Spermidine Uptake by Mitochondria of *Helianthus tuberosus*¹

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**ABSTRACT**

In the present work evidence is provided that spermidine, a polyamine largely present in plant tissues, may be transported, at physiological concentrations, into the matrix space of mitochondria isolated from tubers of *Helianthus tuberosus* L. cv OB1 (Jerusalem artichoke). It is concluded that the movement of spermidine strictly depends on membrane potential, since it is drastically blocked by valinomycin and only slightly sensitive to nigericin. Mg²⁺ and K⁺ inhibit the transport of spermidine in line with the general concept that these cations compete for the same binding sites on the mitochondrial membrane. In contrast to previous data on mammalian mitochondria, spermidine uptake by plant mitochondria does not depend on the presence of inorganic phosphate. This latter result, along with evidence that Ca²⁺ does not affect accumulation of spermidine, indicates that the control of the polyamine uptake mechanism in plant mitochondria is distinct from that of mammalian systems.

The polyamines spermine, spermidine, and putrescine are ubiquitous organic polycations (24). They have been implicated in several cellular functions but their basic mechanism(s) of action has not yet been established (24, 27). In plants, evidence for both long-distance transport (3, 10) and short-distance movement into isolated cells and protoplasts (22, 23) has recently been obtained. It has also been shown that polyamines accumulated by the cells can temporarily be stored into organelles, *e.g.* vacuole (23).

Mitochondria isolated from plant tissues contain variable amounts of polyamines (31). The presence of polyamines inside the mitochondria was originally explained by the observation that these organelles are endowed with the enzyme system which is involved in polyamine synthesis *in situ* (32). Experiments with mammalian mitochondria have later demonstrated that polyamines can also be transported into the mitochondrial matrix space so as to provide a general explanation for the polyamines accumulated inside the mitochondria (28, 30).

In mammalian systems, spermine uptake is an energy-dependent process that requires inorganic phosphate (28). In addition, evidence has been obtained that the spermine taken up by respiring mitochondria was entirely discharged upon addition of either Mg²⁺ or the uncoupler FCCP² (28). This finding implies that spermine can move bidirectionally across the inner mitochondrial membrane.

The dependence of spermine transport on that of Pi has been suggested to reflect the necessity to maintain a proper H⁺ balance since Pi acts as a proton donor to the mitochondrial proton pump (28). Another possibility, which has been taken in consideration only quite recently (30), is that polyamines enter into the mitochondrial matrix space as a response of the generation of a membrane potential. Indeed, under physiological conditions, polyamines exhibit a net positive charge according to the evidence that Mg²⁺ and polyamines compete for the same binding sites on the inner mitochondrial membrane (28).

At present, very little is known about the interaction between polyamines and plant mitochondria (32). Owing to this, a study of some biochemical parameters characterizing the uptake of spermidine by mitochondria isolated from tubers of *Helianthus tuberosus* was performed. The data clearly indicate that spermidine is actively taken up by isolated plant mitochondria with an apparent *Kₐ* that is close to the cytosolic spermidine concentration. This transport is dependent on both respiration and membrane potential. This latter finding is supported by evidence that nigericin, an ionophore catalyzing the electroneutral exchange of K⁺ and H⁺, does not seem to greatly affect the uptake of spermidine.

**MATERIALS AND METHODS**

**Plant Material**

*Helianthus tuberosus* L. cv OB1 (Jerusalem artichoke) was grown and vegetatively propagated in the Botanical Garden of Bologna University. The tubers were harvested at the beginning of dormancy in November and stored in moist sand at 4°C.

**Isolation of Mitochondria**

Mitochondria were isolated according to Douce *et al.* (8) except that the tissues were only grated in two volumes of chilled isolation medium (4°C) and then squeezed through four layers of cheesecloth. The final pellet was resuspended by adding 1 mL of medium containing 0.3 m mannitol, 30 mM Hepes buffer (pH 7.5), without BSA to avoid interferences with the inhibitors added.

