Biochimica et Biophysica Acta, 1107 (1992) 126–130

BBAMEM 75645

Proton potential-dependent polyamine transport by vacuolar membrane vesicles of *Saccharomyces cerevisiae*

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(Received 21 January 1992)

Key words: Polyamine transport; Proton potential; Vacuolar membrane vesicle; (*S. cerevisiae*)

Vacuolar membrane vesicles of *Saccharomyces cerevisiae* accumulated spermine and spermidine in the presence of ATP, not in the presence of ADP. Spermine and spermidine transport at pH 7.4 showed saturation kinetics with K_m values of 0.2 mM and 0.7 mM, respectively. Spermine uptake was competitively inhibited by spermidine and putrescine, but was not affected by seven amino acids, substrates of active transport systems of vacuolar membrane. Spermine transport was inhibited by the H^+-ATPase-specific inhibtors bafilomycin A_1 and *N,N'*-dicyclohexylcarbodiimide, but not by vanadate. It was also sensitive to Cu^{2+} or Zn^{2+} ions, inhibitors of vacuolar H^+-ATPase. Both 3,5-di-*tert*-butyl-4-hydroxybenzilidenemalononitrile (SF6847) and nigericin blocked completely the spermine uptake, but valinomycin did not. [^{14}C]Spermine accumulated in the vesicles was exchangeable with unlabeled spermine and spermidine. However, it was released by a protonophore only in the presence of a counterion such as Ca^{2+}. These results indicate that a polyamine-specific transport system depending on a proton potential functions in the vacuolar membrane of this organism.

Introduction

Polyamines, represented by putrescine, spermidine and spermine, are found to be widely distributed from bacteria through to higher organisms [1]. The significance of these polyamines in cell proliferation is clear, since the depletion of intracellular polyamines by the blockage of synthetic pathways with inhibitors or the mutation of polyamine biosynthetic enzymes halts the cell growth. It can then be restored by the addition of polyamines to the medium [2].

The cellular polyamine level is usually regulated by both its synthetic pathway via ornithine decarboxylase as the rate-limiting enzyme with a very short half-life and by its degradative pathway [2]. It has recently become accepted that movements of polyamines across the cell membrane are also important for regulation of its cellular content [3]. Uptake systems specific for polyamines are reported in bacteria and animal cells [4–7]; we found three polyamine uptake systems, which depend on the proton potential, in *Escherichia coli* [8,9], and a membrane potential-dependent uptake system of polyamines in bovine lymphocytes [10]. As polyamines and their derivatives synthesized in vivo are released into the medium [11], their extrusion pathways must exist.

As polyamines easily bind to cellular anionic constituents such as RNA, DNA and phospholipids [12], it is hard to define the target site(s) crucial for cellular physiology. However, we understand that binding of polyamine to the ribosomal machinery or mRNA plays an important role in protein synthesis in the cytoplasm [13,14].

The cytoplasmic polyamine level is probably influenced by its compartmentalization in various organelles of eukaryotes: a few reports have indicated the existence of the polyamine transport system in mitochondria [15,16]. Vacuoles compose the largest compartment in plants and fungi [17]. It has been proposed that vacuoles maintain cytoplasmic homeostasis of nutrients and ions and act as a metabolic compartment or lytic compartment in intracellular digestive processes [17]. In *Neurospora crassa*, it was reported that spermidine is localized in vacuoles [18]. However, the mechanism of polyamine accumulation into vacuoles has not been investigated.

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Abbreviations: ANS, 1-anilinonaphthalene-8-sulfonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; HPLC, high-performance liquid chromatography; Mes, 2-(*N*-morpholino)ethanesulfonic acid; SF6847, 3,5-di-*tert*-butyl-4-hydroxybenzilidenemalononitrile.

We examined here polyamine transport by vacuolar membrane vesicles of *Saccharomyces cerevisiae*, and found a polyamine specific transport system depending on a proton potential.

