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The Use of Fura-2 Fluorescence to Monitor the Movement of Free Calcium Ions into the Matrix of Plant Mitochondria (*Pisum sativum* and *Helianthus tuberosus*)[1]

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Purified mitochondria isolated from pea (*Pisum sativum* L. cv Alaska) stems and Jerusalem artichoke (*Helianthus tuberosus* L. cv OB1) tubers were loaded with the acetoxymethyl ester of the fluorescent Ca^{2+} indicator fura-2. This made possible the continuous monitoring of free $[Ca^{2+}]$ in the matrix ($[Ca^{2+}]_m$) without affecting the apparent viability of the mitochondria. Pea stem mitochondria contained an initial $[Ca^{2+}]_m$ of approximately 60 to 100 nM, whereas $[Ca^{2+}]_m$ was severalfold higher (400–600 nM) in mitochondria of Jerusalem artichoke tubers. At low extramitochondrial Ca^{2+} concentrations (≥100 nM), there was an energy-dependent membrane potential increase in $[Ca^{2+}]_m$; the final $[Ca^{2+}]_m$ was phosphate-dependent in Jerusalem artichoke but was phosphate-independent in pea stem mitochondria. The data presented indicate that (a) there is no absolute requirement for phosphate in Ca^{2+} uptake; (b) plant mitochondria can accumulate external free Ca^{2+} by means of an electrophoretic Ca^{2+} uniporter with an apparent affinity for Ca^{2+} (K_m approximately 150 nM) that is severalfold lower than that measured by conventional methods (isotopes and Ca^{2+}-sensitive electrodes); and (c) $[Ca^{2+}]_m$ is within the regulatory range of mammalian intramitochondrial dehydrogenases.

The primary physiological function of the Ca^{2+} transport system in mammalian mitochondria is now believed to be the regulation of matrix Ca^{2+} (Denton and McCormack, 1985; Hansford, 1985). The need for such a regulation of $[Ca^{2+}]_m$ resides in the fact that in mammalian tissues there are three soluble intramitochondrial dehydrogenases that can be activated severalfold by increase in $[Ca^{2+}]_m$ within the micromolar range (McCormack and Denton, 1984; McCormack et al., 1989). Recent evidence suggests that this might also be true for certain plant mitochondrial enzymes, e.g. the matrix enzyme NAD(H)-glutamate dehydrogenase from turnip root (*Brassica rapa* L.), shown to be activated between 1 and 10 μM Ca^{2+} (Itagaki et al., 1990). It has also been reported that inside-out submitochondrial vesicles generated from mitochondria of potato tubers (*Solanum tuberosum* L. cv Bintje) catalyze an NADPH oxidation that is half-maximally stimulated by 3 μM Ca^{2+} (Rasmusson and Møller, 1991); this suggests that the activity of a membrane-bound enzyme located inside the inner mitochondrial membrane can be controlled by matrix Ca^{2+}.

In plant mitochondria, Ca^{2+} uptake varies among species and among tissues and ages in the same species (Chen and Lehninger, 1973; Åkerman and Moore, 1983; Martins and Vercesi, 1985; Rugolo et al., 1990; Silva et al., 1992). One of the variable factors is the requirement for phosphate. Indeed, a survey of the capacity of mitochondria from various plant species for respiration-linked Ca^{2+} uptake indicated that phosphate is not required for maximal Ca^{2+} accumulation in mitochondria from corn (*Zea mays*) and mung bean (*Phaseolus aureus*) stems (see, however, Martins and Vercesi, 1985; Carnieri et al., 1987), artichoke (*Cynara scolymus*) buds, and mushrooms (*Agaricus campestris*), whereas it is absolutely necessary for Ca^{2+} uptake in red beet (*Beta vulgaris*) and sweet potato (*Impomoea batatas*) roots, potato (*Solanum tuberosum*) tubers, and onion (*Allium cepa*) stems (Chen and Lehninger, 1973). Conversely, a common and widely established feature of the electrophoretic pathway is its very high K_m for Ca^{2+} (between 1 and 10 μM); this conclusion is based on data obtained with isotopic approaches and/or selective electrodes (Dieter and Marmé, 1983; Martins et al., 1986). However, these techniques have some obvious disadvantages, the most significant being their poor sensitivity at low $[Ca^{2+}]$.

