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Chloromethyltetramethylrosamine (Mitotracker Orange™) Induces the Mitochondrial Permeability Transition and Inhibits Respiratory Complex I

IMPLICATIONS FOR THE MECHANISM OF CYTOCHROME *c* RELEASE*

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We have investigated the interactions with isolated mitochondria and intact cells of chloromethyltetramethylrosamine (CMTMRos), a probe (Mitotracker Orange™) that is increasingly used to monitor the mitochondrial membrane potential ($\Delta\psi_m$) *in situ*. CMTMRos binds to isolated mitochondria and undergoes a large fluorescence quenching. Most of the binding is energy-independent and can be substantially reduced by sulfhydryl reagents. A smaller fraction of the probe is able to redistribute across the inner membrane in response to a membrane potential, with further fluorescence quenching. Within minutes, however, this energy-dependent fluorescence quenching spontaneously reverts to the same level obtained by treating mitochondria with the uncoupler carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone. We show that this event depends on inhibition of the mitochondrial respiratory chain at complex I and on induction of the permeability transition pore by CMTMRos, with concomitant depolarization, swelling, and release of cytochrome *c*. After staining cells with CMTMRos, depolarization of mitochondria *in situ* with protonophores is accompanied by changes of CMTMRos fluorescence that range between small and undetectable, depending on the probe concentration. A lasting decrease of cellular CMTMRos fluorescence associated with mitochondria only results from treatment with thiol reagents, suggesting that CMTMRos binding to mitochondria in living cells largely occurs at SH groups via the probe chloromethyl moiety irrespective of the magnitude of $\Delta\psi_m$. Induction of the permeability transition precludes the use of CMTMRos as a reliable probe of $\Delta\psi_m$ *in situ* and demands a reassessment of the conclusion that cytochrome *c* release can occur without membrane depolarization and/or onset of the permeability transition.

The role of mitochondria in apoptosis has been related to the release of two intermembrane proteins, cytochrome *c* and apoptosis-inducing factor. In the presence of μM dATP or mM ATP, cytochrome *c* is able to activate procaspase 9, followed by activation of procaspase 3 (1, 2). Apoptosis-inducing factor is a protease acting through proteolytic activation of a nuclear endonuclease (3). Cloning has recently revealed that apoptosis-inducing factor is a flavoprotein with predicted mass of 57 kDa that displays a striking homology with bacterial ferredoxin and NADH oxidoreductases (4).

Activation of the apoptotic program by these mitochondrial proteins requires their release into the cytosol, and the mechanism(s) by which this occurs is the subject of intense investigation (see Refs. 5–7 for reviews). One of the most studied targets is the mitochondrial PT,[1] a sudden increase of the inner membrane permeability to solutes that has been extensively studied for at least 40 years (see Refs. 8–11 for reviews). It is generally agreed that the PT is due to opening of a high conductance channel, MTP, which is activated by matrix Ca^{2+} and inhibited by CsA. The MTP possesses regulatory sites that accommodate many mediators of cell death, and its opening *in vitro* is accompanied by depolarization and matrix swelling. A specific question is whether or not a mitochondrial PT is a requisite for the release of apoptogenic proteins, an issue that has generated conflicting results (2–4, 12–19).

Mitochondrial membrane potential difference ($\Delta\psi_m$) in single cells is routinely measured using cationic fluorescent probes in conjunction with microscopy techniques. One of the properties of the ideal probe is minimal or absent passive binding to mitochondrial or other intracellular membranes, which allows its rapid response to changes of $\Delta\psi_m$ (see Ref. 20 for a thorough discussion). However, studies of mitochondrial involvement in cell death often demand the assessment of a series of parameters that may require cell fixation, including assessment of cytochrome *c* release with *in situ* methods based on immunodetection. This is obviously not compatible with the maintenance of $\Delta\psi_m$, because fixation leads to immediate depolarization with release of the $\Delta\psi_m$ probes previously accumulated by energized mitochondria. To circumvent this problem, Kroemer and co-workers (21) introduced the use of chloromethyl derivatives of the fluorescent cationic rosamine probes (Mitotracker™). The rationale is that the positively charged probe will be accumulated by energized mitochondria in response to $\Delta\psi_m$, followed by binding to mitochondrial SH groups via the probe chloromethyl moiety. At this point, the covalently bound probe should not be released despite de-energization and therefore stably "mark" the membrane potential existing prior to disruption of membrane integrity. Macho *et al.* (21) indeed showed

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[1] The abbreviations used are: PT, permeability transition; CMTMRos, chloromethyltetramethyl rosamine; MTP, mitochondrial permeability transition pore; PhAsO, phenylarsine oxide; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone; TMRM, tetramethylrhodamine methyl ester; MOPS, 4-morpholinepropanesulfonic acid; NEM, *N*-ethylmaleimide.

