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Rhodamine 123 as a probe of mitochondrial membrane potential: evaluation of proton flux through F_0 during ATP synthesis

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Abstract

Rhodamine 123 (RH-123) was used to monitor the membrane potential of mitochondria isolated from rat liver. Mitochondrial energization induces quenching of RH-123 fluorescence and the rate of fluorescence decay is proportional to the mitochondrial membrane potential. Exploiting the kinetics of RH-123 fluorescence quenching in the presence of succinate and ADP, when protons are both pumped out of the matrix driven by the respiratory chain complexes and allowed to diffuse back into the matrix through ATP synthase during ATP synthesis, we could obtain an overall quenching rate proportional to the steady-state membrane potential under state 3 condition. We measured the kinetics of fluorescence quenching by adding succinate and ADP in the absence and presence of oligomycin, which abolishes the ADP-driven potential decrease due to the back-flow of protons through the ATP synthase channel, F_0. As expected, the initial rate of quenching was significantly increased in the presence of oligomycin, and conversely preincubation with subsaturating concentrations of the uncoupler carbonyl cyanide *p*-trifluoro-metoxyphenilhydrazone (FCCP) induced a decreased rate of quenching. *N*,*N*′-dicyclohexylcarbodiimide (DCCD) behaved similarly to oligomycin in increasing the rate of quenching. These findings indicate that RH-123 fluorescence quenching kinetics give reliable and sensitive evaluation of mitochondrial membrane potential, complementing steady-state fluorescence measurements, and provide a mean to study proton flow from the mitochondrial intermembrane space to the matrix through the F_0 channel.

Keywords: Mitochondria; Membrane potential; ATP synthase; Proton transport; Rhodamine 123

1. Introduction

Several cationic dyes distribute electrophoretically into the mitochondrial matrix in response to the electric potential across the inner mitochondrial membrane [1–3]. The accumulation takes place as a consequence of their charge and of their solubility in both the inner membrane lipids and the matrix aqueous space. For the above reason, these dyes have been extensively employed to measure the mitochondrial electric potential ($\Delta\psi_{mit}$) exploiting their spectroscopic properties or, alternatively, after isotopic labelling [4–6].

Among these dyes, Rhodamine 123 (RH-123) was first used to measure $\Delta\psi_{mit}$ in intact cells both as a microscopic stain [7,8] and by cytofluorometry by monitoring the increase in fluorescence due to its electrophoretic accumulation in mitochondria [9]. In isolated mitochondria, Emaus et al. [10] first showed that energization induced a red shift and extensive quenching of RH-123 fluorescence, so that dye accumulation could be suggested as a sensitive and specific probe of $\Delta\psi_{mit}$ [10,11].

Although RH-123 and similar dyes are still employed preferentially in cellular studies, their use with isolated mitochondrial suspensions has appeared in several investigations to measure respiration-driven membrane potential [10,12]. Changes of $\Delta\psi_{mit}$ are induced, directly or indirectly, by the proton movements occurring across the mitochondrial inner membrane during oxidative phosphorylation [13,14]: under physiological conditions, there is active proton extrusion by respiration and passive proton intake through ATP synthase during ATP synthesis, besides other possible leak pathways; likewise, membrane polarisation by

Abbreviations: F_1F_0-ATPase, H^+-translocating ATP synthase of F_1F_0 type; F_0, membrane sector part of H^+-translocating ATP synthase; $\Delta\psi_{mit}$, electric membrane potential of mitochondria; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; FCCP, carbonyl cyanide *p*-trifluoro-metoxyphenilhydrazone; RH-123, Rhodamine 123

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addition of respiratory substrates to isolated mitochondria induces RH-123 quenching [10,15], while depolarisation by ADP addition induces fluorescence recovery [10].

Monitoring the changes of $\Delta\psi_{mit}$ as a result of both active and passive proton transports across the mitochondrial inner membrane, by analysing the individual steps in which the protons are moved, can be of great value in understanding physiological and pathological processes in which mitochondrial function is involved at the level of proton movements.

Exploiting $\Delta\psi_{mit}$ in the presence of respiratory substrates and ADP, when protons translocated by the respiratory chain are allowed to diffuse back into the matrix through F_1F_0-ATPase during ATP synthesis, is a measure of the steady state established between respiration-driven $\Delta\psi_{mit}$ formation and ADP-driven $\Delta\psi_{mit}$ dissipation. The different extent of steady-state $\Delta\psi_{mit}$ after addition of succinate and ADP in the presence and absence of oligomycin, which abolishes the ADP-driven proton dissipation through the ATP synthase channel, should be a measure of the contribution of passive proton movement through the ATP synthase channel itself. The lipophilic nature of RH-123 allows it to diffuse through the mitochondrial membrane in response to potential and concentration gradients, being trans-bilayer diffusion of the dye much slower than the actual changes in $\Delta\psi_{mit}$ [3,15]. However, because RH-123 is less lipophilic than other cation dyes, it perturbs only slightly the membrane surface potential and it has similar kinetic constants for influx and efflux from mitochondrial matrix [15]. Due to this reason, RH-123 results a good candidate to measure the actual membrane potential, but it cannot be used to directly measure kinetics of $\Delta\psi_{mit}$ formation. According to Scaduto and Grotyohann [11], RH-123 uptake is in proportion to $\Delta\psi_{mit}$, therefore the rate of fluorescence quenching has also to be a function of $\Delta\psi_{mit}$, as well as the steady-state level of fluorescence decrease.

For this reason, in isolated rat liver mitochondria we investigated the kinetics of fluorescence quenching induced by succinate after addition of ADP, in the presence and absence of oligomycin. The results indicate that measuring the kinetics of fluorescence quenching consequent to RH-123 uptake by isolated mitochondria is a better estimation of $\Delta\psi_{mit}$ than the steady-state RH-123 fluorescence quenching measurement; the results suggest also that the rate of RH-123 fluorescence quenching can be used to study proton intake into the mitochondrial matrix through the F_0-ATPase channel during ATP synthesis.