Therefore, a potential major step forward in the study of mitochondrial Ca^{2+} transport has been afforded by the reports that the fluorescent Ca^{2+} indicator fura-2/AM (Tsien, 1981) can be successfully entrapped within the matrix of mitochondria from mammalian tissues (Lukacs and Kapus, 1987; McCormack et al., 1989) without affecting their apparent viability (McCormack et al., 1989). This has made the continuous monitoring of $[Ca^{2+}]_m$ possible, and a good correlation between this and the Ca^{2+} requirement of matrix enzymes such as the pyruvate and 2-oxoglutarate dehydrogenases has been found (McCormack and Denton, 1984).

In the present paper, we report the first measurement of $[Ca^{2+}]_m$ in plant mitochondria. This study shows that Percoll-purified mitochondria from pea (*Pisum sativum*) stems and Jerusalem artichoke (*Helianthus tuberosus*) tubers accumulate the acetoxymethyl ester of fura-2 with subsequent entrapment of its free acid form. This allowed us to estimate the variations in $[Ca^{2+}]_m$ under different respiratory conditions.

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Abbreviations: $[Ca^{2+}]_{free}$, free Ca^{2+} concentration; $[Ca^{2+}]_m$, matrix free Ca^{2+} concentration; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone.

MATERIALS AND METHODS

Plant Material

Etiolated pea (*Pisum sativum* L. cv Alaska) stems were obtained by growing plants for 7 to 8 d in the dark at 25°C and 70% RH. Jerusalem artichoke (*Helianthus tuberosus* L. cv OB1) was grown and vegetatively propagated in the Botanical Garden of the University of Bologna. The tubers were harvested at the beginning of dormancy (November) and stored in moist sand at 4°C.

Isolation of Purified Mitochondria

Approximately 150 to 200 g of etiolated stems were ground in 300 to 350 mL of 0.3 M mannitol, 20 mM Hepes buffer (pH 7.2), 1 mM EDTA, 1 mM EGTA, 5 mM Cys, 0.1% BSA (isolation buffer) with a mortar. The homogenate was filtered through four layers of gauze and the filtrate was centrifuged at 28,000*g* for 5 min. The pellet was resuspended in half of the initial volume of the isolation buffer by a Potter homogenizer. This fraction was centrifuged at 2,500*g*; the pellet was discharged and the supernatant centrifuged at 28,000*g* for 5 min. The mitochondria were collected at the bottom, resuspended in 2 mL of 0.3 M mannitol, 20 mM Hepes buffer (pH 7.2), and purified on a discontinuous gradient formed by three layers of 40, 21, and 13.5% (v/v) Percoll; mitochondria were collected at the 21/40% interface and washed once by resuspension in 0.3 M mannitol, 2 mM Hepes buffer (pH 7.2). This procedure resulted in approximately 10 to 15 mg of mitochondrial proteins.

The procedure for the isolation of mitochondria from tubers of *H. tuberosus* was similar to that described for pea stem mitochondria except for the following points: (a) the starting material consisted of approximately 300 g of tubers; and (b) mitochondria were purified on a discontinuous Percoll gradient as previously described (Rugolo and Zannoni, 1992).

Loading with Fura-2

Solutions (buffers and substrates) were filtered through a Chelex chelating resin (Bio-Rad) to remove contaminating Ca^{2+} before use.

Percoll-purified mitochondria, suspended at a final concentration of 3 mg/mL in 0.3 M mannitol, 20 mM Hepes buffer (pH 7.2), 0.1% BSA, were incubated with 10 μM fura-2/AM (plus EGTA, as indicated) for 20 min at 29°C with continuous stirring. After 20 min of loading, mitochondria were centrifuged (7 min, 10,000*g*) and resuspended at 15 mg/mL.

Fluorescence Measurements

Fluorescence measurements were performed with a Multiscan-2 spectrofluorimeter (AMKO-LTI) equipped with an alternating dual-wavelength excitation system and interfaced with an Archie-RIVAL 286 computer. The wavelengths used were 350 and 380 nm for excitation and 505 nm for emission. The isosbestic wavelength of 361 nm for excitation was used in the Mn^{2+} quench experiments.

