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Plant mitochondrial electrical potential monitored by fluorescence quenching of rhodamine 123

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Abstract

The suitability of the fluorescent dye rhodamine 123 for qualitative and quantitative determinations of the electrical potential difference ($\Delta\Psi$) in isolated pea (*Pisum sativum* L.) stem mitochondria was evaluated. A fluorescence quenching of rhodamine 123, as a consequence of dye uptake, occurred following mitochondria energization by both external and internal substrates. This quenching was associated to the generation of $\Delta\Psi$, because it was completely released by uncouplers and respiratory inhibitors. The conversion of the proton gradient (ΔpH) into $\Delta\Psi$, induced by nigericin or a permeant weak acid (phosphate), increased the quenching. The uptake of the probe was accompanied by 40 % of unspecific binding in coupled, but not in uncoupled, mitochondria. Rhodamine 123 quenching varied linearly with a K^+-diffusion potential. ADP induced a transient and cyclic change of fluorescence which was associated to ATP synthesis. Consequently, rhodamine 123 did not influence oxygen consumption by mitochondria in both state 4 and 3, thus indicating that, at the concentrations assayed, the probe was not toxic. It is concluded that rhodamine 123, followed by fluorescence quenching, is a suitable probe to study the energetics of isolated plant mitochondria.

Additional key words: membrane potential, mitochondria, *Pisum sativum* L.

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Abbreviations: BSA - bovine serum albumin; Δp - proton motive force; ΔpH - proton gradient; $\Delta\Psi$ - electrical potential difference; EDTA - ethylenediaminetetraacetic acid; FCCP - carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; HEPES - N-(2-hydroxyethylpiperazine-N'-[2-ethanesulfonic acid]); MOPS - 3-(N-morpholino) propanesulfonic acid; P_i - inorganic phosphate; TPP^+ - thiamine pyrophosphate chloride; Tris - tris(hydroxymethyl)aminomethane.

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Introduction

According to the chemiosmotic theory (Mitchell 1966), the oxidation of reduced substrates by plant mitochondria builds up an electrochemical proton gradient and generates a protonmotive force (Δp), whose dissipation drives ATP synthesis and the movement of solutes across this membrane (Douce 1985). Δp is the sum of two interconvertible parameters, the proton gradient (ΔpH) and the electrical potential difference (ΔΨ). However, in mitochondria Δp is mainly in the form of ΔΨ, due to the rapid conversion of ΔpH into ΔΨ, caused by phosphate anions (Douce 1985).

Some fluorescent probes have been applied as optical indicators of ΔΨ. These basically act by two different mechanisms: the permeant dyes (with delocalized charges) work by a potential-dependent accumulation mechanism and these changes take place in a few seconds; impermeant dyes (with localized charges) and the permeants themselves also show optical changes that occur in fractions of a millisecond (Waggoner 1976). Using the technique of fluorescence microscopy, it has been found that several cationic probes (*i.e.* cyanines, safranine O and rhodamines) can be applied to cultured cells, tissues and organs, being able to accumulate into mitochondria *in situ* in response to their ΔΨ (Smith 1990). Among these, safranine O has also been found suitable for monitoring ΔΨ in isolated plant mitochondria, and the spectral response of the probe is linearly related to K^+ diffusion potentials at defined dye:protein ratios (Moore and Bonner 1982). The laser dye rhodamine 123 is a sensitive and specific probe that distributes electrophoretically into the matrix of isolated rat liver mitochondria in response to ΔΨ (Emaus *et al.* 1986). This can be employed to visualize the localization, distribution and movement of mitochondria in plant protoplasts, suspension-cell cultures and root hairs (Wu 1987). In addition, it is useful to study the activity of isolated plant mitochondria by flow cytometric analysis (Petit 1992).

In this paper, we show that rhodamine 123 may also be used to follow the generation and dissipation of ΔΨ in isolated plant mitochondria by fluorescence quenching, and that the optical response of the probe is linearly related to K^+ diffusion membrane potentials.

Materials and methods

Preparation of purified mitochondria: Pea (*Pisum sativum* L. cv. Alaska) stems from 7-d-old etiolated plants were used to isolate crude mitochondria, as previously described (Vianello *et al.* 1994).