2. Materials and methods

2.1. Materials

Carbonyl cyanide *p*-trifluoro-metoxyphenilhydrazone (FCCP), *N*,*N*′-dicyclohexylcarbodiimide (DCCD), cyclosporin A, rotenone, antimycin A, oligomycin, and ADP were obtained from Sigma (St. Louis, MO, USA). Lyophilised yeast hexokinase, essentially salt-free, was purchased from Fluka Chemie GmbH (CH) and Rhodamine 123 from Molecular Probes (Eugene, OR, USA). Chemicals were dissolved, stored, and used according to the instructions from the manufacturers. All other reagents used were of the highest grade available and obtained from regular commercial sources.

2.2. Isolation of mitochondria

Coupled mitochondria were isolated from rat liver immediately after killing anaesthetic-treated animals by decapitation according to a slightly modified method of Kun et al. [16], and avoiding the digitonin treatment. Essentially, tissue homogenate (0.22 M mannitol, 0.07 M sucrose, 0.02 M HEPES, 1 mM K-EDTA, 0.1 mM K-EGTA, pH 7.4 containing 0.4% albumin) was centrifuged at 2000 rpm for 10 min (Sorvall SS34 rotor) to remove nuclei and plasma membrane fragments. Then the supernatant was filtered with gauze and centrifuged at 10 000 rpm for 10 min (Sorvall SS34 rotor) to obtain the mitochondrial pellet. Mitochondria were washed in 0.25 M sucrose, 0.02 M HEPES, 1 mM K-EDTA, and 0.1 mM K-EGTA, pH 7.4 and resuspended in the same buffer at about 60 mg/ml protein.

All buffers used contained K-EGTA to avoid any calcium contamination in mitochondrial preparations that could interfere with membrane potential measurements.

2.3. Protein determination

Protein concentration of mitochondrial preparations was assessed by the biuret colorimetric method [17] in the presence of 0.3% (w/v) sodium deoxycholate. Bovine serum albumin was used as standard.

2.4. Respiration measurements

Respiratory rates of rat liver mitochondria were measured at 30 °C using a Clark-type oxygen electrode essentially according to Aicardi and Solaini [18]. The standard incubation medium was composed of 0.25 M sucrose, 0.05 M HEPES, 0.5 mM EDTA, 4 mM $MgSO_4$, and 5 mM KH_2PO_4, pH 7.4 and mitochondrial concentration was usually below 0.5 mg/ml. State 4 and state 3 (0.2 mM ADP) oxygen consumption rates (nmol O_2/min/mg) were calculated from the first derivative of the oxygraph traces. The respiratory control index (RCI), measured using either glutamate–malate (10 mM/10 mM) or succinate (20 mM) as substrate, was always higher than 8 and 4, respectively.

The effect of RH-123 low concentrations (20–100 nM) on both oxygen consumption rates (state 4 and state 3) and RCI of the mitochondrial preparations was tested after incubation of the mitochondria with the fluorescent cation.

2.5. Spectrofluorometric measurements of $\Delta\psi_{mit}$

Rhodamine was dissolved in ethanol and the concentration was assayed spectrophotometrically at 507 nm (ε_{507} = 101 mM^{-1} cm^{-1}). The ethanol concentration in all incubation mixtures of mitochondria was kept below 0.4% (v/v). Fluorescence measurements were made at 25 °C with a Jasco FP-777 spectrofluorometer using a thermostatic apparatus, to avoid differential temperature-dependent unspecific binding of the fluorescent cation to mitochondria [11].

As suggested by Emaus et al. [10], the experimental work has been performed by exciting RH-123 at 503 nm and detecting the fluorescence emission at 527 nm. During the measurements, the reaction medium containing mitochondria was continuously stirred.

Mitochondrial potential ($\Delta\psi_{mit}$) changes have been evaluated by measuring RH-123 fluorescence quenching under the following conditions: 0.15 mg rat liver mitochondria were added to 0.5 ml buffer (250 mM sucrose, 10 mM HEPES, 100 μM K-EGTA, 2 mM $MgCl_2$, 4 mM KH_2PO_4, pH 7.4) containing an ADP regenerating system (10 mM glucose and 2.5 U hexokinase). Before rhodamine (50 nM) addition, samples were incubated with 33 nM cyclosporin A, 1 μg/ml rotenone, and 0.1 mM ADP. Finally, mitochondria were energized by 20 mM succinate in the presence or absence of oligomycin (0.2 μM) to detect membrane potential changes associated with state 4 and state 3 respiratory conditions, respectively.

2.6. Analysis of the fluorescence quenching kinetics

Time courses of RH-123 fluorescence decay were analysed by means of an exponential decay best fitting using the GraphPad Prism 3.0 software (GraphPad Software). Fluorescence values calculated in the time range 0–60 s were normalised on the initial fluorescence (F_i). On the basis of the fluorescence kinetics obtained, we performed both dynamic and static measurements by evaluating the fluorescence quenching initial rate and the steady-state quenching extent, respectively. The initial rates of RH-123 fluorescence quenching were calculated as first derivative of curves at $t = 0$.

2.7. Statistical analysis

Fluorescence quenching data are presented as means ± S.D. The significance of differences of the RH-123 fluorescence measurements was evaluated by the unpaired t test.

3. Results

Respiring mitochondria generate a proton gradient across the inner membrane, producing a pH gradient and a membrane potential or $\Delta\psi_{mit}$. The $\Delta\psi_{mit}$ represents most of the energy of the proton gradient [19], and using the fluorescent cation RH-123, others have measured this potential [10–12,15]. Lipophilic dyes such as RH-123 accumulate in the mitochondrial matrix driven by the electric gradient following the Nerst equation. The higher the $\Delta\psi$, the more RH-123 is taken up into the matrix. In the aqueous phase, the dispersed RH-123 has an emission peak at 525 nm, whereas when it is highly concentrated within the mitochondrial matrix, RH-123 shows a red shift and a fluorescence quenching [10,11].

3.1. Characterisation of the mitochondrial model

The experimental work has been performed on rat liver mitochondria prepared as detailed under the Materials and methods section.