Measurement of $[Ca^{2+}]_m$

Free $[Ca^{2+}]_m$ was calculated according to Grynkiewicz et al. (1985) using the formula

$$[Ca^{2+}] = K_d \frac{R - R_{min}S_f}{R_{max} - R \cdot S_b}$$

where R is the ratio of the fluorescence at 350 and 380 nm of the matrix fura-2 indicator; R_{min} and R_{max} are the ratios of the fura-2 fluorescence intensities at 350 and 380 nm after permeabilization of mitochondria with 0.03% Triton X-100 followed by addition of 30 mM Tris, 5 mM EGTA (R_{min}) and then 6 mM Ca^{2+} (R_{max}); and S_f/S_b is the ratio of fura-2 emission at 1 nM Ca^{2+} and at saturating Ca^{2+} concentrations (excitation at 380 nm). A K_d of 224 nM was used (Grynkiewicz et al., 1985). In all experiments, approximately 0.3 mg of mitochondria were resuspended in 2 mL of 0.3 M mannitol, 20 mM Hepes buffer (pH 7.2) in a stirred cuvette at 29°C.

Mitochondrial O_2 Uptake and Protein Determination

O_2 uptake was measured in a Yellow Springs model 5331 oxygen electrode at 29°C. The oxygen concentration of the air-saturated buffer at 29°C was 240 μM (Allen and Holmes, 1986). Protein content was determined by following the Bradford procedure (Bradford, 1976) with BSA as the standard.

RESULTS

Loading of Fura-2 into Mitochondria

The acidic form of fura-2 is Ca^{2+} sensitive but membrane impermeant. Conversely, the acetoxymethyl ester of fura-2 (fura-2/AM) is membrane permeable but Ca^{2+} insensitive (Grynkiewicz et al., 1985). For this reason, in order to measure $[Ca^{2+}]_{free}$ inside the mitochondrial matrix space the loaded AM form has to be hydrolyzed to gain Ca^{2+} sensitivity and to be retained inside as the impermeant acidic form (Tsien, 1981; Grynkiewicz et al., 1985). This latter condition can be achieved only when two prerequisites are fulfilled: (a) the absence of unspecific hydrolytic activity in the mitochondrial suspension, but the presence of esterase activity within the matrix space; and (b) capacity of the probe to permeate the mitochondrial membrane.

To assess the question of whether isolated mitochondria possess hydrolytic activity, we first tested the appearance of a Mn^{2+}-sensitive fluorescence signal (Hallam et al., 1988; Sage et al., 1989) in both crude and Percoll-purified mitochondrial suspensions. As previously shown, Mn^{2+} does not quench the fluorescence of the fura-2/AM (Hallam et al., 1988; Sage et al., 1989), but it does quench that of fura-2 at the isosbestic point (361 nm). The results (not shown) indicated that crude mitochondria cannot be loaded with fura-2/AM due to the presence of high amounts of unidentified external esterases. For this reason, all the experiments presented here were obtained with Percoll gradient-purified mitochondria loaded with fura-2 (see "Materials and Methods").

Figure 1 (traces a and b) shows the response of the fluorescence signal at 361 nm upon addition of 300 μM Mn^{2+} in

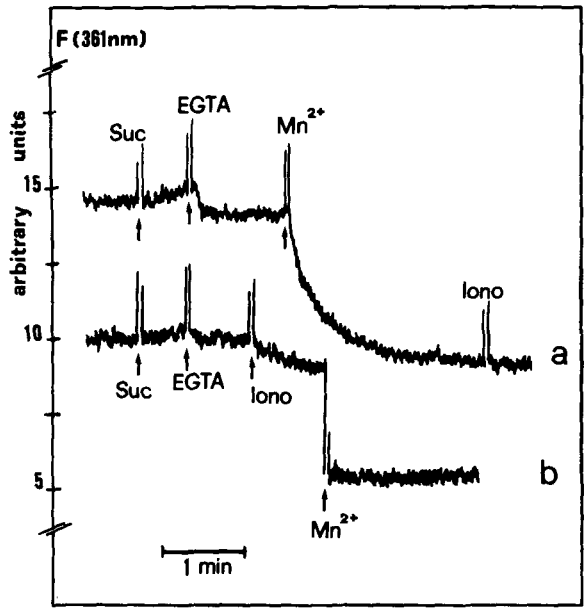

Figure 1. Mn^{2+}-induced quenching of the fluorescence signal at 361 nm in fura-2-loaded mitochondria. Fura-2-loaded mitochondria (0.3 mg/mL final concentration) were incubated in 2 mL of 0.3 M mannitol, 20 mM Hepes (pH 7.2) in the presence of succinate (Suc, 2.5 mM). Where indicated, EGTA (100 μM), Mn^{2+} (300 μM), and ionomycin (Iono, 2 μM) were added.

