Changes of the Fluidity of Mitochondrial Membranes Induced by the Permeability Transition†

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ABSTRACT: The dynamic properties of protein and lipid regions of mitochondrial membranes during the permeability transition (PT) process were studied by following the anisotropy changes of hematoporphyrin (HP) and 1,6-diphenyl-1,3,5-hexatriene (DPH), respectively. We show that opening of the PT pore is accompanied by a remarkable increase of mitochondrial membrane fluidity which is specifically localized to protein sites, while lipid domains are unaffected. The increased membrane fluidity is not related to the collapse of transmembrane potential that follows the PT, as demonstrated by a comparison between the anisotropy properties of permeabilized mitochondria and impermeable, depolarized organelles. Parameters such as osmotic swelling and temperature, which are shown to affect the mitochondrial membrane dynamics in the absence of permeability transition, cannot alone account for the pore dynamical properties. We suggest that the observed increase in fluidity is mainly due to a conformational change of pore-forming protein(s) during the "assembly" of the PT pore.

After being loaded with Ca^{2+}, mitochondria easily undergo a permeability increase (the "permeability transition", PT)[1] to ions and solutes with molecular masses of up to 1500 Da. This leads to the collapse of the proton electrochemical gradient, Ca^{2+} release, loss of matrix components, osmotic swelling, and hydrolysis of any available ATP by the ATPase complex. It is now generally accepted that the permeability transition is due to opening of a large conductance inner membrane pore (*1*, *2*). Many studies indicate that opening of the PT pore is a key event in a variety of toxic, hypoxic, and oxidative forms of cell injury as well as in apoptosis (*3*, *4*).

The mitochondrial PT pore is currently thought to exist due to the formation of dynamic multiprotein ensembles at inner−outer membrane contact sites. Although the molecular identity of the pore is only partially defined, current models point to at least two membrane proteins as putative components of the pore, the inner membrane AdNT and the outer membrane porin, in association with other proteins (*2*, *4*). The transition of the pore from the closed to the open conformation is expected to produce variations in the conformation not only of the pore-forming proteins (which must assemble in the proper way) but also in the dynamic properties of their boundary lipids. Here, we have investigated whether the permeability transition modifies the membrane fluidity, as probed by membrane labeling with fluorescent dyes. We have studied the steady-state fluorescence anisotropy of two probes, 1,6-diphenyl-1,3,5-hexatriene (DPH) and hematoporphyrin (HP). DPH is typically used to probe highly hydrophobic lipid phases of artificial and biological membranes (*5*), while HP mostly interacts with very polar, solvent-accessible regions of the lipid bilayer in liposomes (*6*, *7*) and with protein sites in biological membranes (*8*, *9*). In particular, HP preferentially accumulates in protein regions of the inner membrane in mitochondria (*8*). Interestingly, upon irradiation at 365 nm, HP selectively inactivates discrete pore functional domains which suggests an involvment of the HP-binding sites in the pore structure or regulation (*10*).

We show that, indeed, the mitochondrial permeability transition is accompanied by a remarkable change in the membrane fluidity, as probed by the anisotropy changes of labeled mitochondria. Parameters which influence the membrane fluidity properties such as the temperature as well as the collapse of the transmembrane potential and the osmotic swelling due to the permeability transition have been considered, and their influence on the fluorescence anisotropy of HP and DPH is discussed.

MATERIALS AND METHODS

Liver mitochondria from Wistar rats were prepared using procedures described previously (*11*), and the final pellet was suspended in 0.25 M sucrose to give a concentration of 80−100 mg/mL protein, determined by following the biuret method (*12*). Mitochondria (0.5 mg/mL) were incubated in

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[1] Abbreviations: AdNT, adenine nucleotide translocase; PT, permeability transition; HP, hematoporphyrin IX; DPH, 1,6-diphenyl-1,3,5-hexatriene; 12-AS, 12-(anthroyloxy)stearic acid; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; Pyr G, pyronin G.