The degree of coupling of the mitochondrial preparations was assayed determining the RCI by oxygen consumption measurements. The RCI values of preparations used for experiments were in the range 4–6 and 8–10, using succinate and glutamate/malate as energizing substrate, respectively (not shown). According to several authors [10,11,20–22], RH-123 affects mitochondrial respiration and oxidative phosphorylation, therefore assays to test whether low concentrations, 20–100 nM, could affect either uncoupled (state 4) or ADP-stimulated (state 3) respiratory rates were performed; at the above concentrations, however, the dye was unable to induce changes on the respiratory rates (not shown).

3.2. $\Delta\psi_{mit}$ Assay

The electrochemical potential of the proton gradient generated across the mitochondrial membrane was assessed by monitoring fluorescence quenching of RH-123. Protons were extruded from mitochondria by the respiratory complexes and easily diffused in through F_0. However, a significant fluorescence quenching was maintained at steady state as a balance between activities of respiration and proton flow through F_0. Fig. 1 shows the effect of a series of consecutive additions on the dye fluorescence. Addition of 0.3 mg/ml mitochondria to the medium containing 50 nM RH-123 and an ADP-regenerating system, induced a rapid quenching of the RH-123 fluorescence partially due to uptake of the probe by mitochondria [10,11]. Cyclosporin A was then added to prevent possible dissipation of the membrane potential due to the permeability transition pore opening [23,24], and mitochondrial respiration was stimulated by saturating glutamate/malate addition. A further decrease of fluorescence to a steady state corresponding to apparent state 4 respiration occurred. Addition of ADP induced an enhancement of steady-state fluorescence, which corresponds to state 3 respiration, when the proton gradient significantly decreased due to ADP phosphorylation. Rotenone, a specific inhibitor of NADH dehydrogenase, caused a further increase of fluorescence due to the membrane

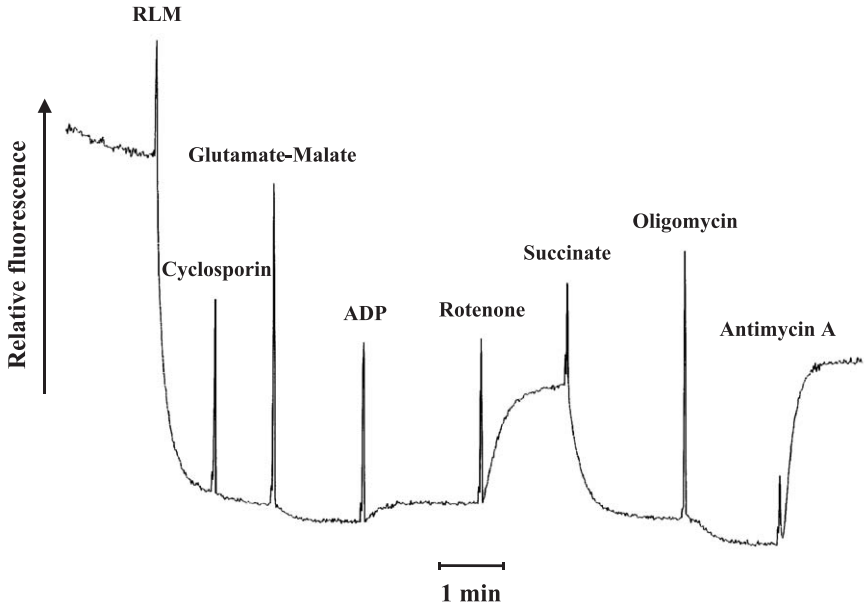

Fig. 1. Time course of RH-123 fluorescence upon addition of several substrates and inhibitors of oxidative phosphorylation. Fluorescence was measured on a spectrofluorometer, by exciting at 503 nm and collecting the emitted fluorescence at 527 nm. 0.3 mg/ml coupled mitochondria was added to a basic reaction medium (respiratory buffer) containing 250 mM sucrose, 10 mM HEPES, 100 μM K-EGTA, 2 mM $MgCl_2$, 4 mM KH_2PO_4 (pH 7.4), 10 mM glucose, 2.5 U hexokinase, and 50 nM RH-123. Further addition, where indicated, was 33 nM cyclosporin A, 10 mM/10 mM glutamate–malate, 100 μM ADP, 1 μg/ml rotenone, 20 mM succinate, 0.2 μM oligomycin, and 1 μg/ml antimycin.

potential dissipation. However, $\Delta\psi_{mit}$ could be recovered by addition of saturating succinate and, according to the literature [25], succinate-energized mitochondria showed a slightly higher steady-state membrane potential than the one induced by glutamate/malate. Finally, inhibition of ATP synthase by oligomycin induced a further increase of the membrane potential due to a block of proton flow through F_0, whereas addition of antimycin A, an inhibitor of complex III of the respiratory chain allowed a recovery of fluorescence caused by membrane potential decrease as a consequence of $\Delta\mu_{H+}$ disappearance. These observations allowed us to investigate dynamic and steady-state RH-123 redistribution across the inner mitochondrial membrane as a consequence of membrane potential changes.

Because the purpose of the present work was mainly to describe a well-reproducible and sensitive method to provide information concerning the contribution of the proton translocation to the membrane potential, changes of RH-123 fluorescence were measured as a function of time in the presence of 0.3 mg/ml coupled mitochondria, with respiration induced by succinate oxidation in the presence of cyclosporin A, rotenone, ADP, and an ADP-regenerating system under conditions of ADP phosphorylation, therefore under conditions of proton influx through F_0. From mitochondrial energization monitored by the dynamic fluorescence quenching of RH-123 reported in Fig. 2A, the time course of F/F_i decay could be derived at different times between 0 and 60 s (Fig. 2B). The curves represent the exponential decay best fitting value as obtained by the GraphPad Prism software (GraphPad Software Incorporated). F_i is the initial fluorescence as derived from the best fitting analysis, and the initial fluorescence quenching rates were calculated as first derivatives at time zero.