Materials and Methods

Strain and culture condition. All the experiments were conducted with *Saccharomyces cerevisiae* haploid strain X2180-1A from the Yeast Genetic Stock Center, Berkeley, CA, USA. Cells were grown in medium (YEPD) containing 1% Difco yeast extract, 2% polypeptone and 2% glucose at 30°C with subsequent shaking at 120 strokes per min.

Chemicals. [^{14}C]Putrescine, spermidine and spermine (4 GBq/mmol) were purchased from DuPont-New England Nuclear. SF6847 was a gift from Dr. Y. Anraku, University of Tokyo, Japan. Other chemicals used were of analytical grade.

Preparation of spheroplast lysate, intact vacuoles and right-side-out vacuolar membrane vesicles. Spheroplast lysate, intact vacuoles and right-side-out vacuolar membrane vesicles, which were essentially free of mitochondrial contamination (no succinate dehydrogenase activity as a marker enzyme for mitochondria), were prepared by the method of Ohsumi and Anraku [19]: in short, intact vacuoles were released into the lysate by an 'osmotic shock' disruption of spheroplasts, and then separated from other organelles and cellular constituents by centrifugation. Only intact vacuoles are floated in 8% Ficoll buffer during centrifugation at $26\,600 \times g$ for 30 min (see ref. 19 for details). The vesicles were perfectly spherical and 0.2–1.0 μm in diameter. An internal water space of vacuolar membrane vesicles of 5.2 μl/mg membrane protein [20] was used for calculation of the concentration.

Transport assay. The standard assay mixture (100 μl) consisted of 25 mM Tris-Mes (pH 7.4), 4 mM $MgCl_2$, 25 mM KCl, 0.5 mM ATP, 0.1 mM polyamine (74 MBq/mmol) and 30–50 μg protein of vacuolar membrane vesicles. The reaction at 25°C was initiated by adding labeled substrate and terminated by diluting the reaction mixture with 5 ml of cold buffer consisting of 10 mM-Tris Mes (pH 6.9), 5 mM $MgCl_2$ and 25 mM KCl. The vacuolar membrane vesicles were recovered on a membrane filter (Millipore, 0.45 μm) and washed with 5 ml of the above buffer. The radioactivity was determined with a liquid scintillation counter.

Polyamine analysis. Polyamine contents in spheroplast lysates, intact vacuoles and vacuolar membrane vesicles were determined with HPLC as described elsewhere [21] after extraction with hot trichloroacetic acid.

Other procedures. Protein was determined by the method of Lowry et al. [22] with bovine serum albumin as standard.

TABLE I

Retention of polyamines in vacuoles

S. cerevisiae X2180-1A grown on YEPD medium were harvested at $3 \cdot 10^7$ cells/ml, and then spheroplast lysate and intact vacuoles were prepared as described in Materials and Methods. Intact vacuoles were then disrupted by osmotic shock to convert to membrane vesicles, and the vesicles and their supernatant were recovered by centrifugation [19]. These fractions were extracted twice with 0.5 ml of 5% trichloroacetic acid, and aliquots were then analyzed for polyamines by HPLC as described previously [21]. Standard error was within the range of 5% in triplicate experiments. PUT, putrescine; SPD, spermidine; SP, spermine. Numbers in parentheses show total polyamine content (in nmol) in each fraction; n.d., not detected.

Fraction	Protein (mg)	nmol/mg protein (nmol)		
		PUT	SPD	SP
Spheroplast lysate	238	3.9	51.6	0.5
Intact vacuoles	1.7	0.8 (1.4)	60.6 (103)	1.9 (3.2)
After precipitation				
Precipitate(vesicles)	0.4	n.d.	72.5 (29)	0.9 (0.4)
Supernatant	1.5	1.1 (1.7)	43.3 (65)	1.9 (2.9)

Results

Retention of polyamines in vacuoles

Table I shows the polyamine contents of spheroplast lysate, intact vacuoles and vacuolar membrane vesicles. Strain X2180-1A was grown on YEPD medium, harvested at $3 \cdot 10^7$ cells/ml, and individual fractions were prepared as described in Materials and Methods. In the fraction of purified vacuoles, putrescine, spermidine and spermine were all detected, although spermidine was preferentially presented. By osmotic disruption, 100% of putresine, 65% of spermidine and 90% of spermine in the vacuoles were released, indicating that all these polyamines were actually retained in vacuoles. Based on α-mannosidase activity as a specific marker enzyme of the vacuolar membrane [20], about 13% of total vacuoles in the spheroplast lysate are recovered by this purification method. This means that 1% of putrescine, 6% of spermidine and 21% of spermine of the lysate are localized in vacuoles although the possibility of a leakage of polyamine from vacuoles by this osmotic shock method cannot be ruled out (Table I): it was reported that about 30% of cellular spermidine is localized in vacuoles of *Neurospora crassa* [18].