The crude mitochondrial fraction was then suspended in 2 cm^3 of a medium containing: 20 mM MOPS-KOH (pH 7.2), 0.3 M mannitol, 1 mM EDTA, 0.1 % (m/v) BSA, and purified on a discontinuous gradient formed by three layers (bottom to top) of 45, 21, 13.5 % (v/v) *Percoll* in 20 mM MOPS-KOH (pH 7.2), 0.5 M sucrose and 0.2 % (m/v) BSA. The gradient was centrifuged at 20 000 *g* for 40 min in a swinging bucket rotor (*Sorvall, HB-4,* Delaware, USA) and the mitochondria were

collected at the 21/45 % interface. Mitochondria were washed from *Percoll* in 250 cm^3 of resuspending medium and centrifuged at 28 000 *g* for 5 min. Purified mitochondria were resuspended in 1 cm^3 of 20 mM HEPES-Tris (pH 7.5), 0.4 M sucrose and 0.1 % (m/v) BSA (fatty acid-free). The suspension contained *ca.* 2 mg (protein) cm^{-3} and was stored on ice.

When mitochondria were purified for rhodamine 123 calibration, sucrose was replaced by 0.25 M KCl in the homogenizing medium, to increase the uploading of K^+. The resuspending and gradient buffer (20 mM MOPS-KOH) was substituted with 20 mM HEPES-Tris (pH 7.2).

Fluorescence measurements: Fluorescence quenching of rhodamine 123 was monitored as fluorescence intensity changes by a *Perkin-Elmer* spectrofluorimeter, *model LS3*. The incubation medium, containing 0.2 mM rhodamine 123, was: 20 mM HEPES-Tris (pH 7.5), 0.4 M sucrose, 0.1 % (m/v) BSA, 5 mM Na/K phosphate, 5 mM $MgCl_2$, 0.2 mg cm^{-3} mitochondrial protein. For rhodamine 123 calibration, mitochondria were incubated in a Na/K phosphate-free buffer. The excitation and emission wavelengths were, respectively, 488 and 526 nm. Safranine O quenching was followed as described earlier (Petrussa *et al.* 1992). The results are expressed as percentage of fluorescence intensity from the fluorescence intensity in the presence of the probes and mitochondria before substrate addition (Q, %). The dye:protein ratio was *ca.* 1 and 25 nmol mg^{-1}(protein) for rhodamine 123 and safranine O, respectively. With this ratio the spectral response of safranine O was linearly related to the electrical potential values (Moore and Bonner 1982).

Oxygen consumption: Oxygen uptake by mitochondria was detected by a Clark-type oxygen electrode (*Model 5331, Yellow Springs Instrument,* Ohio, USA), at 25 °C. The incubation medium was as in fluorescence measurements.

Intramitochondrial K^+ concentration: Mitochondria were resuspended and diluted with *Milli-Q* water. The analysis of K^+ was conducted by a ion-chromatograph equipped *(Model 2000i, Dionex,* USA*)* with a 5 cm *Dionex CG5* cationic guard column plus a 25 cm *Dionex CS12* cationic separation column at room temperature. All determinations were performed under isocratic conditions by using a *Dionex CSRS-I* electrochemical suppressor continuously supplied with *Milli-Q* water and a *Dionex Model CDM-1* conductance detector. The mobile phase consisted of 0.02 M methanesulfonic acid in *Milli-Q* water at a flow rate of 1 cm^3 min^{-1}. A mitochondrial volume of 1 mm^3 mg^{-1}(protein) was considered to calculate K^+ concentration.

Protein determination: The mitochondrial protein was determined as described by Bradford (1976), using the *Bio-Rad (*München, Germany*)* protein assay.

Results

Pea stem mitochondria were incubated with 0.2 μM rhodamine 123 under state 4 conditions (Fig. 1). The addition of both internal (malate plus glutamate or

succinate) and external (NADH) substrates induced an almost instantaneous quenching of rhodamine 123. This indicates that the cationic probe was rapidly accumulated in the mitochondrial matrix in response to the generation of $\Delta\Psi$. The