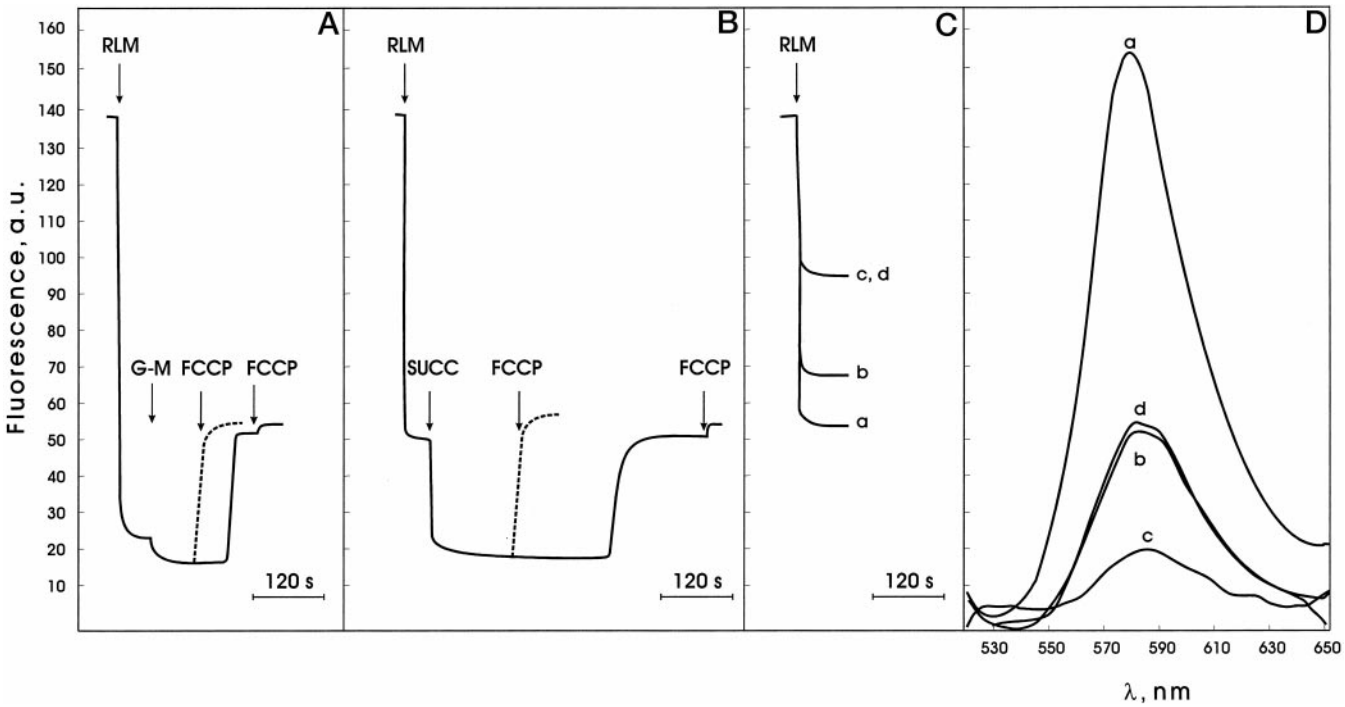

FIG. 1. **Effects of mitochondria on CMTMRos fluorescence.** The incubation medium contained 150 mM KCl, 10 mM Tris-MOPS, 1 mM P_i-Tris, 10 μM EGTA-Tris, 2 μM CMTMRos, 2 μM rotenone (*B* and *D*). The final volume was 2 ml at pH 7.4 and 25 °C. Where indicated (*arrow*), 1 mg of mitochondria (*RLM*) was added (*A–C*). Further additions were as follows: *A*, 5 mM glutamate-Tris plus 2.5 malate-Tris (*G-M*) and 0.4 μM FCCP; *B*, 5 mM succinate-Tris (*SUCC*) and 0.4 μM FCCP. In *C*, mitochondria were pretreated with FCCP alone (*trace a*) or with FCCP plus NEM (*trace b*), PhAsO (*trace c*), or both NEM and PhAsO (*trace d*) as described under "Materials and Methods." *D*, emission spectra of CMTMRos in solution prior to the addition of mitochondria (*trace a*) and in the supernatants obtained after incubation with 0.5 mg × ml^{-1} of mitochondria in the absence of substrate (*trace b*), in the presence of 5 mM succinate-Tris alone (*trace c*), or followed by 0.4 μM FCCP (*trace d*). Mitochondria were separated by centrifugation at 13,000 × *g* for 5 min after an overall incubation time of 2 min.

that accumulation of one of the Mitotracker dyes by thymocytes is somewhat reduced by the uncoupler FCCP. This study represented the basis for the widespread use of Mitotrackers (including those that have never been tested in mitochondria like CMTMRos) as probes of $\Delta\psi_m$ in the course of apoptosis (16).

Because no reports were available on the interactions of CMTMRos with isolated mitochondria, we have carried out a detailed characterization of its effects on basic mitochondrial functions. We show that CMTMRos inhibits the mitochondrial respiratory chain at complex I and that it induces opening of the MTP with concomitant depolarization, swelling, and release of cytochrome *c*. Depolarization of mitochondria *in situ* with protonophores is accompanied by small or negligible changes of CMTMRos fluorescence, depending on the probe concentration, whereas large and lasting fluorescence changes only result from treatment with specific thiol reagents that also decrease probe binding to isolated mitochondria. These results demonstrate that CMTMRos is not a reliable $\Delta\psi_m$ probe and demand a reassessment of the evidence that cytochrome *c* release can occur without membrane depolarization and/or onset of the PT, which has largely been based on the lack of CMTMRos fluorescence changes in the course of apoptosis (15, 16, 19).