the presence or in the absence of 2 μM ionomycin, an ionophore for both Ca^{2+} and Mn^{2+}. It is apparent that under energized conditions (2.5 mM succinate) with no free Ca^{2+} in the suspension medium (100 μM EGTA present), the signal is rapidly quenched by Mn^{2+} (half-time ≤ 1 s) in the presence of ionomycin (trace b), whereas the quench is much slower (half-time ≥ 20 s) in the absence of the ionophore (trace a). The results in Figure 1 demonstrate that deesterification of fura-2/AM occurred within the matrix space, because the Mn^{2+}-induced quenching shows typical uptake kinetics only in the absence of ionomycin. Further experiments (not shown) also indicated that, in agreement with data in rat heart mitochondria (Puskin et al., 1976; Lukacs and Kapus, 1987), the rate of Mn^{2+} uptake was decreased under uncoupled conditions.

Attempts were also made to follow the progressive hydrolysis of fura-2/AM during mitochondrial loading, this measurement being indicative of the fura-2 concentration within the matrix space. At various times, 100 μL of the membrane suspension were diluted to 2 mL (see "Materials and Methods") and excitation spectra were recorded. Hydrolysis of the dye was monitored by the gradual shift in the excitation spectrum from that of the ester (broad peak at 380 nm) to that of the Ca^{2+}-sensitive free acid, peaking at 350 nm. Under the experimental conditions, hydrolysis was complete in 20 min. Figure 2 shows the spectral shift in excitation maximum wavelength due to the variation of $[Ca^{2+}]_{free}$ from 1 nM (2 mM EGTA present) to 1 mM, in a suspension of mitochondria treated with Triton X-100 after a loading time of 20 min (signal corrected for the autofluorescence of unloaded mitochondria). With a loading concentration of 10 μM fura-2/AM, assuming a matrix volume of 1 μL/mg of protein, the internal concentration of fura-2 free acid was estimated to be around 90 to 95 μM, which is sufficient to generate an adequate signal (Cobbold and Rink, 1987) without buffering the changes in $[Ca^{2+}]_m$, as previously shown in mammalian mitochondria (Cobbold and Rink, 1987; Lukacs and Kapus, 1987).

Viability of Fura-2-Loaded Mitochondria

To test the possible inhibitory effect of fura-2 on the mitochondrial respiration, a series of experiments with either loaded or unloaded mitochondria was carried out. The results (not shown) indicated that the respiratory activities, e.g. oxidation of exogenous NADH and succinate, were not affected by fura-2 loading and that the mitochondrial ability to oxidize substrates and generate a membrane potential remained substantially stable for 4 to 5 h after loading.

Measurements of $[Ca^{2+}]_m$ in Isolated Mitochondria

The $[Ca^{2+}]_m$ as determined according to Grynkiewitz et al. (1985) (see "Materials and Methods") was approximately 60 to 100 nM and 400 to 600 nM in mitochondria isolated from pea stems and Jerusalem artichoke tubers, respectively. Although the reason for such a difference is not known, it is noteworthy that the values reported here are within the order of magnitude of those measured in rat heart mitochondria for matrix Ca^{2+}-sensitive enzymes (McCormack et al., 1989).