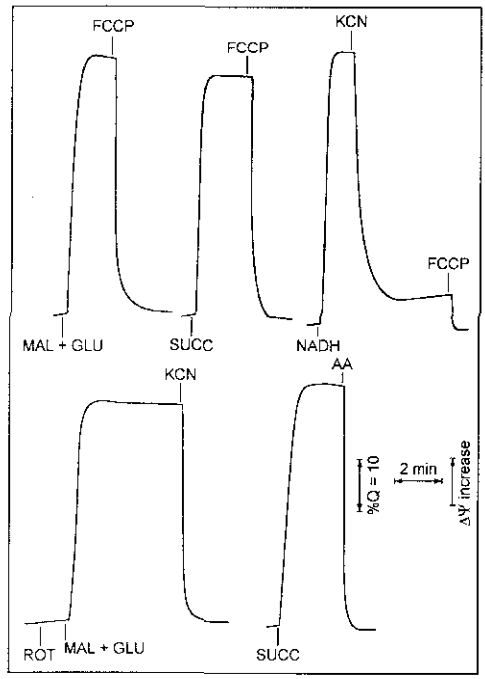

Fig. 1. Fluorescence quenching of rhodamine 123 in purified pea stem mitochondria energized by different substrates. Additions were: 10 mM malate plus 10 mM glutamate (MAL+GLU); 5 mM succinate (SUCC); 1 mM NADH; 1 μM FCCP; 2 mM KCN; 10 μg cm^{-3} antimycin A (AA); 25 μM rotenone (ROT).

accumulated rhodamine 123 was completely released in the medium when a protonophore (FCCP), or metabolic inhibitors (KCN or antimycin A) were added to energize mitochondria. This result suggests that the probe was responding to Δp, or at least to one of its components ($\Delta\Psi$ and ΔpH). Nigericin (*trace A*), which collapses ΔpH and increases $\Delta\Psi$, induced an increase in fluorescence quenching of mitochondria energized by succinate (Fig. 2) or NADH (not shown). Correspondingly, a permeant weak acid (P_i), which causes a similar conversion of ΔpH into $\Delta\Psi$, increased the rhodamine 123 quenching (compare *trace B*, in the presence of P_i and *trace C* in the absence of P_i).

The uptake of rhodamine 123 was confirmed by evaluating the absolute fluorescence changes of the solution containing mitochondria energized by succinate (Table 1). The addition of this substrate caused a decrease of absolute fluorescence that was completely released by FCCP. When the fluorescence was detected after removing the mitochondria by centrifugation, the fluorescence recovered in the

presence of succinate was lower than that detected before centrifugation (*ca.* 40 % decrease). Conversely, in the presence of uncoupler the fluorescence was the same before and after centrifugation. This indicates that in coupled mitochondria (without FCCP) the uptake of rhodamine 123 was accompanied by 40 % unspecific binding of the probe, while this binding was negligible in uncoupled mitochondria.

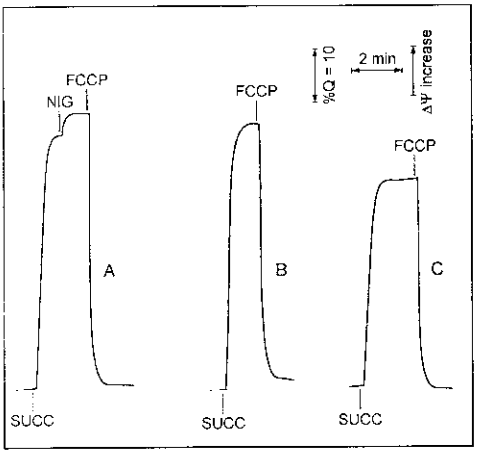

Fig. 2. Effect of nigericin (*trace A*) and phosphate (*trace B*) on fluorescence quenching of rhodamine 123 in purified pea stem mitochondria energized by succinate. Additions were: 5 mM succinate (SUCC); 1 μM FCCP; 2.5 μM nigericin (NIG); *trace C*, medium without 5 mM K/Na phosphate.

Table 1. Uptake of rhodamine 123 (absolute fluorescence, arbitrary units) by purified pea stem mitochondria.