MATERIALS AND METHODS

Liver mitochondria were isolated from Albino Wistar rats weighing about 300 g by standard centrifugation techniques, as described previously (22). Mitochondrial absorbance changes were followed at 620 nm with a Perkin-Elmer Lambda-10 spectrophotometer, and spectrofluorometric measurements were performed with a Perkin-Elmer LS50-B or a Hitachi-Perkin-Elmer 650–40 spectrofluorometer. Oxygen consumption was determined polarographically using a Clark-type oxygen electrode. All assays were performed at 25 °C in instruments equipped with thermostatic control and magnetic stirring.

In the experiments of Fig. 1*C*, mitochondria (25 mg × ml^{-1}) in 0.2 M sucrose, 10 mM Tris-MOPS, pH 7.4, 0.5 mM EGTA-Tris were treated with 400 pmol × mg $protein^{-1}$ FCCP. Identical aliquots were then supplemented with 1 μmol × mg $protein^{-1}$ NEM, 100 nmol × mg $protein^{-1}$ PhAsO, or 1 μmol × mg $protein^{-1}$ NEM plus 100 nmol × mg $protein^{-1}$ PhAsO, and incubations were continued for 1 h on ice. The emission spectra of CMTMRos (see Fig. 1*D*) were collected at λ_{exc} = 505 nm, with excitation and emission slits set at 2.5 nm. Other measurements of CMTMRos fluorescence were performed at λ_{exc} = 525 nm and λ_{em} = 576 nm with excitation and emission slits of 2 and 5 nm, respectively.

Mitochondrial membrane potential was estimated on the Nernst equilibration of rhodamine 123, assuming a matrix volume of 1 μl × mg $protein^{-1}$. Mitochondrial rhodamine 123 uptake was determined under the specified conditions from the fluorescence changes of mitochondrial suspensions containing 0.7 μM rhodamine 123. Because of the presence of CMTMRos during these measurements, variations of rhodamine 123 fluorescence were monitored at the wavelength pair 465–525, with slits set at 2 and 5 nm, respectively. These conditions allowed minimization of the spectral interference between the two probes but could not completely prevent a fluorescence decrease upon the addition of CMTMRos. This abrupt fluorescence change, which was $\Delta\psi_m$-independent and occurred also in fully de-energized mitochondria, was subtracted in the $\Delta\psi_m$ calculations shown in Fig. 4.

MH1C1 rat hepatoma cells were seeded onto uncoated 22-mm-diameter round glass coverslips and grown for 2 days in Ham's F-10 nutrient mixture supplemented with 20% fetal calf serum, in a humidified atmosphere of 95% air 5% CO_2 at 37 °C in a Forma tissue culture water-jacketed incubator. MH1C1 cells grown on each coverslip were then loaded for 45 min at 37 °C with the indicated concentration of the specified probe dissolved in 0.5 ml of Dulbecco's phosphate-buffered saline (Sigma D6650) without penicillin and maintained in the same medium after loading.

Cell fluorescence images were acquired with an Olympus IMT-2 inverted microscope, which was equipped for epifluorescent illumination and included a xenon light source (75 W), a 12-bit digital cooled CCD camera (Micromax, Princeton Instruments), and excitation-emission wavelength filter settings at bandpass 545 ± 25 nm to 590 nm longpass. Images were collected with exposure times ranging between 80 and 100 ms using a 40×, 1.3 NA oil immersion objective (Nikon).

Data were acquired and analyzed using Metamorph software (Universal Imaging). Clusters of several mitochondria (10–30 mitochondria) were identified as regions of interest, whereas nuclear regions were identified as cellular areas without appreciable mitochondrial fluorescence. Background was taken from fields not containing cells. Sequential digital images were acquired every 30 s for 10–20 min, and the average fluorescence intensity of all the regions of interest, of the nuclear regions, and of the background was recorded and stored for subsequent analysis. Mitochondrial fluorescence intensities minus background are reported in Fig. 5 after normalization of the initial fluorescence for comparative purposes, and they represent the mean of 10 regions of interest from three different sets of cells.

CMTMRos and rhodamine 123 were purchased from Molecular Probes (Eugene, OR) and dissolved in Me_2SO. All other chemicals were purchased from Sigma and were of the highest available grade. Cyclosporin A was a generous gift from Novartis (Basel, CH).

RESULTS AND DISCUSSION

Interactions of CMTMRos with Isolated Mitochondria—CMTMRos is a probe that stains mitochondria *in situ*. Because it carries a net positive charge, it can be accumulated by energized mitochondria, which indeed are brightly colored by CMTMRos in living cells in culture (see Fig. 5). Through its chloromethyl moiety, on the other hand, CMTMRos can form covalent bonds with SH groups in proteins, which is the basis for its being "fixable." Because a reliable probe must be able to redistribute depending on $\Delta\psi_m$, we studied the interactions of CMTMRos with isolated mitochondria under different metabolic conditions.