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a sucrose-based medium containing 200 mM sucrose, 10 mM Tris-MOPS, 1 mM P_i, 10 μM EGTA-Tris, 2 μM rotenone, and 3 μg/mL oligomycin (pH 7.4), unless otherwise stated. Succinate (5 mM) was used as the energizing substrate.

Mitochondrial swelling was followed as the decrease in 90° light scattering of the mitochondrial suspension (0.5 mg/mL) at 540 nm, measured with a Perkin-Elmer LS 50 spectrophotofluorimeter equipped with a magnetic stirrer and thermostating control.

Determinations of the mitochondrial membrane potential were based on the changes in the intensity of pyronin G (Pyr G, 3 μM) fluorescence (excitation $\lambda = 520$ nm; emission $\lambda = 580$ nm) as described by Tomov (*13*).

The osmotic pressures of the solutions containing variable concentrations of sucrose (expressed as osmolality) were measured with a Vogel OM 801 vapor pressure osmometer.

Fluidity changes of mitochondrial membranes under different experimental conditions were evaluated by the changes of fluorescence excitation anisotropy (r) of mitochondria-bound dyes. For fluorescence labeling of mitochondria, the following probes were used: hematoporphyrin IX (HP), 1,6-diphenyl-1,3,5-hexatriene (DPH), and 12-(anthroyloxy)stearic acid (12-AS) which monitor the fluidity of different regions of lipid membranes. Stock solutions of the three probes were prepared in absolute ethanol. HP (final concentration of 3 μM) was injected into stirred mitochondrial suspensions (0.5 mg/mL) and the mixture incubated for 2 min before measuring anisotropy. Actually, HP incorporates very rapidly into mitochondria, as indicated by the strong enhancement of the fluorescence emission of the probe, the maximal uptake being achieved in less than 60 s (*8*). DPH and 12-AS require much longer incubation times (>1 h). Thus, to avoid mitochondrial aging, DPH- or 12-AS-labeled mitochondria were prepared by addition of 100 μL of ethanolic dye (2 mM) to each of the 50 mL centrifuge tubes used for the last centrifugation wash of mitochondria (4 mg/mL), according to the procedure described by O'Shea et al. (*14*). A short incubation period (~20 min) was allowed before mitochondria were finally centrifuged. Free probes in the bulk medium do not contribute to the fluorescence anisotropy since they are almost fluorimetrically silent in aqueous media.

Steady-state fluorescence anisotropy values were obtained by measurements of I_{VV} and I_{VH}, i.e., the fluorescence intensities polarized parallel and perpendicular to the vertical plane of polarization of the excitation beam, respectively. The fluorescence anisotropy r is defined by the equation $r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$, where G equals I_{HV}/I_{HH} and is the correction factor for instrumental artifacts.

All the experiments were performed at 25 °C, unless otherwise stated.

All chemicals were of the highest purity commercially available. HP was purchased from Porphyrin Products. DPH, 12-AS, cyclosporin A, carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone (FCCP), and Pyr G were products of Sigma. Incubation conditions and other experimental details are specified in the figure legends.

RESULTS AND DISCUSSION

We have studied the dynamic properties of mitochondrial membranes during the permeability transition process by following the changes in the steady-state fluorescence anisotropy of mitochondria-bound HP and DPH. Actually, the measurements of fluorescence depolarization provide a widespread means of obtaining information about the rotational diffusion freedom of the reporter molecules with respect to both the rate (dynamics) and the range or extent of the rotational motion. This latter reflects the molecular packing (structure) of the membrane components. The extent of depolarization of the exciting polarized light is a measure of the degree to which a population of photoselected molecules lose their original orientation during the lifetime of the excited state. Theoretically, the parameter r can change from a value of 0, in the case of a fully isotropic environment (e.g., in aqueous solvent), to a maximum of 0.4, in the case of frozen solutions where the motion cannot occur. In lipid membranes, however, the minimal anisotropy is a finite value because of the orientational constraints due to the macromolecular structure incorporating the fluorophore. Moreover, the upper limit of anisotropy is less than 0.4 for all the fluorophores studied so far, including HP (limiting $r = 0.3$; our unpublished results) and DPH (limiting $r = 0.362$; *5*). Fluorescence decay times (τ) of HP and DPH in lipid membranes are around 16 (*6*) and 11 ns (*5*), respectively, which provide an appropriate time window for studying the dynamics of membrane components (*5*).