ATP-dependent polyamine uptake by vacuolar membrane vesicles

Anraku and his colleagues have been extensively studying the energetics of yeast vacuoles, and have demonstrated ATP-driven transport systems of Ca^{2+} and amino acids [19,23,24]. All of these systems are actually driven by a proton potential (about 180 mV,

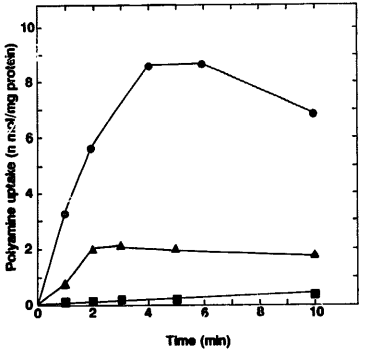

Fig. 1. Time course of ATP-dependent polyamine uptake by vacuolar membrane vesicles. Uptakes of spermine (●), spermidine (▲) and putrescine (■) into the vesicles were assayed under standard conditions in the presence of 0.5 mM ATP. Each point represents the average of triplicate experiments. Standard error was within the range of 10%.

intravacuolar positive) generated by a vacuolar H^+-translocating ATPase, and are suggested to be an nH^+/Ca^{2+} antiporter or nH^+/amino acid antiporters [19,23,24]. We examined the effect of ATP on accumulations of [^{14}C]putrescine, spermidine and spermine into vacuolar membrane vesicles (Fig. 1). The uptake activities of spermidine and spermine were greatly stimulated by ATP, but the activity of ATP-dependent putresine uptake was very low. In the absence of ATP, no significant uptake of polyamines was observed. At 5 min after the addition of ATP, established concentration gradients of spermidine and spermine across the vacuolar membrane were approximately 4 and 17, respectively, indicating ATP-dependent active transport of these polyamines by vacuolar membrane vesicles. When the experiments were performed at 0°C, no accumulation of these polyamines was observed even in the presence of ATP.

Specificity of the uptake system

Table II shows the kinetic parameters of ATP-dependent polyamine uptake by vesicles. Considering the values of K_m and V_{max} for the uptake activities of putrescine, spermidine and spermine (Fig. 1), spermine was the substrate with the highest affinity. Spermidine uptake was inhibited by putrescine (data not shown) and spermine, and spermine uptake was inhibited by spermidine and putrescine. Inhibitions by these

TABLE II

Kinetic parameters of ATP-dependent polyamine uptake

Uptake assays were performed as described in Materials and Methods. Kinetic constants were estimated from the initial velocity of uptakes. The K_i values of competitive inhibition were determined by varying the substrate concentration in the presence of a fixed inhibitor concentration. Standard error was within the range of 10% in triplicate experiments. SP, spermine; SPD, spermidine; PUT, putrescine.

Substrate	K_m (mM)	V_{max} (nmol min^{-1} mg protein)$^{-1}$	K_i (mM)
Putrescine	2.0	0.8	–
Spermidine	0.7	6	0.1 (SP)
Spermine	0.2	7	0.8 (SPD), 1.1 (PUT)

polyamines were apparently all competitive; the K_i values of these polyamines are shown in Table II.

Eight transport systems of amino acid are found in vacuolar membrane [24]. Seven of these are driven by a proton potential generated by H^+-ATPase [20]. To determine whether polyamine uptake is mediated by a specific transport system distinct from these systems, the effect of these seven amino acids on spermine uptake by vesicles was examined (Table III). Spermine uptake was hardly inhibited by the addition of these amino acids, suggesting that an ATP-dependent transport system specific for polyamines exists on the vacuolar membrane.