Ca^{2+} Movements across the Inner Membrane under Various Conditions

Figure 3 shows the effects of Pi, FCCP, EGTA, and ionomycin on the movements of Ca^{2+} ions (uptake and release)

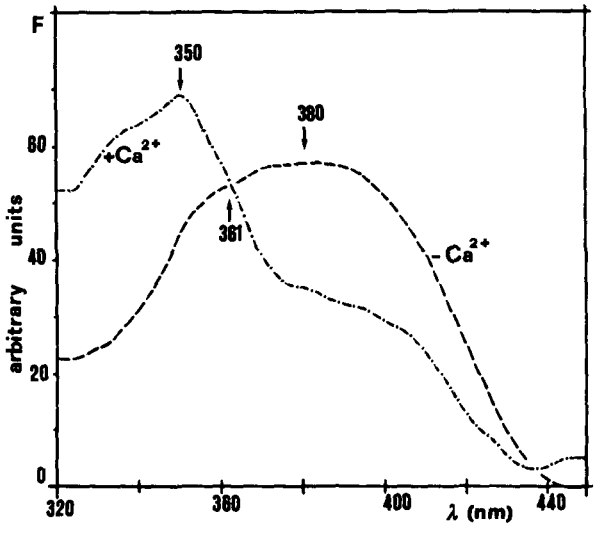

Figure 2. Fluorescence spectra of fura-2-loaded mitochondria in the presence and in the absence of Ca^{2+}. ·····, Spectrum at saturating Ca^{2+} concentrations; - - - -, spectrum at approximately 1 nM Ca^{2+}. Spectra were recorded in 0.03% Triton X-100-treated mitochondria. See text for details.

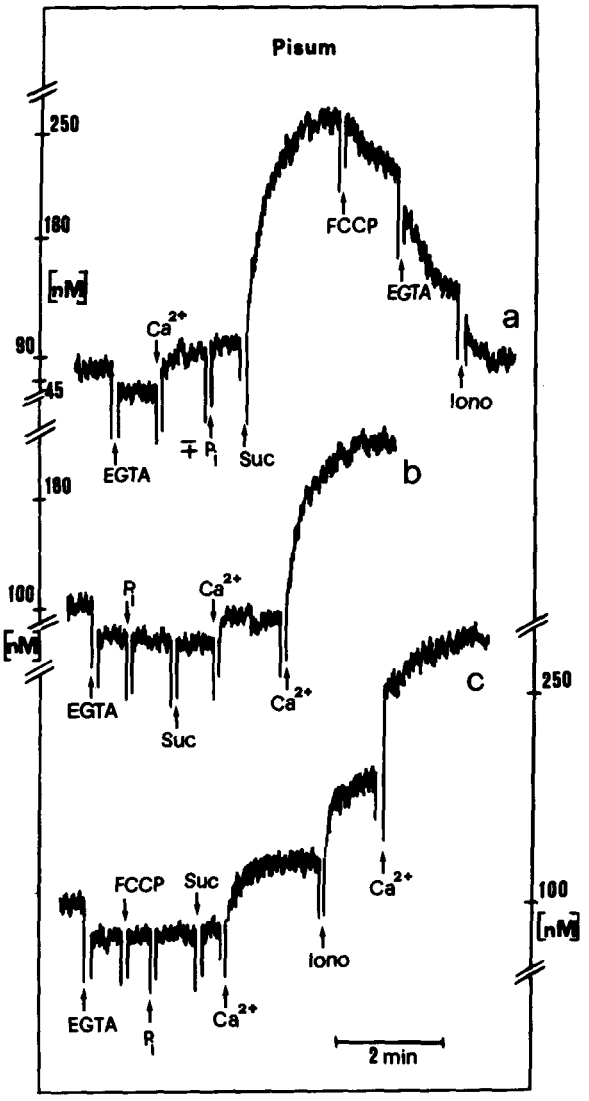

Figure 3. Ca^{2+} movements into fura-2-loaded mitochondria isolated from *P. sativum* stems. Fura-2-loaded mitochondria (0.3 mg/mL) were resuspended in 2 mL of 0.3 M mannitol, 20 mM Hepes (pH 7.2). Where indicated, succinate (Suc, 2.5 mM), Pi (0.4 mM), ionomycin (Iono, 2 μM), and FCCP (0.75 μM) were added. Note that the label "∓" in trace a indicates that the signal trace is not modified in the presence or in the absence of phosphate. The Ca^{2+} additions were as follows: in a, 150 nM; in b, first addition 55 nM, second addition 110 nM (final concentration); in c, first addition 150 nM, second addition 400 nM (final concentration).