The initial rate of the fluorescence decay in the presence of oligomycin increased from 0.20 to 0.33 $(\Delta F/F_i)$/s/mg protein and the quenching extent at steady-state level increased from 2.67 to 2.96 $(\Delta F/F_i)$/mg protein. The enhancement of both the fluorescence quenching rate and the steady-state quenching induced by oligomycin was 65% and 11%, respectively. Furthermore, a comparative analysis of the data of both fluorescence quenching extent and fluorescence quenching rate of 15 different mitochondrial preparations, besides supporting the above data, showed that the mean of the fluorescence quenching rate of oligomycin-treated mitochondria (0.32 ± 0.03 $(\Delta F/F_i)$/s/mg protein ± S.D.) with respect to its control [0.21 ± 0.02 $(\Delta F/F_i)$/s/mg protein ± S.D.] (Fig. 3A), has a higher significance difference ($P < 0.001$) than the mean of the steady-state quenching values, as measured in the presence or absence of oligomycin [3.14 ± 0.36 and 2.81 ± 0.32 $(\Delta F/F_i)$/mg protein ± S.D., respectively, with $P < 0.05$] (Fig. 3B).

Previous studies have shown that dye fluorescence response to mitochondrial $\Delta\psi$ is related to the dye/protein ratio [6,10]; therefore, a titration of 50 nM RH-123 during succinate-driven respiration was carried out with coupled mitochondria in the small range from 0.15 to 0.45 mg/ml protein. Increase of $\Delta F/F_i$ was observed when mitochondria were added to the dye, as expected (Fig. 4B). However, $\Delta F/F_i$ values appeared scattered with respect to the best linear fitting, with a correlation coefficient $r = 0.74$. On the contrary, plotting the rate of fluorescence quenching induced by respiration, $(\Delta F/F_i)$/s, as a function of protein

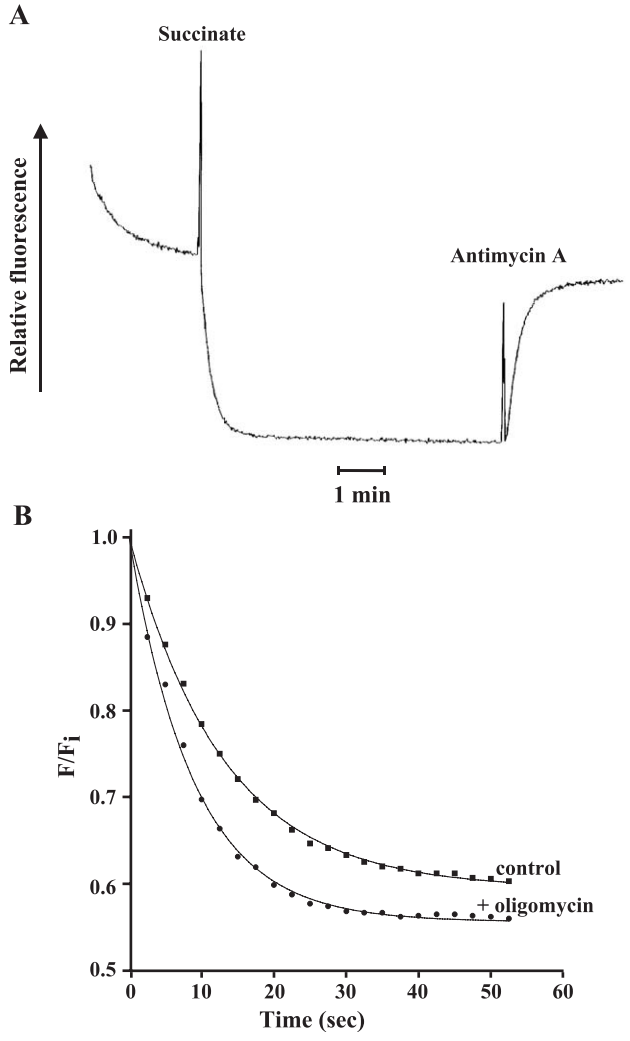

Fig. 2. Mitochondrial energization as monitored by fluorescence quenching of RH-123. (A) Time course of fluorescence change upon addition of 50 nM RH-123 to 0.3 mg/ml rat liver mitochondria suspended in the respiratory buffer containing 33 nM cyclosporin A, 1 μg/ml rotenone, and 100 μM ADP (pH 7.4). Where indicated, 20 mM succinate and 1 μg/ml antimycin were injected. (B) Normalised fluorescence quenching curves obtained as described in details under the Materials and methods section. Experimental data normalised on the initial fluorescence value (F_i) were superimposed on the curves.

concentration (Fig. 4A), a correlation coefficient of 0.99 was calculated.

3.3. Quantitative estimation of $\Delta\psi_{mit}$ by measuring RH-123 fluorescence quenching rate

To accurately quantify $\Delta\psi_{mit}$ by using measurements of fluorescence quenching rate, the relationship between transmembrane potential and fluorescence quenching rates must be properly calibrated. Incubating non-respiring mitochondria in the presence of RH-123 and increasing concentrations of K^+, the addition of valinomycin (0.2 μM) induced an increase of the initial rate of fluorescence quenching. Fluorescence quenching rate was plotted against membrane potential, as calculated according to the Nernst equation, and assuming an internal free K^+ concentration of 20 mM, as recently assessed by Zoeteweij et al. [26] (Fig. 5). By using the GraphPad Prism 3.0 software (GraphPad Software), a linear dependence of $(\Delta F/F_i)$ s^{-1} mg^{-1} from membrane potential was found. The calibration curves intersected the $\Delta\psi_{mit}$ axis at 40 mV, indicating a residual membrane potential for non-energized mitochondria, as pointed out previously [10]. From Fig. 5, for succinate-induced respiration in the presence of ADP (state 3), a $\Delta\psi_{mit}$ of 146 mV was obtained, and oligomycin addition produced a hyperpolarization, increasing $\Delta\psi_{mit}$ up to 199 mV (electric potentials corresponding to mean values of 15 different determinations, from Fig. 3A), a consequence of inhibiting