Effect of Pore Opening on the Membrane Fluidity As Measured by Fluorescence Anisotropy of Labeled Mitochondria. In a typical experiment (Figure 1), mitochondria (0.5 mg/mL) energized with succinate (5 mM) in the presence of rotenone (2 μM) were suspended in the sucrose-based medium described above at 25 °C. The 90° light scattering of the mitochondrial suspension at 540 nm was continuously monitored (Figure 1A), and after 90 s, 150 μM $CaCl_2$ was added. Calcium accumulation induced mitochondrial permeabilization (curve a) which could be totally prevented (curve b) by the specific PT inhibitor cyclosporin A. In parallel experiments, it was ascertained that incubation of mitochondria with HP, DPH, or 12-AS did not affect the phenomenon and its kinetics (data not shown).

Figure 1B shows the effect of mitochondrial PT on the membrane fluidity, as measured by the fluorescence excitation anisotropy of 3 μM HP (excitation $\lambda = 520$ nm; emission $\lambda = 626$ nm) under the same experimental conditions used in Figure 1A. After calcium accumulation, pore opening induced a decrease in the anisotropy of the dye (curves a) which appears to parallel the light scattering change. The strict correlation between the decrease of HP anisotropy and the increase of permeability due to pore opening is demonstrated by the inhibitory effect of cyclosporin A (curves b). Opening of the PT pore by treatment with diethyl pyrocarbonate (a histidine reagent) or diamide (a thiol oxidant) (*2*, *15*) caused changes in HP anisotropy similar to those observed after induction of the PT pore by Ca^{2+} and P_i (data not shown).

In contrast to the results obtained with HP, no change in anisotropy was observed for DPH-labeled mitochondria during pore opening (data not shown). DPH molecules lie close to the very apolar region near the center of the bilayer. The distribution of this dye is heterogeneous, with a significant fraction of molecules lying with their long axis perpendicular to the preferred direction of the acyl chains (*5*). To ascertain whether the orientational order of the apolar dye in the membrane plays a role in probing changes of lipid