The experiments of Fig. 1 report the kinetics of CMTMRos fluorescence quenching following addition of mitochondria and of different respiratory substrates (preliminary experiments indicated that fluorescence quenching is not accompanied by shifts in the emission spectrum of CMTMRos; results not shown). In the absence of rotenone, the addition of mitochondria caused extensive fluorescence quenching that could only be marginally enhanced by addition of the respiratory substrates glutamate and malate (Fig. 1*A*). Shortly after the addition of mitochondria, however, the fluorescence signal increased spontaneously to a level that could only be marginally modified by FCCP (this sequence of events was also observed in the absence of added substrates; results not shown). Addition of mitochondria in the presence of rotenone was followed by a smaller fluorescence quenching, which was enhanced by energization with succinate (Fig. 1*B*). Also in this case, after a few minutes the fluorescence signal spontaneously returned to the level preceding the addition of substrate and could not be further modified by FCCP. This same level of fluorescence was obtained upon the addition of mitochondria treated with FCCP (Fig. 1*C*, *trace a*) or by adding FCCP prior to the spontaneous fluorescence increase (Fig. 1, *dashed traces* in *A* and *B*). Finally, the CMTMRos fluorescence quenching observed upon the addition of mitochondria in the presence of FCCP (Fig. 1*C*, *trace a*) could be reduced by pretreatment with NEM (Fig. 1C, *trace b*) or with the hydrophobic dithiol reagent PhAsO (Fig. 1C, *trace c*), no additive effects being observed with the two compounds together (Fig. 1*C*, *trace d*).

To study the relationship between fluorescence quenching and binding of CMTMRos to mitochondria, we incubated a solution of 2 μM CMTMRos with mitochondria that had been (i) de-energized with rotenone, (ii) energized with succinate, or (iii) energized with succinate, followed by de-energization with FCCP (*i.e.* conditions like those illustrated in Fig. 1*B* for the fluorescence-quenching experiments). Mitochondria were then separated by centrifugation, and the residual fluorescence of the supernatants was measured and compared with that of CMTMRos prior to the addition of mitochondria. Fig. 1*D* shows that rotenone-treated mitochondria bound a large fraction of CMTMRos (*trace b*, compare with *trace a*), that energization with succinate caused further CMTMRos binding (*trace c*, compare with *trace b*), and that treatment with FCCP released only the dye that had been accumulated upon energization (*trace d*, compare with *trace c*).

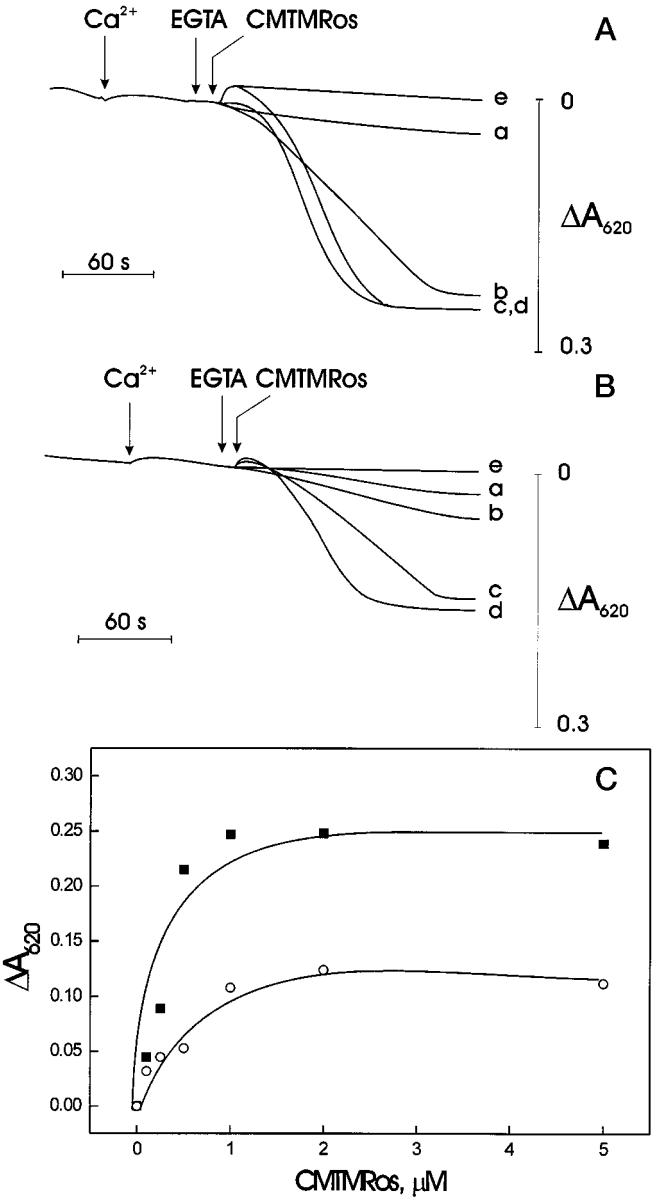

FIG. 2. **CMTMRos induces opening of the permeability transition pore in isolated mitochondria.** The incubation medium contained 150 mM KCl, 10 mM Tris-MOPS, 1 mM P_i-Tris, 10 μM EGTA-Tris, 0.25 μg × ml^{-1} oligomycin and 5 mM glutamate-Tris plus 2.5 mM malate-Tris (*A*) or 5 mM succinate plus 2 μM rotenone (*B*). The final volume was 2 ml at pH 7.4 and 25 °C. The experiments were started by the addition of 1 mg of mitochondria (not shown). In both panels, where indicated 30 μM Ca^{2+}, 0.5 mM EGTA-Tris and 0.25 μM (*traces a*), 0.5 μM (*traces b*), 2 μM (*traces c*), or 5 μM (*traces d* and *e*) CMTMRos were added. In the experiments of *traces e*, 2 μM CsA was present. The data in *C* were obtained from *A* (*squares*) or *B* (*circles*). Note that only selected traces have been reported in *A* and *B* for the sake of clarity.