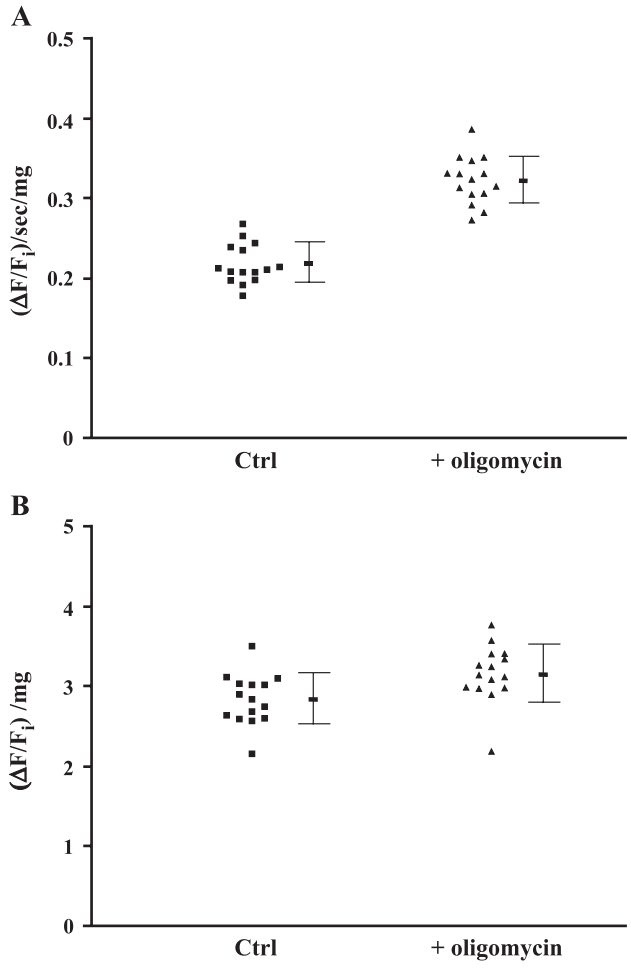

Fig. 3. Reliability and sensitivity of two fluorescence quenching indexes: rate (A) and value at steady state (B), under two different metabolic conditions (ADP phosphorylation and in the presence of oligomycin). Mitochondria were suspended in the respiratory buffer as detailed in the legend to Fig. 2. The kinetics of RH-123 fluorescence quenching induced by succinate energization of mitochondria were recorded. For each single trace, both the initial rate of quenching and the steady-state quenching extent were calculated and expressed as mean ± S.D. Statistical analysis refers to experiments with 15 different mitochondrial preparations, and under the two conditions assayed, the initial rate of quenching and its extent resulted with different significance, evaluated as $P<0.0001$ and $P<0.015$, respectively.

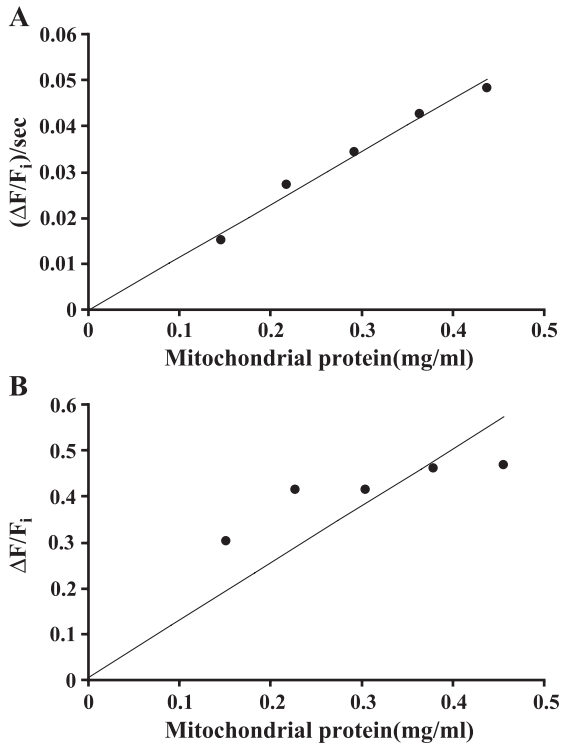

Fig. 4. Relationship between RH-123 fluorescence quenching indexes and rat liver mitochondria concentration. (A) Plot of the fluorescence quenching rate versus mitochondrial protein concentration, and (B) plot of the steady-state fluorescence quenching values as obtained under the same experimental control conditions. Analysis by linear regression assay showed a correlation coefficient (r) of 0.992 and 0.742 for the fluorescence quenching initial rate and the steady-state quenching, respectively.

the dissipation of the proton gradient and ATP synthesis by the F_1F_0-ATPase. These results are in agreement with data previously reported using other methods [25,27,28].

3.4. Validation of the initial rate of respiration-induced RH-123 fluorescence quenching as an index of proton transport through F_0

Measurement of the proton transport activity of F_0, particularly in the direction from the mitochondrial intermembrane space to the matrix, is difficult to achieve. However, if a $\Delta\psi$-sensitive fluorescent dye distribution through the mitochondrial membrane can be associated with active F_0 molecules, the parameter associated with its distribution can reasonably be assumed as an index of this transport activity. The reliability and sensitivity of the method to detect membrane potential changes associated with alteration of the proton pumping activity of the F_1F_0-ATPase has been investigated by testing the effects of two well-known specific inhibitors of the F_0 proton channel under controlled conditions [29,30]: oligomycin and *N*,*N*′-dicyclohexylcarbodiimide. Fig. 6 shows the effect of oligomycin on the respiration-induced initial fluorescence quenching rate of RH-123. At low inhibitor concentrations, the rate increased sharply as oligomycin concentration increased until a plateau was reached at about 50 nM. Therefore, the fluorescence quenching rate showed a hyperbolic dependence on oligomycin concentration. The same data were plotted in the double-reciprocal form (Fig. 6, inset), from which the intercept of the ordinate with the straight line, obtained by interpolating the exper-