**Respiratory Activities**

Respiratory activities were measured polarographically at 28°C using a Yellow Springs model 5331 oxygen meter with

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² Abbreviations: FCCP, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; NIG, nigericin; VAL, valinomycin; ΔΨ, membrane potential ΔG𝐻⁺, electrochemical proton gradient.
a jacketed Gilson Oxygraph reaction chamber (modified to a 1.6 mL volume). Mitochondria were suspended to about 0.2 mg of protein mL in 0.3 mM mannitol, 10 mM Heps buffer (pH 7.2), 5 mM MgCl2, 5 mM KH2PO4, 0.1% BSA.

Outer Membrane Integrity

Substrate-dependent reduction of exogenous Cyt c cannot occur in mitochondria that retain their outer membrane. The integrity of the external mitochondrial membrane was tested by measuring the stimulation of the NADH (1 mM) and/or succinate (2 mM) dependent respiration upon addition of exogenous horse heart Cyt c (50 μM). In all preparations, no antimycin A-insensitive respiration was present. The data presented were obtained with mitochondrial preparations with a percentage of intactness of approximately 85 to 90%. The P/O ratios obtained with NADH and succinate as respiratory substrates were close to 1.3 and 1.6, respectively. The respiratory control was unaffected by salicyl hydroxyacid.

Mitochondrial Matrix Volume

Determination of the mitochondrial matrix volume was performed as described by Nicholls and Lindberg (20). Non-respiring mitochondria (approximately 4–5 mg of protein) were incubated in a medium containing 0.3 mM mannitol, 10 mM Heps (pH 7.5), 2 μM rotenone, 2 μM oligomycin, 37 kBq/mL of 3H2O (marker for water space), and 3.7 kBq/mL of [14C]sucrose (marker for nonsomatic space). Radioactive counting was as described under the following section. This procedure gave a mitochondrial matrix volume of approx 0.7 μL/mg protein.

Polyamine Uptake Experiments

Mitochondria were incubated at 28°C in a thermostatic bath with continuous shaking in a medium containing 0.3 M mannitol, 10 mM Heps (pH 7.5), 2 μM rotenone, and 2 μM oligomycin. In all samples, except controls, 4 mM succinate was added as respiratory substrate, and after 30 s the polyamine was added. This was a mixture of labeled [14C]spermidine (5.55 kBq in 3 μL; specific activity: 3.74 GBq/mmol) and unlabeled spermidine to reach a total final concentration of 100 μM. Other additions are indicated in the descriptions of the various experiments. The final volume of the incubation mixture was 150 μL for each determination, containing approximately 0.2 mg of mitochondrial proteins. When the desired time had elapsed, the mixture was transferred in 400 μL tubes already containing 20 μL 10% (w/v) HC104 and 100 μL silicone oil (Wacker-Chemie GmbH W.G.) AR 200:AR 20 (10:4, v/v); the tubes were then immediately centrifuged at 13,250g for 1 min in a Beckman microfuge B. The microtubes were snapfrozen in liquid nitrogen and the tips, containing the mitochondria, were excised with a razor blade; then, 0.5 mL distilled water and 4.5 mL scintillation cocktail (Beckman Ready Gel) were added. Radioactivity was determined in a Beckman LS 1800 scintillation spectrometer.

Polyamine Analysis

Polyamines were extracted, separated, and detected by the method of direct dansylation as described by Smith and Best (25) using precoated silica gel 60 TLC plates with a concentrating zone (Merck). The solvent was chloroform:triethylamine (5:1, v/v). Fluorescent spots were scraped off the plates and extracted with 2 mL acetone. Fluorescence was measured with a Jasco FP 550 fluorescence spectrometer (excitation at 360 nm, emission at 505 nm). Quantification was performed by comparison with dansylated standards chromatographed under the same conditions, and the identity of the spots was confirmed by cochromatography.