These experiments indicate that CMTMRos fluorescence quenching by mitochondria is due to binding of the probe and that two components can be clearly identified: (i) one component is energy-independent, in that it is also observed in de-energized mitochondria (treatment with rotenone prior to the addition of succinate, Fig. 1*B*; pretreatment with FCCP, Fig. 1C); this component largely (but not exclusively) results from binding to SH groups, in that it can be significantly prevented

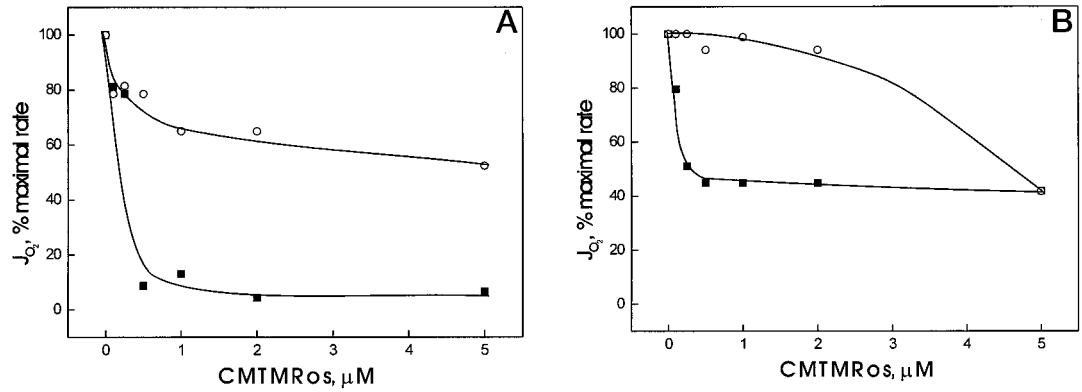

FIG. 3. **Effects of CMTMRos on uncoupled respiration in isolated mitochondria.** The incubation medium was the same as that in Fig. 2 (*A* and *B*). The experiments were started by the addition of 1 mg of mitochondria (final volume, 2 ml; pH 7.4; 25 °C) in the absence (*squares*) or presence (*circles*) of 2 μM CsA. After 1 min 30 μM Ca^{2+} was added, followed after 1 min by the indicated concentrations of CMTMRos and after 4 min by 0.2 μM FCCP. Values on the *ordinate* refer to the normalized rate of respiration following the addition of FCCP. Absolute values were 101 nanoatoms oxygen × mg $protein^{-1}$ × min^{-1} for respiration supported by glutamate plus malate (*A*) and 161 nanoatoms oxygen × mg $protein^{-1}$ × min^{-1} for respiration supported by succinate in the presence of rotenone (*B*).

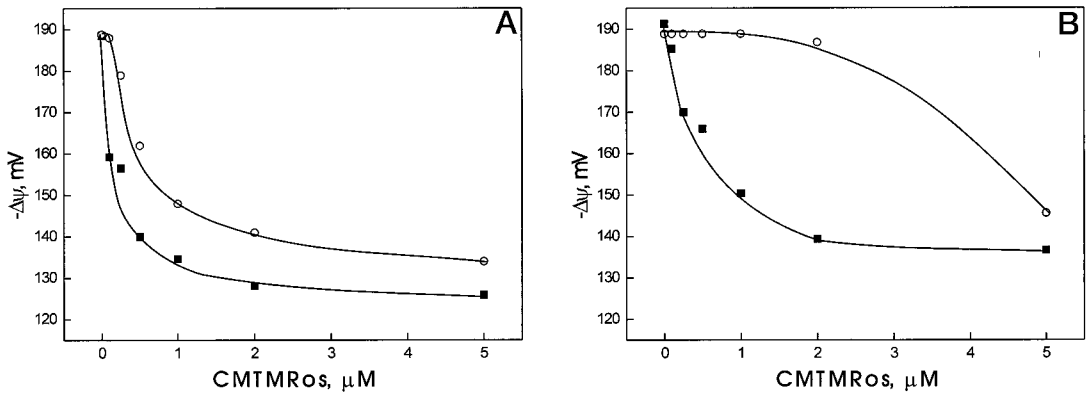

FIG. 4. **Effects of CMTMRos on the membrane potential in isolated mitochondria.** The incubation medium was the same as that in Fig. 2 (*A* and *B*), and it was supplemented with 0.7 μM rhodamine 123. The experiments were started by the addition of 1 mg of mitochondria (final volume 2 ml; pH 7.4; 25 °C) in the absence (*squares*) or in the presence (*circles*) of 2 μM CsA. After the rhodamine fluorescence signal had stabilized, 30 μM Ca^{2+} was added followed by the indicated concentrations of CMTMRos. Values on the *ordinate* refer to the $\Delta\psi_m$ calculated from the rhodamine 123 fluorescence changes induced by CMTMRos when a new steady state fluorescence reading had been achieved (see "Materials and Methods" for further details).

by NEM and PhAsO (Fig. 1C); (ii) one component is energy-dependent, in that it is observed only in mitochondria energized with endogenous substrates or with added glutamate plus malate (Fig. 1*A*) or with succinate (Fig. 1*B*); this component of CMTMRos quenching is readily reversed by de-energization with FCCP (*dashed traces* in Fig. 1, *A* and *B*), suggesting that it is measuring $\Delta\psi_m$. The major problem identified in these experiments is that within minutes of the energy-dependent accumulation of CMTMRos mitochondria undergo spontaneous release of the probe, suggesting that they have depolarized.