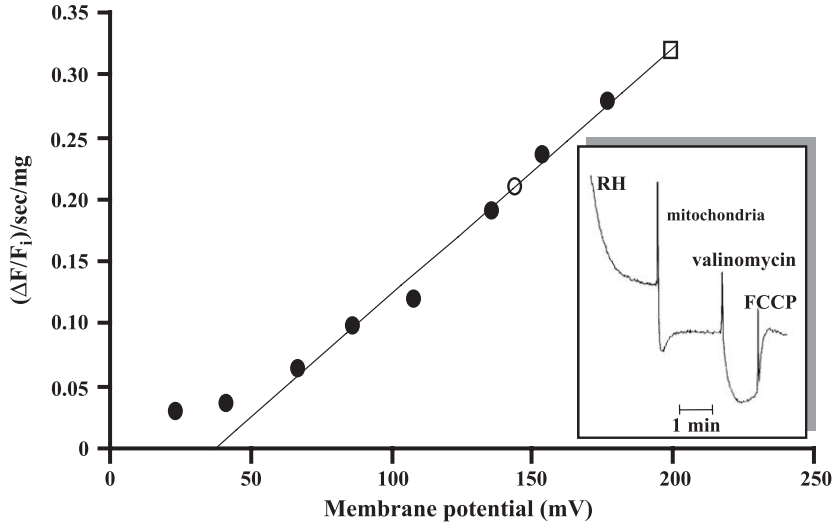

Fig. 5. Correlation between initial fluorescence quenching rates of RH-123 and K^+-diffusion potential. Experimental conditions were: 0.15 mg mitochondria suspended in 0.5 ml of the respiratory buffer in the presence of 50 nM rhodamine, 0.5 μg of both rotenone and antimycin. The fluorescence quenching rates were obtained adding 0.2 μM valinomycin (inset: fluorescence traces at 0.02 mM K_o^+). The $[K_i^+]/[K_o^+]$ ratio was varied by increasing KCl from 0.02 to 8 mM (concomitantly, mannitol was decreased from 39.96 to 24 mM). The initial rates of fluorescence quenching $[(\Delta F/F_i)/s/mg]$ were extrapolated to the time of valinomycin addition. The $\Delta\psi_{mit}$ values reported in the abscissa were calculated by applying the Nernst equation to the $[K_i^+]/[K_o^+]$ ratio. Open symbols show where $\Delta\psi_{mit}$ of mitochondria either incubated under phosphorylation conditions (○) or hyperpolarized by preincubation with oligomycin (□) fall in the calibration curve.

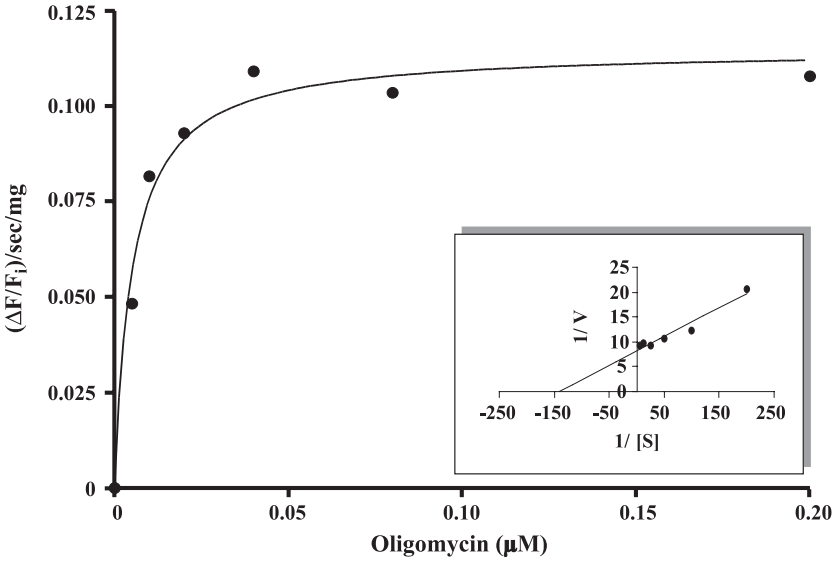

Fig. 6. Titration of RH-123 fluorescence quenching rate with oligomycin. Initial rate values were measured after succinate energization of 0.3 mg/ml respiring mitochondria suspended in the respiratory buffer containing 33 nM cyclosporin A, 1 μg/ml rotenone, 100 μM ADP, 50 nM RH-123, and oligomycin at the indicated concentration. The rate estimated in the absence of oligomycin was subtracted from each value determined at the different inhibitor concentrations. The inset represents the double-reciprocal plot, from which a maximal rate of 0.12 ($\Delta F/F_i$)/s/mg protein was calculated.

imental points, gave the value of 8.33 $[(\Delta F/F_i)/s/mg]^{-1}$, corresponding to a maximal rate of 0.12 ($\Delta F/F_i$) s^{-1} mg $protein^{-1}$. The intercept of the straight line with the abscissa was at -141 μM^{-1} corresponding to a concentration of 7.1 nM oligomycin necessary for 50% fluorescence quenching rate increase. Because the ATP synthase/oligomycin stoichiometry is 1 to 1 and the binding of the inhibitor is rapid and irreversible, the expected concentration of ATP synthase in the fluorometer cuvette should be 14.2 nM, a figure consistent with data previously reported [31]. Incidentally, the above observation confirms that the oligomycin concentration (0.2 μM) used in the experiments described above was competent to completely inhibit the ATP synthase in the cuvette. Similar results were obtained when DCCD, which covalently binds to Glu 58 of the *c*-subunit of the F_0-ATPase sector, substituted for oligomycin (Fig. 7). In the present case, the intercepts of the straight line with the axes in the double-reciprocal plot gave a maximal rate value of 0.11 ($\Delta F/F_i$) s^{-1} mg $protein^{-1}$ and 130 nM DCCD necessary for 50% fluorescence quenching rate