in pea stem mitochondria actively respiring succinate. Trace b indicates that mitochondria containing a basal $[Ca^{2+}]_m$ of approximately 60 nM do not accumulate Ca^{2+} externally added at a concentration of 55 nM (trace b, first addition), whereas the Ca^{2+} uptake is quite evident in the presence of a final concentration of 110 nM Ca^{2+} (trace b, second addition). Trace a, which represents the results of typical experiments performed in the presence or in the absence of Pi, shows quite clearly that Ca^{2+} uptake in pea stem mitochondria is Pi independent (labeled as ∓) but energy dependent. There was indeed a negligible accumulation of Ca^{2+} in the absence of succinate (trace a) and also in the presence of the uncoupler FCCP (trace c); this is in line with the fact that addition of FCCP to Ca^{2+}-loaded mitochondria (trace a) induced a substantial Ca^{2+} efflux. As expected, this latter effect was stimulated by EGTA plus ionomycin (trace a). Under uncoupled conditions (trace c), addition of ionomycin induced a passive uptake of Ca^{2+} that reached the same level obtained in the absence of FCCP and ionomycin (trace b) (0.24 pmol Ca^{2+} mg^{-1} protein). Figure 4 shows the double-reciprocal ($1/v$ versus $1/[S]$) Lineweaver-Burk plot of the rate of mitochondrial free Ca^{2+} accumulation under energized conditions (data obtained in the absence of Pi). The Ca^{2+} influx rate was measured as initial variation of $[Ca^{2+}]_m$ as a function of variable external $[Ca^{2+}]_{free}$ (Fig. 4, inset). Notably, the apparent K_m for free Ca^{2+} of the electrophoretic uniporter is approximately 7 times lower than that determined in mung bean mitochondria (Åkerman and Moore, 1983).

We have previously shown that Ca^{2+} uptake by Jerusalem artichoke mitochondria depends on membrane potential and it requires Pi (Rugolo et al., 1990). This conclusion was obtained by following the distribution of $^{45}Ca^{2+}$ under respiratory conditions in which the concentration of externally added Ca^{2+} ions (100 μM) was 10^3 times higher than the estimated cytosolic $[Ca^{2+}]$ (see Bush and Jones, 1990). To test our previous conclusion and to compare it with the data obtained in fura-2-loaded pea steam mitochondria, the basal $[Ca^{2+}]_m$ of Jerusalem artichoke mitochondria was lowered to 60 to 100 nM by addition of 2 mM EDTA during the loading procedure. Figure 5 shows the effects of Pi, FCCP, antimycin A, EGTA, and ionomycin on the movements of Ca^{2+} ions in such Ca^{2+}-depleted, fura-2-loaded mitochondria from Jerusalem artichoke tubers. The most striking difference between pea and Jerusalem artichoke mitochondria is that in the latter case the Ca^{2+} uptake is markedly enhanced by 0.4 mM Pi

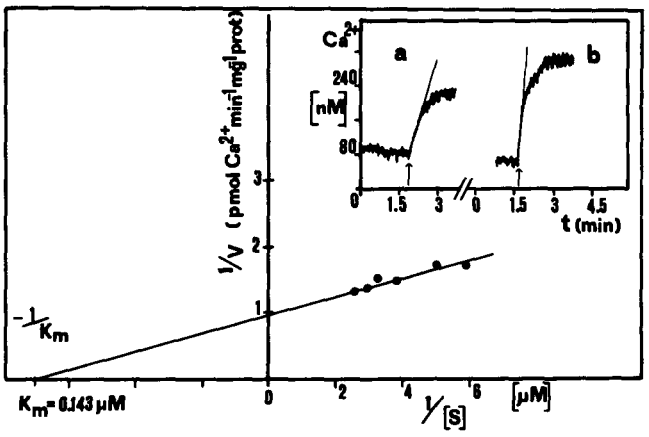

Figure 4. Lineweaver-Burk plot of the Ca^{2+} influx into the matrix space of pea stem mitochondria as monitored by fura-2. The rise in matrix Ca^{2+} was determined as described in "Materials and Methods." V_{max} for free Ca^{2+} accumulation into the matrix space was 1 pmol min^{-1} mg^{-1} protein. Inset, Two examples of Ca^{2+} influx kinetics as a function of variable exogenous Ca^{2+} concentrations (addition in a, 140 nM Ca^{2+}; addition in b, 260 nM Ca^{2+}). Assay conditions are as for Figure 3 (see text for details).