In principle, mitochondrial depolarization can be caused by inhibition of respiration, increased membrane permeability to protons (or charged species in general), or both. One of the most studied endogenous targets for permeability changes is the MTP, an inner membrane high conductance channel whose opening leads to collapse of the $\Delta\psi_m$ and to solute equilibration within the limits of the channel's exclusion size of approximately 1,500 Da (see Ref. 11 for a recent review). MTP opening can be conveniently studied based on the apparent absorbance change of mitochondria, which decreases when the pore opens and mitochondria swell (11). The experiments of Fig. 2 document the volume changes following the addition of CMTMRos to mitochondria energized with either glutamate plus malate (Fig. 2*A*) or succinate in the presence of rotenone (Fig. 2*B*). Following the accumulation of a small Ca^{2+} load that did not cause MTP opening *per se*, EGTA was added to prevent Ca^{2+} release via the uniporter (23), followed by increasing concentrations of CMTMRos. CMTMRos caused a decrease of absorbance (Fig. 2, *A* and *B*, *traces a–d*) that was fully prevented by CsA (Fig. 2, *A* and *B*, *traces e*) and therefore mediated by MTP opening. CMTMRos was a more effective inducer with glutamate and malate (Fig. 2*A* and *closed symbols* in Fig. 2*C*) than with succinate as the substrate (Fig. 2*B* and *open symbols* in Fig. 2*C*), suggesting specific interactions with respiratory complex I, which is a well characterized element of MTP modulation (24, 25). It is important to note that CMTMRos caused a PT at submicromolar concentrations, *i.e.* well within the range that is used in cellular studies.

We next assessed the effects of CMTMRos on mitochondrial electron transfer by measuring the rate of uncoupled (maximal) respiration at increasing CMTMRos concentrations. Depolarization favors opening of the MTP (26), which causes (i) depletion of pyridine nucleotides (with secondary inhibition of electron transfer from NAD^+-dependent substrates to complex I) and (ii) swelling with gradual cytochrome *c* depletion (with secondary inhibition of electron transfer between the bc_1 and aa_3 cytochromes). These measurements, illustrated in Fig. 3, were therefore performed both in the presence (*open symbols*) and absence (*closed symbols*) of CsA, to sort out the primary effects of CMTMRos on electron transfer from the secondary effects mediated by events caused by MTP opening. CMTMRos inhibited electron transfer at complex I (Fig. 3*A*, *open symbols*),

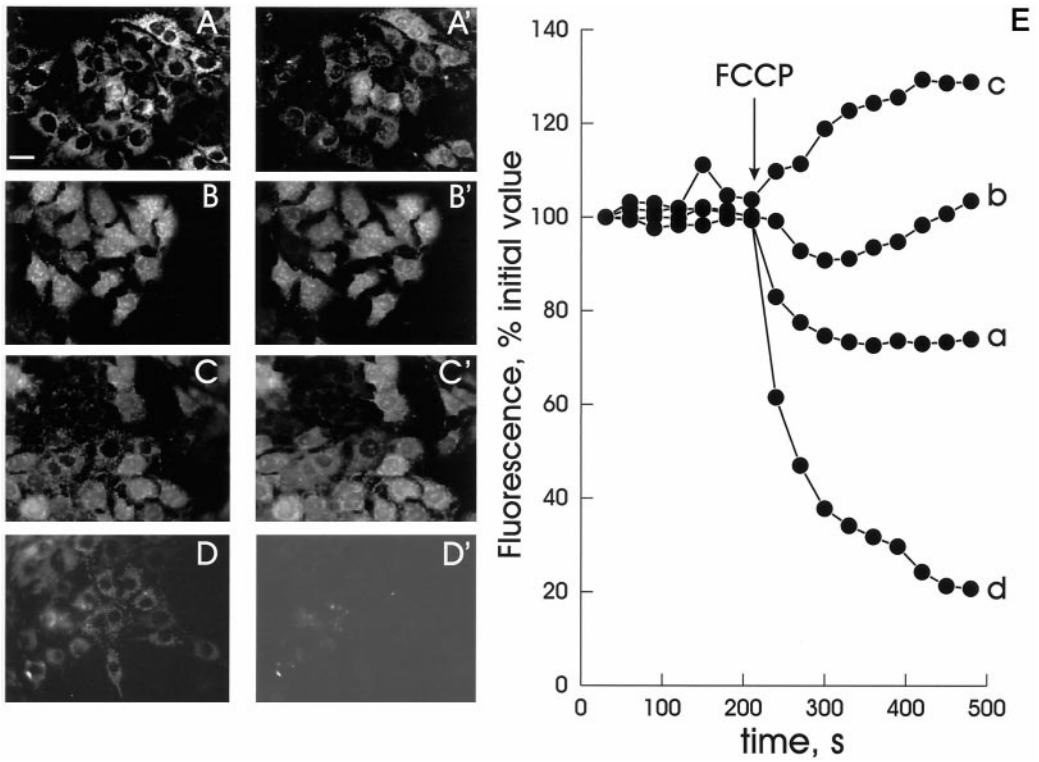

FIG. 5. **Fluorescence changes of CMTMRos and TMRM in living cells; effects of FCCP.** MH1C1 cells were loaded with 25 nM (*A*, *A′*, and *trace a*), 75 nM (*B*, *B′*, and *trace b*), or 150 nM (*C*, *C′*, and *trace c*) CMTMRos, or with 25 nM TMRM (*D*, *D′*, and *trace d*), and fluorescence images were collected at 30-s intervals. *A–D* show the images taken before the addition of FCCP, whereas *A′–D′* show the images of the same cells 5 min after the addition of 2 μM FCCP. *Bar*, 17 μm. *E* reports the changes of fluorescence intensity over mitochondrial regions of interest determined as described under "Materials and Methods." Where indicated (*arrow*), 2 μM FCCP was added.