Protein Assay

Protein content was measured by the method of Lowry (15) with BSA as a standard.

RESULTS

Respiratory Electron Transport and Spermidine-Uptake

Mitochondria from tubers of Helianthus tuberosus readily oxidize NADH and most of the citric acid cycle intermediates (not shown). In all the experiments reported here, Na-succinate was used as respiratory substrate since it was consumed at a rate compatible with the feasibility of the polyamine uptake assay (see "Materials and Methods"). A typical spermidine uptake experiment is shown in Figure 1. As expected, a very rapid removal of the polyamine from the medium was seen during the first 15 s (time 0, approximately 4 nmol/mg protein). This uptake is likely to reflect the binding of spermidine to mitochondrial membranes (26), since it was sen
sitive to uncouplers (see below, Fig. 4) and/or respiratory chain inhibitors (Fig. 1, curve b) and it was time independent. Owing to this, in all experiments hereafter presented, the uptake data were corrected for spermidine binding by subtraction of controls incubated either in the presence of substrate plus FCCP or in the absence of substrates. Notably, following the spermidine-binding process a further significant uptake of spermidine was observed, the saturation level being achieved in about 2 min (approximately 5 nmol/mg protein) (curve a, control). This latter slow uptake was completely blocked by antimycin A (0.5 μM) (curve b, AA), and it was not affected by 0.1 mM inorganic phosphate (curve c, Pi). This latter result indicates that, in contrast to previous observation in mammalian systems (28), the uptake of spermidine by plant mitochondria is phosphate-independent. In mitochondria isolated from H. tuberosus, the inhibition of the respiratory chain by antimycin A (0.1–0.2 μM) is accompanied by a marked decrease of the membrane potential and of the rate of phosphorylation (16). From this it is apparent that the slow phase of the spermidine uptake is an energy-dependent process which is likely to be coupled to transport of spermidine inside the mitochondria.

**Kinetics of Spermidine Transport**

The kinetics of spermidine uptake by actively respiring Jerusalem artichoke mitochondria (shown in Fig. 2) indicated that the initial rate of spermidine transport into internal matrix space approached a saturation level at a polyamine concentration close to 200 μM. The apparent \( K_m \) (inset of Fig. 2) was 89 μM. In Table 1, the amounts of spermidine, spermine, and putrescine in both mitochondria and entire cells of H. tuberosus tubers are summarized (data expressed as nmol/mg protein). Interestingly enough, spermidine is the most abundant polyamine in both mitochondria and in the entire cells.

**Uptake of Spermidine as Function of Mg\(^{2+}\), K\(^+\), and Ca\(^{2+}\)-Concentrations**

Under physiological conditions polyamines exhibit a net positive charge. For this reason, positively charged ions are expected to inhibit the uptake of spermidine by mitochondria since they compete for the same binding sites on the membrane. As shown in Figure 3, this expectation was verified for Mg\(^{2+}\) and K\(^+\) ions, whereas Ca\(^{2+}\) did not have any significant effect on spermidine uptake. Notably, the concentrations of cations used were close to the current accepted cytosolic contents.

**Effects of Mobile-Carrier Ionophores on Spermidine Uptake**

Energy-transducing membranes such as mitochondria lack a native K\(^+\) permeability. This is accomplished by valinomycin and nigericin, the two ionophores being mobile carriers that catalyze the electrical unipart of K\(^+\) and the electroneutral exchange of K\(^+\) for H\(^+\), respectively (19). Valinomycin is
therefore used to estimate or to abolish membrane potential, whereas nigericin abolishes ΔpH across the membrane. As a consequence, a complete suppression of the total ΔΩH⁺ is obtained through combination of the two ionophores. A similar uncoupling effect is caused by the lipophilic weak acid FCCP. This uncoupler shuttles across the membrane in either the protonated and deprotonated form; in so doing it collapses the ΔΩH⁺.