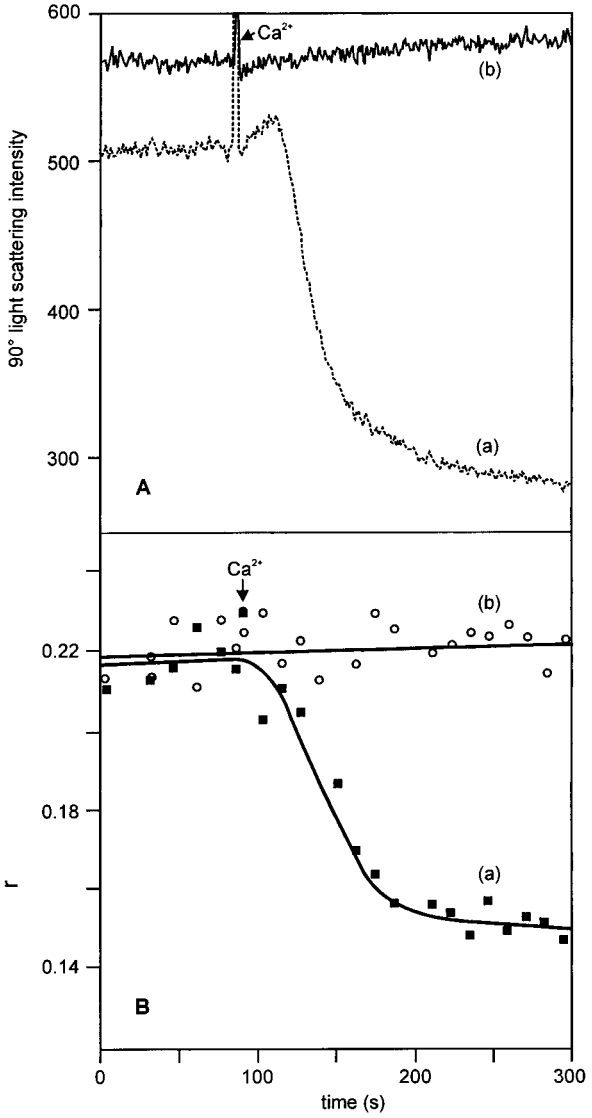

FIGURE 1: Kinetics of the mitochondrial permeability transition (A) and anisotropy change (B) of HP-labeled mitochondria. (A) Kinetics of the 90° light scattering decrease. One milligram of mitochondrial protein was incubated at 25 °C in 2 mL of a medium containing 200 mM sucrose, 10 mM Tris-MOPS, 10 μM EGTA-Tris, 1 mM P_i, 3 μg/mL oligomycin, 5 mM succinate, and 2 μM rotenone (pH 7.4). Swelling was initiated by the addition of 150 μM $CaCl_2$ (arrow) (curve a). In curve b, the initial medium was supplemented with 1 μM cyclosporin A. Light scattering intensities are expressed in arbitrary units. (B) Kinetics of the HP anisotropy change. The experimental conditions were as described for panel A except that 3 μM HP was added to the medium and allowed to incubate for 2 min with mitochondria before addition of Ca^{2+}. HP fluorescence excitation anisotropy (*r*) was monitored at 520 nm (emission $\lambda = 626$ nm).

domains during the permeability transition process, we repeated the experiments using 12-(anthroyloxy)stearic acid (12-AS) which is known to intercalate parallel to the phospholipid acyl chains (*5*). Also with 12-AS, however, no fluidity changes could be detected at the onset of the PT (data not shown).

Effect of Transmembrane Potential on the Membrane Fluidity. It is well-known that a change in the electric field across a biological membrane can change its dynamic behavior (*5*, *14*, *16*). In particular, changes of potential gradients across the membrane of phospholipid vesicles and living cells are accompanied by appreciable modifications

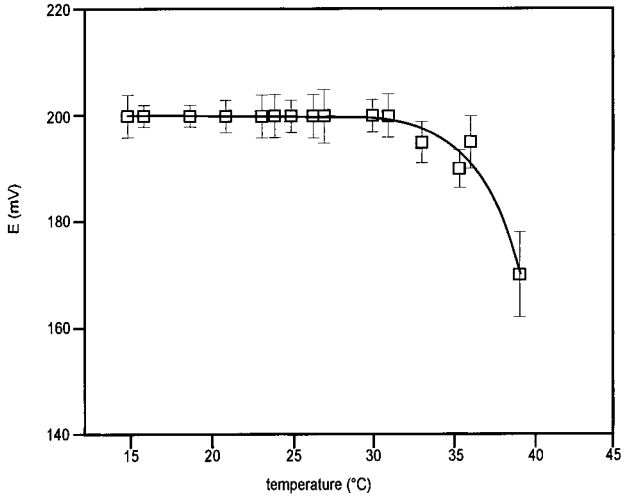

FIGURE 2: Mitochondrial transmembrane potential (E_{mV}) as a function of temperature. At each temperature, the fluorescence emission of a mitochondrial suspension (0.5 mg/mL) in the sucrose-based medium containing 3 μM Pyr G was determined at 580 nm (excitation $\lambda = 520$ nm). The fluorescence intensities of the dye were recorded before (mitochondria deenergized with 2 μM rotenone) and after (energized mitochondria) addition of 5 mM succinate. The ratio between the two fluorescence intensities was used to calculate the membrane potential by using the reference curves of Tomov (*13*). The data depicted in the figure are the average of three independent experiments. The vertical bars represent the range of experimental deviation.

in the lipid bilayer rigidity and thickness, in the conformation of membrane proteins, and in the accessibility of reactive chemical groups. Thus, the collapse of the transmembrane potential during pore opening could contribute to the changes of anisotropy we have observed (Figure 1B).