Properties of polyamine uptake

Table IV shows the nucleotide specificity of spermine uptake. Spermine transport was not only stimulated by ATP but also by GTP, UTP and CTP in this order. No transport of spermine was observed by the addition of ADP. This preference corresponded well to the nucleotide specificity of the vacuolar membrane H^+-ATPase [20]. The effect of various ATPase in-

TABLE III

Effect of various L-amino acids on ATP-dependent spermine uptake

The initial rates of spermine uptake were determined under standard conditions. The concentrations of labeled spermine and unlabeled amino acid were 0.1 and 2 mM, respectively. Each point represents the mean of duplicate experiments.

Amino acid	Relative activity (%)
None	100
Arginine	102
Lysine	83
Histidine	138
Phenylalanine	99
Tyrosine	84
Glutamine	116
Isoleucine	127

TABLE IV

Nucleotide specificity of spermine uptake

Initial rates of spermine uptake were determined under standard conditions. The concentration of nucleotide was 0.4 mM. Each point represents the mean of duplicate experiments.

Nucleotide	Relative activity (%)
ATP	100
GTP	86
UTP	45
CTP	32
ADP	0

hibitors and ionophores on ATP-dependent spermine uptake was also examined (Table V). Vesicles preincubated with 10 μM bafilomycin A_1 [25] or 0.1 mM *N,N'*-dicyclohexylcarbodiimide, a specific inhibitor for the vacuolar H^+-ATPase, showed no spermine uptake activity. By contrast, vanadate, an inhibitor of the E_1E_2 type ATPase, slightly activated spermine uptake. Cupric and zinc ions, which strongly inhibit H^+-ATPase [20], blocked the activity completely. Calcium ions also inhibited the uptake, but to a lesser degree than Cu^{2+} and Zn^{2+}. When magnesium ions were removed from the reaction mixture, the uptake activity was completely lost, indicating that they are essential for the uptake activity. These results suggest that the vacuolar H^+-ATPase participates in the spermine uptake by membrane vesicles. Moreover, spermine uptake was severely inhibited by the protonophores SF6847 and CCCP, as well as by an electroneutral K^+/H^+ antiporter, nigericin, indicating that it depends on a proton potential. However, valinomycin did not inhibit spermine uptake activity (Table V). The same effect of these reagents on ATP-dependent spermidine uptake was obtained (data not shown). All these results suggest that active polyamine transport by membrane vesicles is driven by a proton potential generated by the vacuolar H^+-ATPase.

TABLE V

Effect of various reagents on ATP-dependent spermine uptake

Assays were performed under standard conditions and the activity without addition (control) was taken as 100%. Membrane vesicles were preincubated with bafilomycin A_1 or DCCD at 25°C for 10 min before assay. Other reagents were added just before assay. Each point represents the mean of duplicate experiments.

Addition	Concentration (mM)	Relative activity (%)
ATPase inhibitors		
Bafilomycin A_1	0.01	9
DCCD	0.2	0
Sodium vanadate	0.1	152
Divalent cations		
$MgCl_2$	0.1	100
$CaCl_2$	0.1	55
$CuCl_2$	0.1	0
$ZnCl_2$	0.1	0
Ionophores		
SF6847	0.001	12
CCCP	0.02	6
Nigericin	0.001	23
Valinomycin	0.01	98

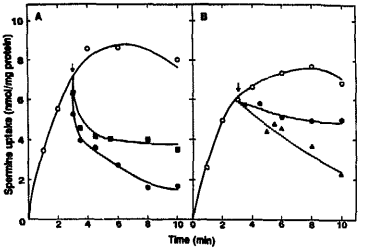

Fig. 2. Efflux of [^{14}C]spermine from vacuolar membrane vesicles. (A) Effect of unlabeled polyamine. ATP-driven spermine uptake (○) was carried out under standard conditions with 0.1 mM [^{14}C]spermine. At the arrow, 1 mM spermine (●) or 2 mM spermidine (■) was added to portions of the reaction mixture. (B) Effect of SF6847 and Ca^{2+}. ATP-driven spermine uptake (○) was carried out as the same as in Fig. 2A. At the arrow, 2 μM SF6847 (●) or SF6847 plus 2 mM $CaCl_2$ (▲) was added to portions of the reaction mixture.