In Figure 4, the effects of valinomycin, nigericin, and FCCP on spermidine uptake are shown. The data indicate that the transport of spermidine inside the mitochondria was completely blocked by 3 μM FCCP and 10 nM valinomycin, whereas 100 nM nigericin slightly affected the spermidine accumulation. It is important to note that in all experiments 30 mM KCl was present. These experimental conditions have been chosen deliberately in order to meet two opposite requirements, namely: (a) valinomycin and nigericin require the presence of an high overall concentration of K⁺ in the assay to fully prevent the formation of either a transmembrane pH gradient or a membrane potential, respectively; (b) an excess of KCl tends to blur the effect of ionophores since it drastically inhibits the uptake of spermidine (see Fig. 3).

**DISCUSSION AND CONCLUSIONS**

The current study demonstrates that the polyamine spermidine, in addition to its binding to the membrane, may be actively transported inside of isolated plant mitochondria (*Helianthus tuberosus*). According to this conclusion, the main features of this transport can be summarized as follows.

(a) The spermidine uptake depends on respiratory electron flow, since antimycin A, a specific inhibitor of the b/c1 complex, prevents the accumulation of spermidine by mitochondria (Fig. 1).

(b) The spermidine is taken up by respiring mitochondria in an energy-dependent process, since the uncoupler FCCP abolishes the uptake (Fig. 4).

(c) The uptake of spermidine is driven (mainly) by the membrane potential, since the ionophore nigericin, which abolishes the transmembrane pH difference, slightly affects the spermidine transport (see also below).

(d) Spermidine uptake is inhibited by Mg²⁺ and K⁺, whereas it is not affected by inorganic phosphate (Pi) (Fig. 1).

Another important point to underline is that the concentration of spermidine used in our experiments (100 μM) is very close to that measured intracellularly (24). This consideration supports, therefore, the concept that spermidine uptake may be of general importance in the physiology of plant cells.

In mammalian systems, FCCP was shown to prevent spermine uptake and to induce the release of much of the accumulated polyamine (28). This finding implies that spermine can move bidirectionally across the inner mitochondrial membrane and that spermine uptake is an energy-dependent process. In this connection, it is noteworthy that under physiological conditions, polyamines are largely protonated and exhibit a net positive charge. This consideration suggests that spermidine uptake depends on the extent of the electrical potential across the membrane, which is in agreement with our finding that spermidine uptake by plant mitochondria is completely blocked by valinomycin (shown in Fig. 4). Owing to this, the partial nigericin-induced inhibition of spermidine uptake (Fig. 4, KCl+NIG) was somewhat unexpected since it is in contrast with recent evidence in rat liver mitochondria in which a nigericin-stimulated spermine uptake was observed (30). Clearly, this particular point needs further investigation.

Another striking difference between plant and animal mitochondria concerns the different effect induced by Pi on the uptake of polyamines. Indeed, spermine uptake by rat liver mitochondria strongly depends on the presence of anions such as phosphate and/or acetate (28, 30). Conversely, spermidine uptake by plant mitochondria is unaffected by inorganic phosphate (this work). This apparent discordancy might be due to the fact that in plant mitochondria the membrane potential is very high (220–240 mV) (7, 9) compared to that in animal mitochondria (150–180 mV) (2, 14). As a consequence, ΔΩH⁺ of plant mitochondria arises mainly from the membrane potential, the proton gradient being at most 0.2 pH unit (ΔΩH⁺ = −12 mV). This phenomenon is probably due to the presence of an active H⁺/K⁺ exchanger, originally claimed by Hensley and Hanson (13) to explain the release of respiration with little swelling by corn mitochondria (12). Indeed, application of elementary principles of cell homeostasis predicts (11) that chemiosmotic mitochondria could not survive without a K⁺/H⁺ antiporter. The K⁺/H⁺ antiporter of animal mitochondria catalyzes both electroneutral K⁺/H⁺ and Na⁺/H⁺ exchange across the inner membrane, and it is activated by matrix swelling (11). In plants, Na⁺ is not an essential element (halophytes are an exception), and it is likely to presume that plant mitochondria are endowed with an active cation antiporter that is involved mainly in K⁺/H⁺.
exchange. The direct consequence of the presence in plant mitochondria of a negligible proton gradient, compared to that of animal mitochondria (0.4–0.8 pH unit), is that polyamine uptake by plant mitochondria strongly depends on Δψ. Paradoxically, although polyamines also are transported electrochemically in animal mitochondria, in this latter system the uptake is indirectly controlled by the proton gradient. In fact, in rat liver mitochondria the rate of spermine uptake increases sharply and nonohmically by increasing the membrane potential from 180 mV to 200 mV (30). Since the same activation is observed upon addition of phosphate, acetate and nigericin during state 4 succinate respiration, it can be concluded that spermine uptake by mammalian mitochondria reflects the reduction of the pH gradient.