Additions	Before centrifugation	After centrifugation
0.2 μM rhodamine 123	165	161
+ 5 mM succinate	81	47
+ 5 mM succinate + 1 μM FCCP	166	164

K^+ diffusion potential was induced by the addition of 20 nM valinomycin to de-energized mitochondria, suspended in a K^+-free medium (Fig. 3, *inset*). This addition induced a K^+ efflux from the mitochondria that determined fluorescence quenching of the dye as a result of the generation of a K^+-diffusion potential. After equilibration, the potential was collapsed by the subsequent addition, step by step, of K^+ in the range from 0.1 to 8 mM (Moore and Bonner 1992). These data were plotted *versus* $\Delta\Psi$ values calculated according to the Nernst equation, on the basis of an estimated mitochondrial matrix K^+ concentration of 94 mM. The variation of $\Delta\Psi$ was linearly related to per cent quenching in the 65 - 210 mV range ($r^2 = 0.99$). The intercept on the x-axis occurs at a potential value of *ca.* 65 mV, probably corresponding to an energy-independent Donnan potential (Fig. 3). In the light of this result the variation

of fluorescence, associated to the oxidation of reduced substrates (Fig. 1), was linked to the generation of an electrical potential of 200 - 210 mV.

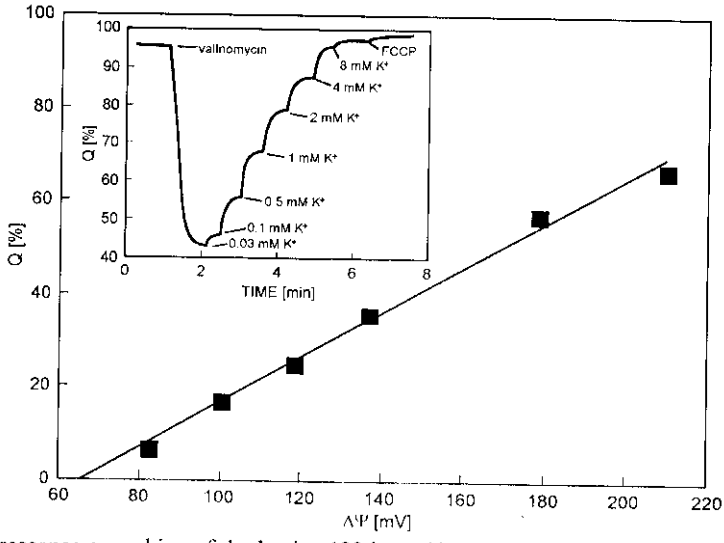

Fig. 3. Fluorescence quenching of rhodamine 123 in purified pea stem mitochondria as a function of potassium diffusion potential.

Since rhodamine 123 could inhibit mitochondrial metabolism, particularly mitochondrial ATPase activity (Mai and Allison 1983, Modica-Napolitano *et al.* 1984, Emaus *et al.* 1986, Bullough *et al.* 1989), experiments were performed to verify if ADP-induced dissipation of $\Delta\Psi$ may be monitored by this probe to follow ATP synthesis. The successive additions of ADP to mitochondria, energized by both malate plus glutamate or succinate, induced a transient and cyclic change of fluorescence that was completely inhibited by oligomycin (Fig. 4, *traces A, B*). These results indicate that at least this concentration of rhodamine 123 did not interfere with ATP synthesis. This conclusion was confirmed by showing that this probe could be employed to follow $\Delta\Psi$ formation in pea mitochondria energized by ATP (*trace C*).

The effects of rhodamine 123 on mitochondrial metabolism were further examined (Table 2). The laser dye did not influence oxygen consumption in either state 4 or

Table 2. Effect of rhodamine 123 concentration on oxygen consumption [nmol (O_2) mg^{-1}(protein) s^{-1}] and on the initial rate of ATP-dependent safranine O quenching [%Q mg^{-1}(protein) min^{-1}] in purified pea stem mitochondria (RCR - respiratory control ratio, n.d. - not detected).

Rhodamine 123 [μM]	Oxygen consumption state 4	state 3	RCR	Safranine O quenching
None	3.1	9.7	3.14	287
0.2	3.2	9.5	2.94	281
1.0	3.2	9.4	2.89	234
10	3.3	8.7	2.64	n.d.
20	3.2	8.3	2.55	n.d.

state 3 in the range from 0.2 to 20 μM. Correspondingly, the initial rate of ATP-dependent fluorescence quenching of safranine O was not significantly altered by 0.2 or 1 μM rhodamine 123. Higher concentrations were not tested because of an interference between the two dyes.