To magnify the expected changes due to the variation of the membrane potential, we compared the anisotropy values of HP-labeled mitochondria, in the energized state (with succinate and rotenone) and the depolarized state, obtained either in the absence of the energizing substrate (impermeable, nonrespiring mitochondria) or after inducing pore opening by Ca^{2+} loading (permeabilized mitochondria). Since the temperature is a known effector of membrane fluidity, the measurements were extended to a wide range of temperatures (15−38 °C) which includes physiological values for most mammalian cells. The range of temperatures investigated allows maintenance of a membrane potential, as verified by measurements with pyronin G according to the method of Tomov (*13*) (Figure 2).

In the absence of the permeability transition, HP-loaded mitochondria in the energized state exhibited similar anisotropy values up to about 25 °C, after which *r* decreased at increasing temperatures (Figure 3, curve a). Essentially the same results were obtained when the temperature was decreased from 38 to 15 °C (data not shown), indicating that the process is reversible. Deenergized membranes (curve b) exhibited a less pronounced dependence on temperature with values of *r* that were higher than those obtained for energized organelles over the range of temperatures being investigated. Although the decrease in *r* after energization of mitochondria appeared to be rather slight below 25 °C, the reproducibility of the data was achieved by measuring, at each temperature, the HP anisotropy on the same mitochondrial suspension before and after addition of succinate, in three sets of

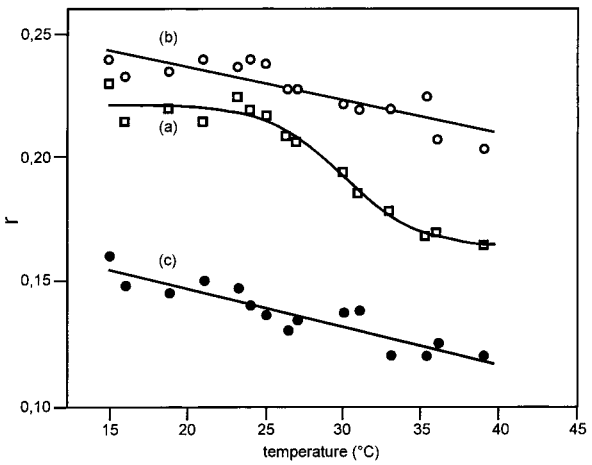

FIGURE 3: Anisotropy of HP-labeled mitochondria as a function of temperature. In curve a (□), mitochondria (0.5 mg/mL) suspended in the sucrose-based medium described in Figure 1 were energized with succinate (5 mM). In curve b (○), mitochondria were suspended in the sucrose-based medium without succinate. In curve c (●), energized mitochondria in the sucrose-based medium were allowed to undergo the permeability transition with 150 μM $CaCl_2$. Labeling with HP was as reported in the legend of Figure 1. HP anisotropy was excited at 520 nm (emission $\lambda = 626$ nm). The data depicted in the figure are the average of three independent experiments. The precision of the data (SD) is ±10%.