The lack of effect induced by Ca\(^{2+}\) on polyamines uptake in both plant and mammalian mitochondria, although not completely understood, deserves some consideration. Indeed, it has widely been established that mitochondrial Ca\(^{2+}\) accumulation is an energy-dependent process (1, 6); Ca\(^{2+}\) uptake is therefore expected to interfere with the uptake of polyamines. In rat liver mitochondria, Ca\(^{2+}\) accumulation is linked to a ruthenium red-sensitive uniporter, and it has been shown to drastically reduce Δψ (from 202 to 140 mV) (14). Interestingly, the membrane potential is rapidly restored by addition of phosphate (14). From this, it is apparent that polyamine uptake by mammalian mitochondria is likely to be unaffected by Ca\(^{2+}\) provided the presence of phosphate. Unfortunately, previous studies on polyamines transport have overlooked this important point (28). In plant mitochondria, Ca\(^{2+}\) accumulation is not via a simple electrochemical uniport since it is dependent on phosphate (1, 6). The uptake mechanism is slow with a rate which is at least two orders of magnitude lower than the mammalian counterpart (1, 4–6). In addition, Ca\(^{2+}\) uptake in the presence of phosphate causes a very small decrease in Δψ (≈10 mV), indicating that Ca\(^{2+}\) enters in the form of a Ca\(^{2+}\)/phosphate complex carrying less than two positive charges (5, 18). These data clearly suggest that Ca\(^{2+}\) itself is not expected to limit the polyamines uptake by plant mitochondria.

The physiological role of spermidine uptake by plant mitochondria is, at present, obscure due to the fact that these organelles are endowed with the enzymes devoted to the polyamine synthesis in situ (32). In mammalian systems, spermine modifies the permeability of the inner mitochondrial membrane in such a way that Ca\(^{2+}\) and phosphate movements are significantly repressed (21). Since these movements are associated with mitochondrial efflux of Mg\(^{2+}\) and adenine nucleotides, spermine tends to prevent the fall of Δψ induced by Ca\(^{2+}\) and Pi cycling (29). This latter phenomenon appears to be unlikely in plant mitochondria in view of the slow Ca\(^{2+}\)/phosphate uptake and the small decrease in Δψ observed under these physiological conditions. A recent study by Marcote et al. (17) demonstrates that polyamines (spermidine, spermine, and putrescine) at physiological concentrations induce the transport of the precursor of ornithine carbamoyltransferase into isolated rat liver mitochondria. In this particular case, the role of polyamines would be that of stabilizing the mitochondrial structures involved in the transport system through a closer approximation of the outer and inner membranes (17). Although a similar function might be predicted also for mitochondrial and cytosolic polyamines of plant cells, this particular point remains open to experimental verification. Experiments aiming at defining the presence of an exchange mechanism between mitochondrial and cytosolic polyamines are in progress.

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**LITERATURE CITED**

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