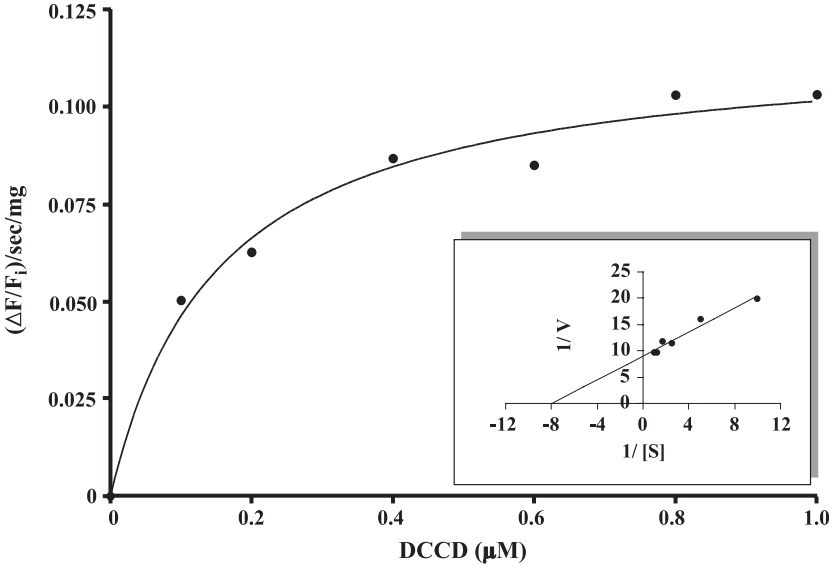

Fig. 7. Titration of RH-123 fluorescence quenching rate with DCCD. Initial rate values were obtained after addition of 20 mM succinate to 0.3 mg/ml mitochondria suspended in the respiratory buffer containing 33 nM cyclosporin A, 1 μg/ml rotenone, 100 μM ADP, 50 nM RH-123, and DCCD at the indicated concentrations. The rate estimated in the absence of DCCD was subtracted from the values determined at different inhibitor concentrations. The inset represents the double-reciprocal plot, from which a maximal rate of 0.11 ($\Delta F/F_i$)/s/mg protein was calculated.

increase. This concentration of DCCD, higher than that of oligomycin, was expected because the rate of DCCD binding to the *c*-subunit of the ATP synthase is low [32] and our incubation time was restricted to minutes to avoid loss of mitochondrial integrity and coupling. It has to be noticed that the maximal fluorescence quenching rate values calculated through rhodamine uptake titration with the two F_0 inhibitors are very similar [0.12 and 0.11 ($\Delta F/F_i$) s^{-1} mg protein^{-1}], confirming the high reliability of the fluorescence index to detect the mitochondrial membrane potential and to measure the molar fraction of active F_0 channels.

The effect of a subsaturating concentration of an uncoupler, which dissipates the proton gradient across the inner mitochondrial membrane, on the initial rate of the RH-123 fluorescence quenching is shown in Fig. 8. Addition of 40 nM FCCP to the sample before succinate energization reduced the quenching initial rate from 0.180 to 0.145 ($\Delta F/F_i$) s^{-1} mg protein^{-1}, as calculated on the basis of the exponential best fitting analysis. These figures support the view that the method described is strongly associated with $\Delta\psi$ changes due to proton transport through the mitochondrial inner membrane, because oligomycin and FCCP, having opposite effects on the electrochemical transmembrane potential, resulted in a significant enhancement (+80%) and decrease (−20%) of the initial rate of rhodamine quenching formation, respectively; a finding that confirms the specificity of this method.

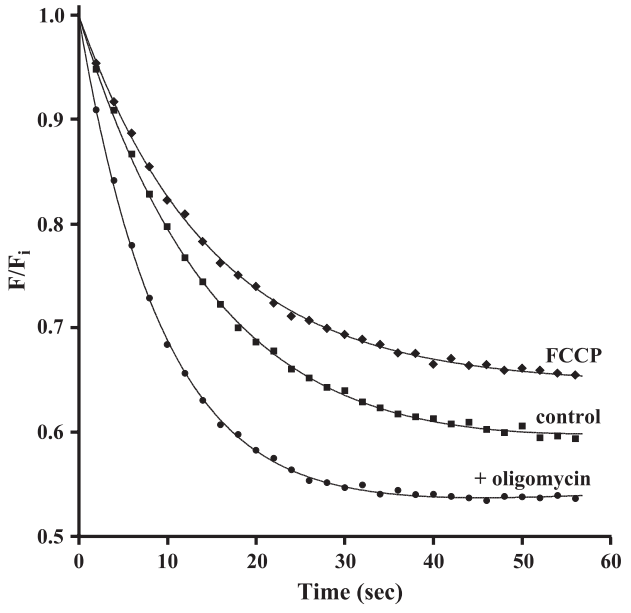

Fig. 8. Effect of both the uncoupler FCCP and the ATP synthase inhibitor oligomycin on the RH-123 fluorescence quenching rate. Normalised fluorescence decays obtained by experimental quenching, as monitored after succinate energization of 0.3 mg/ml mitochondria suspended in the respiratory buffer (pH 7.4) containing 33 nM cyclosporin A, 1 μg/ml rotenone, 100 μM ADP, 50 nM RH-123, and where indicated 40 nM FCCP or 0.2 μM oligomycin. Experimental data as normalised on the initial fluorescence value (F_i) were superimposed on the curves. The initial rate of quenching in the presence of FCCP was nearly 20% decreased compared to control, whereas oligomycin enhanced the rate about 80% with respect to control.

4. Discussion

The use of RH-123 and similar probes for measuring mitochondrial membrane potential ($\Delta\psi_{mit}$), both in intact cells by microscopic and cytofluorometric methods and in isolated mitochondria by standard spectrofluorometric methods, is of great interest because $\Delta\psi_{mit}$ represents a sensitive parameter of the effectiveness of the mitochondrial bioenergetic function. The measure in intact cells is based on the fluorescence enhancement due to uptake of the probe in the cell and hence in the mitochondria, whereas, in isolated organelles, the potential-dependent uptake induces fluorescence quenching. The reason for the quenching is probably in the aggregation or stacking of the dye after accumulation [6,10,11,33] so that total fluorescence in the assay is decreased. Incidentally, the fact that dye accumulation induces fluorescence quenching suggests that the fluorescence enhancement observed in the cytofluorometric determinations must be less than expected by the potential-dependent accumulation.