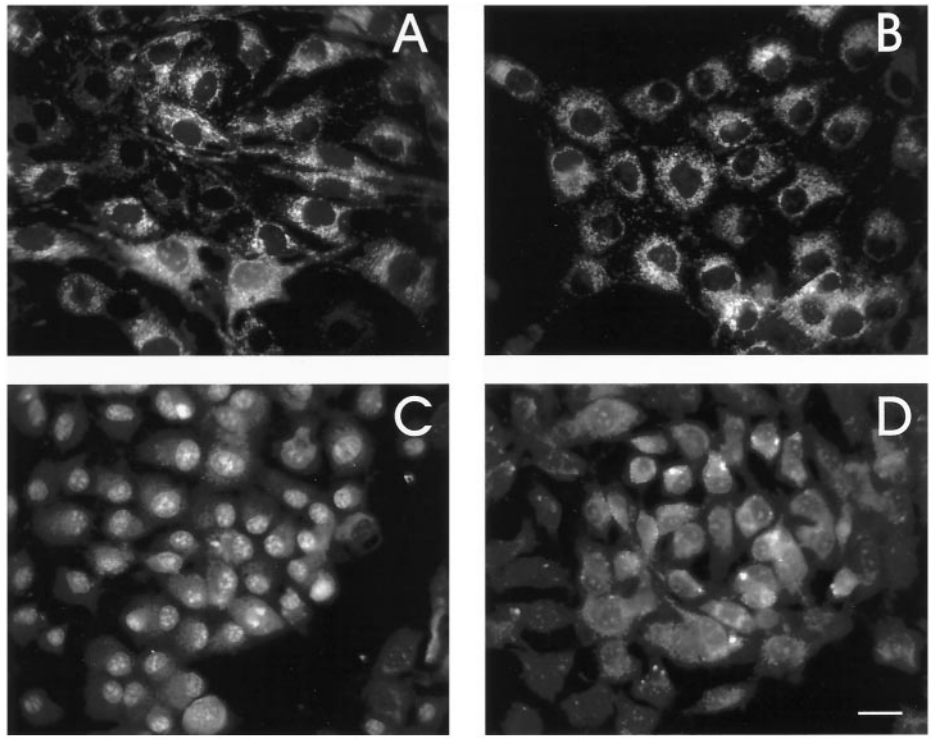

FIG. 6. **Effects of FCCP and of thiol reagents on CMTMRos fluorescence in living cells.** MH1C1 cells grown on coverslips were treated for 10 min with 0.01% (v/v) Me_2SO (*A*), 2 μM FCCP (*B*), 1 mM NEM (*C*), or 0.3 mM PhAsO (*D*) at 25 °C in Dulbecco's phosphate-buffered saline, washed twice with growth medium, and finally treated with 150 nM CMTMRos. Fluorescence images were then recorded. *Bar*, 21 μm. 0.01% Me_2SO was chosen for the control experiment because this was the highest concentration of solvent, which was reached in the experiment with PhAsO.

and respiratory inhibition was dramatically potentiated by omission of CsA (Fig. 3*A*, *closed symbols*). On the other hand, respiration through complexes II + III was much less affected by CMTMRos (Fig. 3*B*, *open symbols*), inhibition of respiration being observed only at 5 μM CMTMRos (Fig. 3*B*, *open symbols*), whereas the early inhibition observed in the absence of CsA (Fig. 3B, *closed symbols*) was due to MTP-dependent loss of cytochrome *c* and could be prevented by 10 μM added cytochrome *c* (results not shown). These effects of CMTMRos (inhibition of complex I and, at higher concentrations, of complexes II and III; induction of MTP opening) can easily explain the spontaneous increase of CMTMRos fluorescence observed

in Fig. 1 as being due to CMTMRos-dependent mitochondrial depolarization.

This issue was addressed directly in protocols where $\Delta\psi_m$ was measured with rhodamine 123 (a probe that displayed a tolerable overlap with the CMTMRos spectrum). Fig. 4 shows that in the absence of CsA submicromolar concentrations of CMTMRos caused a dramatic depolarization of mitochondria irrespective of whether complex I or complex II electron donors were used (Fig. 4, *A* and *B*, *closed symbols*), although depolarization was more effective in the former case. In the presence of CsA, depolarization was still observed with submicromolar concentrations of CMTMRos with glutamate plus malate as the substrates, whereas higher concentrations were needed with succinate. These experiments confirm that CMTMRos dramatically impairs maintenance of the $\Delta\psi_m$ through effects that can be explained by a combination of respiratory inhibition and MTP opening.