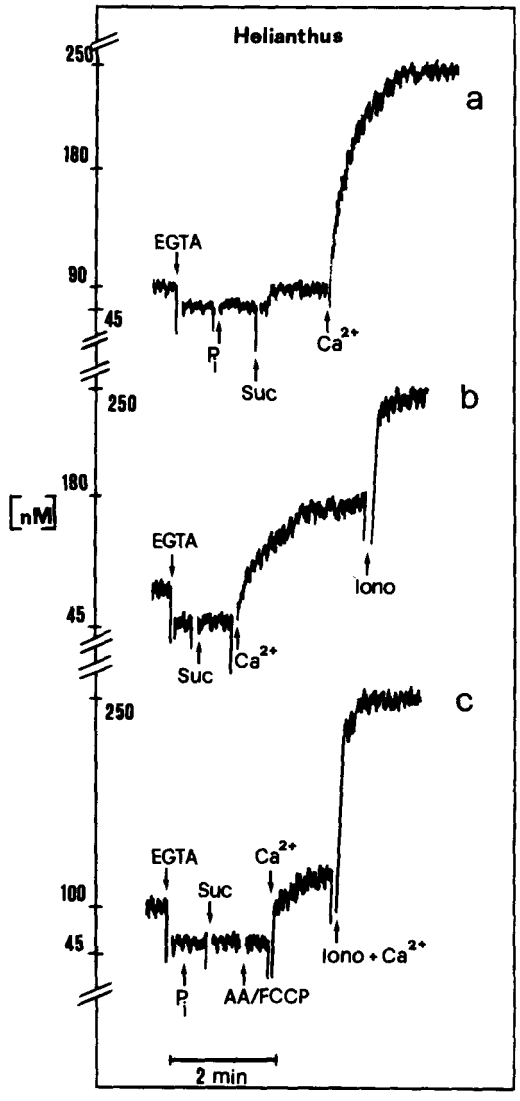

Figure 5. Ca^{2+} movements in fura-2-loaded mitochondria isolated from tubers of *H. tuberosus*. Assay conditions are as in Figure 3. Where indicated, antimycin A (AA, 4 μM) and FCCP (0.75 μM) were added. The Ca^{2+} additions were as follows: a, 150 nM; b, 150 nM; c, first addition 150 nM; second addition (Iono plus Ca^{2+}) 400 nM (final concentration).

(compare traces a and b). Traces a, b, and c also show that there is no Ca^{2+} uptake under deenergized conditions (plus antimycin A and/or FCCP, trace c); further experiments indicated that, as previously shown in pea stem mitochondria (Fig. 4), the apparent affinity for Ca^{2+} of the uniporter is far below the micromolar range, with K_m approximately equal to 200 nM (not shown).

DISCUSSION AND CONCLUSIONS

The capacity to load fura-2 into intact purified plant (*P. sativum* and *H. tuberosus*) mitochondria under conditions where their respiratory activities are not impaired offers clear advantages in the study of the regulation and role of matrix Ca^{2+} and mitochondrial Ca^{2+} transport.

One major objection to this type of approach would be that the $[Ca^{2+}]_m$ as determined by in vitro experiments, i.e. in isolated mitochondria, does not reflect the actual in vivo mitochondrial concentration, i.e. in intact cells and tissues. However, it has previously been demonstrated that the total mitochondrial Ca^{2+} content in situ, as determined by electron-probe x-ray microanalysis (Somlyo et al., 1985), was in the range of 0.5 to 2.0 nmol mg^{-1} protein, a concentration close to that obtained in mitochondria isolated by different fractionation techniques (Reinhart et al., 1984; Assimacopoulos-Jeannet et al., 1986).