To determine whether accumulated spermine in vesicles is in a free, osmotically active form, the efflux of spermine was examined (Fig. 2). The spermine accumulated in the vesicles was exchangeable with a 10-fold excess of nonradioactive spermine (Fig. 2A). Spermidine also prompted the exchange diffusion of spermine, but to a lesser extent. However, spermine was not in a completely free form, since SF6847 did not readily release it (Fig. 2B). When Ca^{2+} ions were added as counter ion to the suspension, the efflux of spermine became accelerated (Fig. 2B).

Discussion

In this work, we used vacuolar membrane vesicles in which the intravacuolar polyamines were depleted (Table I), instead of the fragile vacuoles, and found a new active transport system of polyamines in vacuoles of *S. cerevisiae*. This system was ATP-dependent and polyamine-specific. Judging from the sensitivity of spermine uptake to various ATPase inhibitors and ionophores, this system depends on a proton potential generated by H^+-ATPase [20]. We expect that the polyamine uptake system may be a substrate/nH^+

antiporter as reported in the uptake systems of arginine and Ca^{2+} [19,23]. However, we must consider that accumulated spermine in vesicles is probably not in a free form; spermine at a steady-state level was not released even when the proton potential was dissipated by the addition of the protonophore SF6847 (Fig. 2B). Accumulated spermine probably binds with some anionic constituents in the vesicles. Dürr et al. [26] measured the amounts of amino acids and polyphosphates in the vacuoles of yeast, and found a linear correlation of their contents, from which they suggested that polyphosphates act as ion exchange resins to conserve amino acids stably in the vacuolar space [26]. Also in the case of *N. crassa*, it is pointed out that polyphosphates sequester spermidine as the anionic binding sites within vacuoles [18,27]. Thus, it is quite possible that most of polyamines in vacuoles (Table I) are tightly bound with polyphosphates: the membrane vesicle fraction still retained about 30 nmol P_i of polyphosphate per mg protein, although most intravacuolar polyphosphates were released during the preparation of the membrane vesicles [20]. Accumulation of polyamines into the vesicles should be due to the combined effect of active polyamine uptake driven by a proton potential and binding of polyamines with the anionic sites in the vesicles. In order to discuss the precise energetics of polyamine transport, we will require an analysis by reconstitution of the purified transport system (transporter) into proteoliposomes.

There are very few reports which show the presence of the polyamine transport system in eukaryotic organelles [15,16,28]. Toninello et al. [15,16] reported a spermine/phosphate co-transport system in rat liver mitochondria. However, the physiological function of this system is obscure. In *S. cerevisiae* and *N. crassa*, vacuoles actually retain polyamines, especially spermidine, under the conditions examined (Table I and [18]). In one mutant of *N. crassa*, whose growth is highly sensitive to putrescine added to the medium, the content of putresine in vacuoles was observed to be remarkably increased [29]. Recently we found a culture condition under which polyamine toxicity for growth of the wild-type strain of *S. cerevisiae* was induced: spermine was the most toxic of the three (Kakinuma, Y., Masuda, N. and Igarashi, K., manuscript in preparation). Under this condition, more polyamines were accumulated by the cells than were required for the growth; the vacuolar polyamine level was concomitantly increased. Vacuoles may serve as a storage system(s) of polyamines to provide a homeostatic reserve or as a detoxification system to prevent the effects of excess free polyamines in the cytoplasm. The active polyamine transport system reported here possibly functions to facilitate the accumulation of polyamines in vacuoles. Experiments are now in progress to examine the physiological significance of the vacuolar polyamine transport sysytem in more detail.

Acknowledgments

We thank Dr. Y. Anraku for his encouragement. We also thank Dr. Y. Ohsumi and Dr. E. Uchida for their valuable discussions. This work was supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

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