Interactions of CMTMRos with Intact Cells—CMTMRos is being increasingly used to monitor $\Delta\psi_m$ in living cells (15, 16, 19, 27). The problems posed by cellular measurements are different from those encountered in studies with isolated mitochondria. In studies with isolated mitochondria very high intramitochondrial concentrations are reached, which in turn result in extensive self-quenching of the dye fluorescence (Fig. 1). In principle, this can be exploited to estimate $\Delta\psi_m$, whose magnitude is indeed related to *quenching* of the total suspension fluorescence. Conversely, when mitochondria are studied with the same probe in cytofluorimetric or imaging protocols, the magnitude of $\Delta\psi_m$ is estimated from the *intensity* of the cell fluorescence. We have therefore studied the interactions of CMTMRos with living cells and assessed its response to depolarization and sulfhydryl reagents in imaging protocols where conditions were optimized for staining of mitochondria *in situ*.

In the experiments of Fig. 5, fluorescence changes were studied in cultures of MH1C1 cells grown on coverslips and loaded with CMTMRos concentrations ranging between 25 and 150 nM or with 25 nM TMRM, a widely used $\Delta\psi_m$ probe (28). In all cases the fluorescence displayed a punctate pattern typical of a mitochondrial distribution, which was stable with time. The addition of FCCP was followed by fluorescence changes that critically depended on the CMTMRos concentration. A small and stable fluorescence decrease was observed at 25 nM CMTMRos (Fig. 5, *A*, *A′*, and *trace a*), while essentially no change was detected at 75 nM (Fig. 5, *B*, *B′*, and *trace b*) and 150 nM CMTMRos (Fig. 5, *C*, *C′*, and *trace c*). Even under the most favorable conditions (25 nM CMTMRos), the fluorescence response to FCCP was a minimal fraction of that displayed by TMRM (Fig. 5, *D*, *D′*, and *trace d*).

The interaction of CMTMRos with mitochondria in intact cells was addressed further in the experiments of Fig. 6. MH1C1 cells were incubated with 150 nM CMTMRos (Fig. 6*A*). As expected, the punctate pattern of cellular fluorescence was not affected by pretreatment with FCCP (Fig. 6*B*), whereas pretreatment with NEM (Fig. 6*C*) or PhAsO (Fig. 6*D*) significantly changed the staining pattern. In both cases the cytoplasmic staining became diffuse, and nuclei became fluorescent with brigth nucleolar-type staining, an effect that was particularly prominent in the case of NEM (Fig. 6*C*). These results indicate that mitochondrially associated CMTMRos fluorescence displays a prevalent energy-independent component in intact cells as well and that mitochondrial binding largely occurs at SH groups.

Conclusions—In this paper we have shown that CMTMRos is a powerful inducer of the mitochondrial PT and a respiratory inhibitor that is particularly efficient with respiratory complex I. Once MTP opening has occurred, further respiratory inhibition takes place because of depletion of pyridine nucleotides and of cytochrome *c*. As a result of these effects, CMTMRos causes mitochondrial depolarization; yet most of the probe cannot be released because it binds in an energy-independent way. Rather than reporting changes of $\Delta\psi_m$, variations of CMTMRos fluorescence are more likely to reflect modification of mitochondrial and cellular SH groups. The major conclusion of the present study is therefore that CMTMRos cannot be used as a reliable $\Delta\psi_m$ probe in living cells, a consideration that has profound implications for the current debate on the role of mitochondria in cell death and in particular on the mechanism of cytochrome *c* release in apoptosis. Indeed, a PT caused by CMTMRos could have contributed to cause cytochrome *c* release *in situ*, and the ensuing depolarization may simply not have been detected (15, 16, 19) by the lack of fluorescence changes of the probe. It must also be stressed that CMTMRos still stains mitochondria in cells that have been treated with fully uncoupling concentrations of FCCP (Fig. 6*B*). Our results invalidate conclusions about the time sequence of events between mitochondrial depolarization and cytochrome *c* release that are based on the use of CMTMRos irrespective of whether the probe is added before or after the apoptotic stimulus.

Not all of the studies on the relationship between $\Delta\psi_m$ and cytochrome *c* release have employed CMTMRos, but we have reasons to think that the conclusions of many studies may not be as univocal as it has been assumed. For example, the widely used 3,3′-dihexyloxacarbocyanine iodide is an inhibitor of complex I, and at concentrations higher than 40 nM it may measure changes of the plasma membrane rather than of the mitochondrial membrane potential (20). Quite importantly, most studies have overlooked the fact that $\Delta\psi_m$ can easily be maintained by hydrolysis of glycolytic ATP by the mitochondrial F_1F_0 ATPase even when respiration has been completely inhibited by release of all of cytochrome *c*, a simple explanation of why a $\Delta\psi_m$ decrease is not detected by probes, including those that do not make covalent bonds (2, 12, 19). Finally, it must be stressed that apparent cytochrome *c* release can be caused by the techniques used to disrupt cells prior to organelle separation. It has been reported that despite respiratory inhibition, cytochrome *c* release could not be detected in the course of apoptosis induced by Fas ligation when Jurkat cells were disrupted by nitrogen cavitation rather than mechanical homogenization (29). It appears that an unequivocal assessment of the mechanism(s) of cytochrome *c* release and its relationships with $\Delta\psi_m$ will require further work.

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