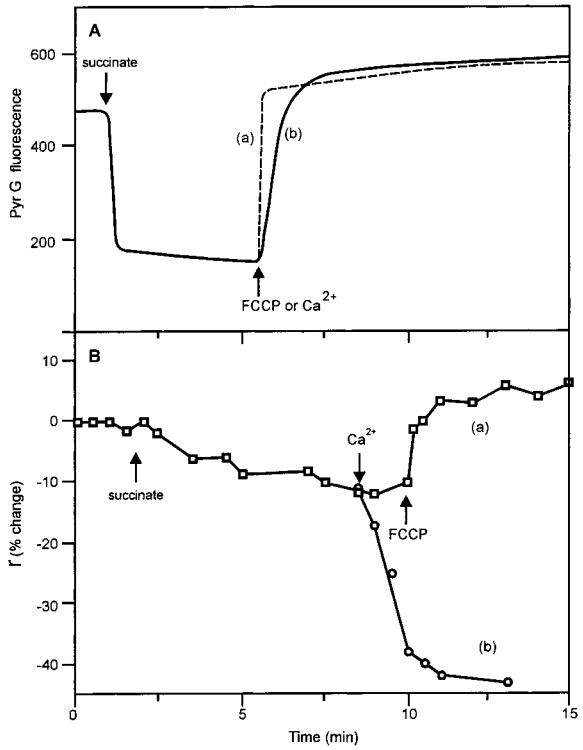

FIGURE 4: Dependence of PyrG fluorescence (A) and HP anisotropy (B) on the mitochondrial membrane potential and permeability transition. (A) Mitochondria (0.5 mg/mL), incubated with 3 μM Pyr G, were suspended in the sucrose-based medium in the absence of an energizing substrate. Pyr G fluorescence (in arbitrary units) was followed by exciting at 520 nm (emission $\lambda = 580$ nm). Where indicated (arrows), succinate (5 mM), FCCP (0.3 μM) (curve a), or $CaCl_2$ (150 μM) (curve b) was added. (B) The experimental conditions were the same as described for panel A except that mitochondria were incubated for 2 min with HP and the dye fluorescence anisotropy at 520 nm (emission $\lambda = 626$ nm) was followed. The data were reported as the percent change in the *r* of substrate-deprived mitochondria.

experiments. On the contrary, when mitochondrial membranes were depolarized by induction of the permeability transition (curve c), the *r* values were markedly lower than those monitored for energized organelles. This opposite tendency in the two cases of membrane depolarization is clearly strenghtened in the experiment whose results are depicted in Figure 4 where mitochondria were fully depolarized by the uncoupler FCCP. We used a concentration (0.3 μM) of FCCP which induced changes in the fluorescence of Pyr G (*13*) nearly coincident with that obtained after opening the PT pore (see Figure 4A). Then, as shown in Figure 4B for a typical experiment, we followed the change in anisotropy of the bound HP in the different energy states of the membrane. Energization by succinate of rotenone-treated mitochondria caused a ~10% decrease in *r* which was reverted by addition of FCCP. After the uncoupler had been supplied, the value of *r* exhibited by mitochondria was comparable to that exhibited by substrate-deprived organelles. The same result was obtained when FCCP was added directly to rotenone-deenergized mitochondria (not shown). On the contrary, full collapse of the membrane potential after the PT (obtained by addition of the inducer Ca^{2+}) led to a ~40% decrease in *r*. These experiments clearly indicate that the collapse of transmembrane potential is not a major factor in inducing changes of the HP mobility during the PT.

Measurements of anisotropy in the 15−38 °C range were also carried out for DPH-labeled mitochondria (Figure 5) with the aim of checking possible temperature-dependent fluidity changes of the lipid phase after pore opening. In the absence of the permeability transition, the temperature-dependent anisotropy trend of energized DPH-labeled mitochondria (curve a) was similar to that obtained for HP-labeled mitochondria. Again, deenergization of DPH-labeled organelles induced an increase in membrane rigidity (curve b). Upon induction of the permeability transition, the anisotropy−temperature curve (curve c) nearly coincided with that obtained for depolarized nonrespiring mitochondria which confirmed the insensitivity of lipid regions to fluidity changes during the PT even at high temperatures.

Experiments with 12-AS-labeled mitochondria did not reveal anisotropy changes at the onset of the PT until 38 °C (data not shown).

The experiments so far described suggest the following. (i) In the absence of the permeability transition, changes in transmembrane potential can modulate the dynamic properties of both protein regions (as probed by HP) and lipid domains (as probed by DPH) of mitochondrial membranes. Over the range of temperatures being studied, imposition of a potential gradient across the membrane results in a "looser" membrane structure. Our results are in agreement with those obtained by Lakos et al. (*17*) for the DPH-labeled cytoplasmic membrane of mouse thymus cells. In contrast, O'Shea et al. (*14*, *18*) found an increase in the lipid rigidity of mitochondrial membranes, as measured by DPH anisotropy, upon imposition of transient electric fields of various magnitudes. Preliminary results indicate that this discrepancy can be ascribed to a difference in the medium composition (work in progress). (ii) Induction of PT causes a substantial change in the membrane fluidity which cannot be attributed to the collapse of the transmembrane potential. Rather, mitochondrial membranes depolarized by pore opening are more "fluid" than polarized organelles, while an opposite