The determination of $\Delta\psi_{mit}$ by means of the above probes, both in cells and in isolated mitochondria, is commonly achieved by steady-state fluorescence measurements after equilibration of the probe in the mitochondrial compartment. Because the uptake of the cationic probe is proportional to the driving force, this study aimed to demonstrate whether the kinetics of uptake could be exploited as an indication of $\Delta\psi_{mit}$ itself. The study clearly shows that the kinetic method applied to isolated rat liver mitochondria is endowed with better sensitivity and reproducibility with respect to the static fluorescence quenching measurements. The slightly higher reliability of the kinetic method could be due to the fact that the $\Delta\psi$-independent component of the quenching, presumably due to unspecific binding of the probe to mitochondrial components, more strongly affects the static, rather than the dynamic, measurements.

Consistent with previous reports [20–22], RH-123 caused specific inhibition of ADP-stimulated respiration and ATP synthesis, and it could induce mitochondrial swelling [10]. However, our experiments could be performed at concentrations of rhodamine one [10,11] or two [34] orders of magnitude lower than those previously reported, and no evidence of coupled/uncoupled mitochondrial function inhibition under our conditions could be observed. Rather, clear-cut effects of either inhibitors (oligomycin, DCCD) of F_1F_0-ATPase activities or uncouplers (FCCP) on $\Delta\psi_{mit}$, were shown in the present study.

The only previous dynamic method for evaluation of the energetic state of mitochondria was exploited in cells by cytometry [35]. The authors measured the initial velocity of accumulation of RH-123 in mitochondria rather than the end-point of fluorescence change; they pointed out that the

dynamic method, being more rapid, reduces the probability of observing adverse effects due to probe concentration and long incubation times of the cells with the dye.

The sensitivity of the kinetic method described in our study allowed to clearly observe relatively small $\Delta\psi_{mit}$ changes: the transition from state 4 to state 3 respiration (addition of ADP) determines a potential decrease of 22–26 mV [10,27]; in the presence of oligomycin, state 4 $\Delta\psi_{mit}$ significantly increases by 5–14 mV, as reported by Scott and Nicholls [36] and Dykens and Stout [3]. The kinetic behaviour of the RH-123 fluorescence quenching here reported was in line with the referred changes.

When we treated mitochondria with the protonophore FCCP, which dissipates the H^+ gradient across the inner mitochondrial membrane, or with the F_1F_0-ATPase/H^+-pump inhibitor oligomycin (or DCCD), which prevents transport of H^+ back into the matrix from the intermembrane space, we found that the quenching rate decreased or increased, respectively. This finding clearly indicates the specificity of the method. Therefore, the effects of subsaturating concentrations of oligomycin, DCCD and FCCP on the initial velocity of RH-123 fluorescence quenching allows us to state that (i) the method is sensitive also to small changes of mitochondrial membrane potential, (ii) the method is able to detect even minute alterations of proton flux through ATP synthase, and (iii) the method can be used to quantitatively evaluate the contribution of F_0 proton transport to $\Delta\psi_{mit}$ formation (or dissipation).

This study strongly supports the use of RH-123 as a sensitive and reliable probe of membrane potential in isolated mitochondria because the method is simple and direct, and can be employed using a standard fluorometer. However, optimal conditions of assay have to be found and used. In fact, several factors must be taken into account: the mitochondrial preparations must be optimal and freshly prepared, and the protein to probe concentration ratio must be chosen and kept within small ranges in any experiment, because an abrupt change in the protein concentration will affect the distribution ratio of the probe across the membrane, and hence the observed fluorescence changes, as first observed by Zanotti and Azzone [6] using safranine as a probe, and subsequently discussed by Scaduto and Grotyohann [11], who used several fluorophores, including RH-123.

The method here presented has two advantages over the method based on the measurements of steady-state RH-123 fluorescence, described earlier [10], particularly when comparing $\Delta\psi_{mit}$ of different mitochondrial preparations: it is well reproducible and it is more sensitive, as shown in Fig. 3. However, it has to be noticed that the latter has a great advantage if one has to evaluate the action of effectors on $\Delta\psi_{mit}$ of a given mitochondrial preparation, because $\Delta\psi_{mit}$ can be recorded continuously on the same sample. Therefore, the two methods can complement each other and allow the use of RH-123 to provide information on $\Delta\psi_{mit}$ both in the same mitochondrial sample and in samples from different preparations. This is necessary when the behaviour of $\Delta\psi_{mit}$ in mitochondria isolated from tissues of patients and controls has to be monitored. Of course, applying a method based on measurements of fluorescence (both static and dynamic) requires that one is aware of the effects residual $\Delta\psi_{mit}$ of that particular mitochondrial preparation has on those measurements. Moreover, when applying the kinetic method here presented, one has also to verify whether under the experimental conditions chosen, mitochondrial substrate transport affects the measurements.

However, considering the above described advantages, the kinetic method may be used to reveal $\Delta\psi_{mit}$ decrease in most models of apoptosis; therefore, it might provide information on the early events of changes of the mitochondrial inner membrane permeability [37,38] that usually precedes the release of caspase and nuclease activating proteins, playing a major role in the apoptotic process [39].

Moreover, and this is a novelty, our method can specifically be employed to show dysfunction of proton pumping capacity by either the respiratory complexes and the ATP synthase, and this is one of the studies we are at present carrying out (manuscript in preparation). The exploitation of the method, therefore, might be particularly useful in the field of mitochondrial pathologies, to establish whether the cell energy defect should be ascribable to alteration of the protonophoric activity of the enzymes involved in oxidative phosphorylation. In particular, considering the objective difficulties existing in the study of proton movements from the intermembrane space to the matrix across the ATP synthase complex, our method might allow to shed light on the pathogenesis of the NARP syndrome (neuropathy, ataxia and retinitis pigmentosa), caused by a point mutation at nucleotide 8993 of mitochondrial DNA and affecting the ATPase-6 gene; a debated aspect of the above pathology is whether the observed fall of ATP synthesis is an expression of decoupling of the ATP synthase enzyme from the membrane potential or, as proposed by many authors, it is the consequence of a block of proton transport across the F_0 channel [40–42].

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