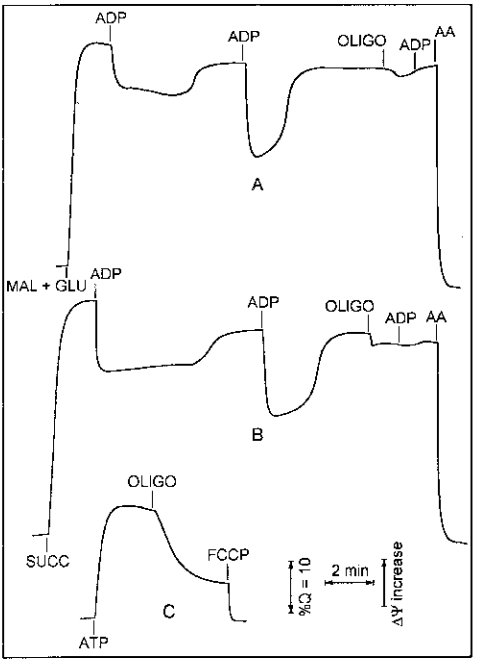

Fig. 4. ADP-induced fluorescence quenching decrease of rhodamine 123 in purified pea stem mitochondria energized by malate plus glutamate or succinate. Additions were: 150 μM ADP; 0.5 μg cm^{-3} (*traces A, B*) or 5 μg cm^{-3} oligomycin (OLIGO; *trace C*); 1 mM ATP. Other additions were as in Fig.1.

Discussion

The above results show that the permeant laser dye rhodamine 123, similarly to what was found in rat liver mitochondria (Emaus *et al.* 1986), can be used, by following fluorescence quenching, to monitor $\Delta\Psi$ and ATP synthesis in isolated plant mitochondria. The energy-dependent $\Delta\Psi$ changes, associated to this quenching, are demonstrated by the very fast dissipation induced by protonophores and respiratory chain inhibitors. In addition, the effects of nigericin and P_i show that $\Delta\Psi$ increases by collapsing ΔpH and that Δp, in the presence of P_i, is almost completely in the form of $\Delta\Psi$. The uptake of rhodamine 123 is, however, accompanied by a binding of the dye to mitochondria that, as previously shown (Emaus *et al.* 1986), is higher in coupled than in uncoupled organelles. Nevertheless, the binding recovered in coupled pea stem mitochondria (*ca.* 40 %) is less than that observed in rat liver mitochondria. The

response of rhodamine 123 is linearly related to a K^+ diffusion potential and, therefore, this method can also be used for quantitative estimation of the membrane electrical potentials. Using this plot, the calculated $\Delta\Psi$ is *ca.* 200 - 210 mV, a value which falls within the range of $\Delta\Psi$ estimated in plant mitochondria by radioactive tracers or ion-selective electrodes.

It is known that rhodamine 123 may cause a fairly specific inhibition of ADP-stimulated respiration, probably by inhibiting ATPase activity, as a consequence of its accumulation in the mitochondrial matrix in response to the electrical potential (Mai and Allison 1983, Modica-Napolitano *et al.* 1984, Emaus *et al.* 1986, Bullough *et al.* 1989). Nevertheless, our results demonstrate that ATP synthesis may be followed as ADP-induced changes of fluorescence and that this probe is not toxic towards isolated plant mitochondria at a concentration up to 20 μM. The concentration of rhodamine 123 used in these experiments being only 0.2 μM, it is possible to exclude side-effects under these conditions. In any case, the toxicity of rhodamine 123 in plant cells appears to be quite low (Wu 1987).

The major potential-sensitive fluorescent dyes hitherto employed to monitor the activity of isolated or *in situ* plant mitochondria are cyanines, safranine O and rhodamines. Cyanine dyes have been found to be suitable to visualize mitochondria by fluorescence microscopy and to evaluate their activity in intact cells (Matze and Matze 1986, Liu *et al.* 1987) and protoplasts (Reich *et al.* 1986). Conversely, safranine O has been used only to detect $\Delta\Psi$ of isolated mitochondria (Moore and Bonner 1982). Rhodamine 123 has been found to be a very sensitive probe to monitor, by fluorescence microscopy, $\Delta\Psi$ in mitochondria of plant protoplasts, suspension-culture cells and root hairs (Wu 1987). In addition, this dye can be employed to study the activity of isolated plant mitochondria by flow cytometric analysis (Petit 1992) or, as shown in this paper, by fluorescence quenching. The low phytotoxicity of rhodamine 123 (Wu 1987) together with the possibility to use it with either isolated (Petit 1992) or *in situ* mitochondria (Wu 1987), renders it a very versatile probe to evaluate the energetic metabolism by different fluorescent techniques.

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