Lukacs and Kapus (1987) reported that in fura-2-loaded rat and heart mitochondria, the $[Ca^{2+}]_m$ rose from 0.2 to 1.8 μM as the total Ca^{2+} rose from 0.5 to 6 nmol mg^{-1} protein, and Davis et al. (1987) reported increases of $[Ca^{2+}]_m$ from 0.1 to 2.0 μM as the total Ca^{2+} content rose from 0.5 to 2 nmol mg^{-1} protein. Both studies reported a nonlinear relationship between total Ca^{2+} content and $[Ca^{2+}]_m$ over this range, with a tendency to larger increases in $[Ca^{2+}]_m$, with respect to total Ca^{2+}, at higher values (Davis et al., 1987; Lukacs and Kapus, 1987). Our results show that in fura-2-loaded mitochondria isolated from pea stems and Jerusalem artichoke tubers, the $[Ca^{2+}]_m$ increases from approximately 0.1 to 0.25 μM under steady-state respiratory conditions. This $[Ca^{2+}]_m$ range is within the regulatory range of the intramitochondrial dehydrogenases in mammalian systems (Denton and McCormack, 1985; McCormack et al., 1989), but it is 1 order of magnitude lower than the K_m (Ca^{2+}) of the matrix enzyme NAD(H)-glutamate dehydrogenase from turnip root (between 1 and 10 μM) (Itagaki et al., 1990) and of the internal membrane-bound NAD(P)H dehydrogenases of potato tuber mitochondria (3 μM) (Rasmusson and Møller, 1991). In this respect, it is worth noting that the $[Ca^{2+}]_m$ of freshly isolated Jerusalem artichoke mitochondria is between 0.4 and 0.6 μM; in the latter case, however, we have been successful in detecting the rise in $[Ca^{2+}]_m$, as determined by the fluorescent dye fura-2, only after a dye-loading procedure in the presence of EGTA so as to decrease the initial $[Ca^{2+}]_m$ to approximately 50 nM (Fig. 5). These data indicate that (a) the in vivo $[Ca^{2+}]_m$ can be two to three times higher than the value reached by respiration in vitro (0.25 μM) and that (b) the $[Ca^{2+}]_m$ can be different between plant species, since in freshly isolated pea stem mitochondria, $[Ca^{2+}]_m$ is between 0.06 and 0.1 μM. These considerations again substantiate the proposal that the main function of the mitochondrial Ca^{2+} transport system is to regulate matrix Ca^{2+}, and hence the enzymes and oxidative metabolism, although the more clear-cut evidence of the role of $[Ca^{2+}]_m$ has been obtained in mammalian mitochondria (McCormack and Denton, 1984; Carafoli, 1988).

The present studies extend our previous finding that Ca^{2+} uptake by Jerusalem artichoke mitochondria requires Pi (Rugolo et al., 1990), since the $[Ca^{2+}]_m$ reached its maximal value only upon addition of 0.4 mM Pi under energized conditions (Fig. 5, a and b). Conversely, Ca^{2+} uptake by pea stem mitochondria, although dependent on membrane potential, is not significantly affected by Pi (Fig. 3, trace a). In both cases, however, significant variations in $[Ca^{2+}]_m$ were observed upon Ca^{2+} additions higher than 100 nM. In this respect, it should be noted that because of the mechanisms

regulating $[Ca^{2+}]_m$, Ca^{2+} buffering by the indicator tends not to have much effect on basal or steady-state levels of $[Ca^{2+}]_m$ (McCormack and Crompton, 1991). Ca^{2+} buffering by the indicator can, however, alter the kinetics of Ca^{2+} changes and buffer transient $[Ca^{2+}]_m$ responses. Although we are aware of these intrinsic limitations in the method, the results in Figure 4 suggest that the affinity of the uniporter for Ca^{2+} can be lower than that previously determinated by conventional methods (between 1 and 30 μM) (Åkerman and Moore, 1983; Martins and Vercesi, 1985). This finding is of particular interest because it is now well established that the cytoplasmic $[Ca^{2+}]_{free}$ is approximately 0.1 to 0.2 μM and oscillates around micromolar concentrations only under pathological conditions and for very short periods of time (Bush and Jones, 1990; Read et al., 1992).

In conclusion, the present work reports, for the first time in higher plant mitochondria, variations in the matrix free Ca^{2+} as determined by the fluorescent dye fura-2. The data presented demonstrate that in plant mitochondria, both Ca^{2+} uptake and $[Ca^{2+}]_m$ can differ considerably between plant species. On the other hand, the similarities found between the $[Ca^{2+}]_m$ of plant and animal mitochondria suggest that the poorly defined picture of the Ca^{2+}-regulated mitochondrial enzymes in plants requires more systematic investigation.

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