was added to the active site of the *Synechocystis* PotD model prior to putrescine docking. Putrescine was docked to the model in a similar binding mode as in the crystal structure of *E. coli* PotF (Fig. 3c; see color insert). *Synechocystis* PotD residues Leu40, Glu209, Trp267, Gln270, Trp293, and Asp295 are involved in binding putrescine, while Asp206 and Asn269 are too far away to be able to bind. Glu209 interacts with the N2 atom of putrescine via a water molecule.

Induction of PotD. Growth of *Synechocystis* cells in medium containing either 0.5 mM putrescine or spermidine led to an increase in PotD, about 1.6- and 2.8-fold, respectively (Fig. 4a). A slight decrease in PotD was observed in the presence of 0.5 mM spermine. As previous studies showed an increase in polyamine uptake under osmotic stress [14, 15], we further tested whether PotD levels could change under osmotic stress. A small increase in PotD by about 40 and 30% was observed when *Synechocystis* cells were stressed with 0.55 M NaCl and 0.3 M sorbitol, respectively (Fig. 4b). There was no synergistic effect on the increase of PotD by the simultaneous presence of polyamine and either NaCl or sorbitol (Fig. 4c).

DISCUSSION

In the present work we have studied the *in vitro* binding capacity with respect to polyamines of the binding protein of *Synechocystis* to clarify the characteristics of polyamine transport. Sets of genes involved in polyamine uptake have been identified in the annotated genome of *E. coli* so far [25]. Unlike *E. coli* and other bacterial species genes, the gene encoding the binding component subunit of *Synechocystis* (PotD) is not physically linked to those encoding the hydrophobic components, suggesting alternative regulatory pathways controlling the balanced expression of the transport system components [25]. The transporters in cyanobacteria consist of several polypeptide subunits, but their structure is not much related to the corresponding *E. coli* ones. Moreover, the low sequence identity between the PotD in *E. coli* and *Synechocystis* subunits does not allow identification of the *Synechocystis* ones using homology searches. Hence, it is necessary to clarify the details of the transport mechanism in *Synechocystis* since it is not obvious that the functions of PotD are identical in *E. coli* and *Synechocystis*.

The *potD* gene (*slr0401*) was cloned and overexpressed in *E. coli* as a recombinant His-tagged protein (rPotD). The SDS-PAGE gel showed overexpression of the His-tagged PotD, with apparent molecular mass of 43 kDa, which is slightly higher than the theoretical full-length native form of PotD (41 kDa) previously reported in other bacteria [4, 26]. The rPotD is capable of binding putrescine, spermidine, as well as spermine, although the affinity for putrescine is about 2-fold lower than that for spermidine. The K_d values for polyamine binding reported here are different from those previously reported by Brandt et al. [19] using the surface plasmon resonance (SPR) method. The SPR measurements involved immobilization of rPotD on the surface of modified gold and, as a result, the protein could not maintain its native form [27]. In contrast, the binding studies using radioactive polyamines are quite sensitive, and the native form of rPotD was maintained during the experiment. Polyamines can effectively bind to the binding site of rPotD giving rise to reliable K_d values.

Comparison of rPotD affinity for different polyamines reveals that both spermidine and spermine, which similarly bound to rPotD according to the results of docking experiments (Figs. 3a and 3b), had higher affinity than putrescine. Our results are in agreement with those

on *E. coli* PotD, which favors the binding of spermidine (K_d = 3.2 μM) rather than putrescine (K_d = 100 μM) [10]. On the contrary, *E. coli* PotF shows a high binding affinity to only putrescine (K_d = 2.0 μM) and does not bind other polyamines [11]. Likewise, TpPotD of *Treponema pallidum* (PotD homolog) also exhibits a higher binding affinity for putrescine (K_d = 10 nM) than spermidine (K_d = 430 nM) [28]. These results may indicate different characteristics of polyamine binding sites among different organisms.

L-Amino acids such as arginine, asparagine, glutamic acid, or lysine hardly inhibited spermidine binding. Spermidine showed higher inhibition (58%) of spermine binding activity than did spermine on spermidine binding activity (35%) (Table 2). This lent further support for the preference of rPotD to bind spermidine rather than spermine. The less favorable binding of spermine to rPotD might be related to the four positively charged nitrogen atoms leading to hindered structure of spermine.

A unique network of hydrogen bonds presumably limits the binding cavity of putrescine-preferential *E. coli* PotF, making it too small to bind the larger polyamines. The conserved water molecule plays a special role in preventing putrescine from protruding deep into the binding cavity [11]. *Escherichia coli* PotD seems to lack the hydrogen bond network, resulting in a more flexible binding cavity and, thus, being able to bind both spermidine and putrescine, the latter with lower affinity [11]. Regarding the binding preference, the binding cavity of *Synechocystis* PotD is more similar to the binding cavity of *E. coli* PotD. The docking experiments conducted with putrescine, spermidine, and spermine showed that the binding site of *Synechocystis* PotD is capable of accommodating all three polyamines. The fact that *Synechocystis* PotD has more favorable interactions with spermidine than with putrescine provides an explanation for the higher affinity binding of spermidine among different organisms.

Spermidine was more effective than putrescine in the induction of PotD in *Synechocystis*. In *E. coli*, spermidine was also shown to better induce the transcription of the TPO5 gene than putrescine [29]. The TPO5 protein has a role in the excretion of polyamine with higher capacity for putrescine than spermidine. For *Synechocystis*, the increase in PotD content by spermidine is necessary for the acclimation of cells to external spermidine, accompanied by uptake and accumulation of spermidine. Since high intracellular spermidine is inhibitory to cell growth [15], we found that the increase in PotD is beneficial to the cells due to the ability of PotD to excrete spermidine into the medium (data not shown). This would enable the cells to maintain a low level of intracellular spermidine when exposed to high external concentration of spermidine.

Taken together, the results from the present study reveal the capacity of the rPotD of *Synechocystis* sp. PCC 6803 to bind specifically to polyamines, and the docking results provide an explanation for the preferred spermidine binding. Further work on the interaction between PotD and other proteins as well as the characterization of genes and the transporter involved in polyamine transport in *Synechocystis* sp. PCC 6803 is in progress.

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