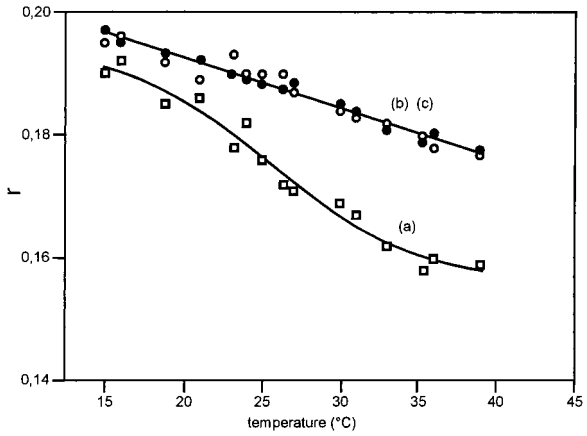

FIGURE 5: Anisotropy of DPH-labeled mitochondria as a function of temperature. In curve a (□), mitochondria (0.5 mg/mL) suspended in the sucrose-based medium described in Figure 1 were energized with succinate (5 mM). In curve b (○), mitochondria were suspended in the sucrose-based medium without succinate. In curve c (●), energized mitochondria in the sucrose-based medium were allowed to undergo the permeability transition with 150 μM $CaCl_2$. Labeling with DPH was as reported in Materials and Methods. DPH anisotropy was excited at 340 nm (emission $\lambda = 460$ nm). The data depicted in the figure are the average of three independent experiments. The precision of the data (SD) is ±10%.

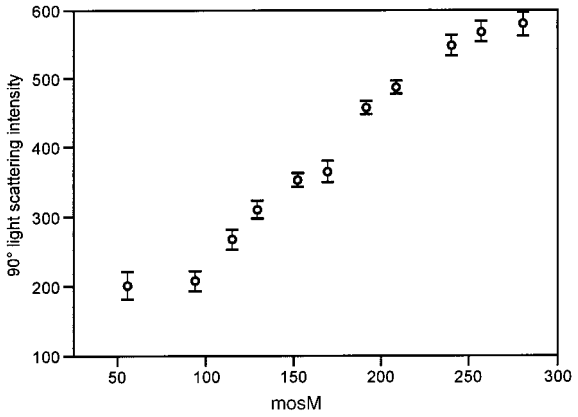

FIGURE 6: Mitochondrial swelling as a function of the medium osmolality. Mitochondria (0.5 mg/mL) labeled with 3 μM HP and energized with 5 mM succinate were suspended in a medium containing 10 mM Tris-MOPS, 10 μM EGTA-Tris, 1 mM P_i, 3 μg/mL oligomycin, 2 μM rotenone (pH 7.4), and different amounts of hypertonic sucrose, resulting in the indicated osmolalities. The 90° light scattering intensities (in arbitrary units) were monitored after incubation for 2 min. Each point is the average of at least three determinations. The vertical bars represent the range of experimental deviation.

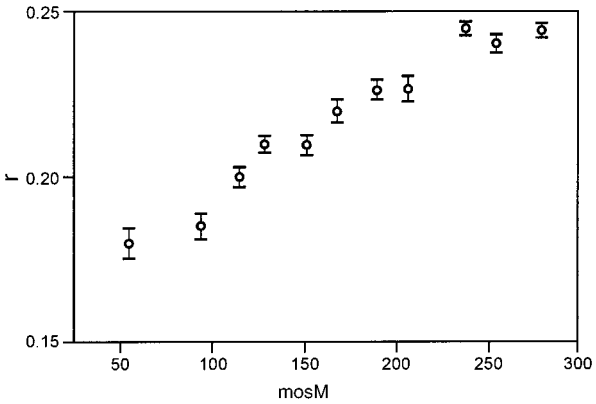

FIGURE 7: Anisotropy of HP-labeled mitochondria as a function of the medium osmolality. Experimental conditions were as described in the legend of Figure 6. HP anisotropy was excited at 520 nm (emission $\lambda = 626$ nm).

tendency is observed for depolarized membranes in nonrespiring mitochondria. (iii) The fluidity changes induced by the PT are confined to restricted areas of the mitochondrial membranes, which are not probed by DPH or 12-AS over a wide range (15−38 °C) of temperatures. Only protein regions which are typically probed by HP are affected. The specific inhibition by cyclosporin A of the HP fluidity changes induced by pore opening, observed in the temperature range of 15−38 °C (Figure 1B and data not shown), suggests that the HP-binding sites belong to the protein(s) involved in pore formation. This conclusion is in agreement with previous results on the inhibitory effects of irradiated HP on the mitochondrial PT pore (*10*). (iv) The anisotropy changes of HP during pore opening could be ascribed to a change in the fluorescence lifetime induced, for example, by the change in the dielectric constant of the medium. However, HP lifetime is almost unaffected by the nature or physical properties of the microenvironment, mainly depending on the dye aggregation state (*19*). Under all our experimental conditions, HP was present as a monomer ($\tau \sim 16$ ns) (*6*, *8*). In addition, the HP fluorescence quantum yield remained unchanged after pore opening (data not shown). Because of the well-known proportionality between quantum yield and τ, this fact supports the constancy of the lifetime after the PT.

Effect of Osmotic Swelling on Membrane Fluidity. In addition to the collapse of transmembrane potential, the swelling of the mitochondrial matrix during pore opening could play an important role in the observed increase in membrane fluidity. In general, the response of mitochondria to a decrease in osmotic pressure is a rearrangement of the inner membrane from the native, efficiently packaged to random structures (*20*) which implies a reorganization of the lipid bilayer as well, as demonstrated by the formation of kink structures in the acyl chains (*21*). In addition, upon induction of the permeability transition, mitochondria manifest a disruption of the outer membrane integrity which leads to the release of intermembrane proteins. The effects of swelling induced by changes in osmotic pressure on membrane fluidity have been compared to those induced by the permeability transition. For this purpose, HP-labeled mitochondria were suspended at 25 °C in a hypotonic medium (56 mosM), after which the osmolality was adjusted with sucrose to the values indicated in Figures 6 and 7. After incubation for 2 min, the 90° light scattering intensities (Figure 6) and the corresponding anisotropies (Figure 7) were monitored. The value of osmolality necessary to obtain a degree of swelling similar to that of permeabilized organelles was 125 mosM. This extent of swelling induced a decrease in anisotropy from ~0.225 (200 mosM medium) to ~0.21 (125 mosM medium) (Figure 7). Thus, the contribution of swelling to the total decrease in r (up to ~0.14, Figures 1B and 3) after opening of the PT pore is about 18%. During the permeabilization process, however, the decrease in anisotropy associated with swelling is almost fully counterbalanced by the increase associated with full depolarization (see Figures 3 and 4). In conclusion, a decrease in the osmotic pressure results in an increase in membrane fluidity. This

effect, however, cannot alone account for the drastic decrease in membrane rigidity observed at the onset of pore opening.

CONCLUSION

The transition from the impermeable to the permeabilized state of the inner mitochondrial membrane after opening of the PT pore is accompanied by a remarkable increase in mitochondrial membrane fluidity, which is localized to specific domains of the mitochondrial membranes, namely, protein regions which are typically probed by HP. On the other hand, very apolar lipid regions of mitochondrial membranes probed by DPH (or 12-AS) are not affected. The specificity of HP in sensing changes in membrane dynamics during pore opening can be attributed to a strategic location of the dye in protein sites of the inner mitochondrial membrane which appear to participate in pore formation.

Finally, the increase in fluidity is not correlated with parameters that generally affect the membrane dynamics such as transmembrane potential or temperature changes; furthermore, volume changes account for the phenomenon to a minor extent. We suggest that the fluidity increase is mostly secondary to a conformational change of pore-forming protein(s) during the "assembly" of the PT pore. If this is the case, the anisotropy changes of HP can provide a very effective tool for monitoring the pore during its formation.

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