Mitochondrial Contact Sites

LIPID COMPOSITION AND DYNAMICS*

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Two membrane fractions of intermediate density between inner and outer mitochondrial membranes were isolated by density gradient centrifugation from osmotically lysed mitochondria and mitoplasts of liver. These fractions were characterized by the presence of both monoamine oxidase and cytochrome c oxidase activities and bound hexokinase. 1) The content of the fractions in proteins and lipids was assessed by biochemical determination. Thin-layer and gas-liquid chromatography showed that the two contact site-enriched fractions contain predominantly phosphatidylcholine (81%), phosphatidylethanolamine (27%, half-saturated), and cardiolipin (27%, fully saturated). 2) The dynamics of the fractions were assessed by fluorescence polarization techniques using 1,6-di-phenyl-1,3,5-hexatriene as a probe and by fluorescence decay measurements. We have verified that differences in static anisotropy cannot be exclusively attributed to differences in fluorescence lifetimes. On the contrary, the results indicated an increased lipid mobility in "inner membrane contact sites," which is probably related to a lower cholesterol to phospholipid ratio, as well as a lower saturation of the fatty acyl chains when compared with "outer membrane contact sites." Taken all together, the spectroscopic measurements confirm the biochemical results, leading to the idea that the two populations of contact sites have different physicochemical properties, which are probably mainly determined by the membrane from which they are derived. They constitute microdomains enriched either in inner or outer mitochondrial membranes.

Contact sites between the two mitochondrial membranes were first described by Hackenbrock (1) in thin sections of liver mitochondria. They were further characterized in freeze-fractured mitochondria (2) and were found to increase during active oxidative phosphorylation (3, 4). This was taken as an indication that contact sites play an important role in the regulation of the mitochondrial metabolism. For example, in liver and brain mitochondria, hexokinase binds preferentially to outer membrane pores located in the contact sites (5, 6). These structures may therefore be of great importance for functional coupling of kinases to the inner compartment, and it can be expected that other kinases are located in these mitochondrial areas. This has been recently reported for the creatine kinase and the nucleoside diphosphate kinase also (7). In addition, the import of proteins and phospholipids from the cellular cytoplasm into mitochondria is expected to occur via regions where the outer and the inner membranes are very close to each other (8, 9). Contact sites between the two membranes are also involved in channeling metabolites and ions to the inner compartment (4) since the active Ca2+ uptake and Na+-dependent efflux of Ca2+ were impaired upon removal of porin-bound hexokinase (10).

Up to now, very limited information has been available on the nature of the lipids involved in the formation of mitochondrial contact sites (5). We recently reported the separation of two contact site-enriched fractions in liver mitochondria (11) differing in their cholesterol and dolichol contents. Since a semi-fusion model has already been proposed (2) in which nonblayer phospholipids may be involved in the formation of contacts between the mitochondrial membranes, the aim of the present study was to present a detailed analysis of the lipids of the mitochondrial contact sites and to search for a possible link between the lipid composition and dynamics of the contact sites. The results are discussed in terms of microheterogeneity and lipid microdomains in these submitochondrial compartments.

EXPERIMENTAL PROCEDURES

Chemicals—All chemicals were purchased from Sigma. Silica Sep-Pak columns were from Waters Associates (Milford, MA) and silica gel plates from Merck (Darmstadt, FRG). Fluorescent probes were all obtained from Molecular Probes Inc. (Eugene, OR).

Enzyme Assays—The determinations of monoamine oxidase (EC 1.4.3.4), cytochrome c oxidase (EC 1.9.3.1), NADPH cytochrome c reductase (EC 1.6.2.5), and hexokinase (EC 2.7.1.1) were carried out as previously reported (11).

Preparation of Mitochondria and Contact Sites—Mitochondria were isolated from mouse livers (11), and submitochondrial particles were prepared by a swelling procedure as follows. The purified mitochondria were exposed to a first swelling by incubation in 10 mM phosphate buffer (pH 7.4), with subsequent addition (after 20 min at 4 °C) of 61.5% sucrose in order to obtain a 0.45 M sucrose medium. A centrifugation at 10,000 × g enabled us to separate mitoplasts (pellet) from outer membranes (supernatant). The outer membranes were sedimented at 145,000 × g. The mitoplasts were then subjected to another swelling in 1 mM phosphate buffer for 20 min at 4 °C, followed by the addition of 61.5% sucrose as described above. After 20 min, the mixture was centrifuged at 100,000 × g for 60 min. This latter pellet and the outer membrane pellet were layered above two separate 38-ml sucrose density gradients varying from a density of 1.29 to 1.06 g/ml and were centrifuged for 3 h in a Spinco SW 27/1 rotor at 18797
the long periods of the data accumulation. The contribution of stray light was estimated, with solutions of small unilamellar vesicles (27,000 rpm). Subsequently, the gradients were divided into 1-ml fractions, which were characterized by marker enzymes. Contact site fractions (see “Results”) were layered again on a discontinuous sucrose gradient (50, 47.5, 45, 42.5, and 40% sucrose) before lipid composition and fluorescence polarization studies.

**Binding of Hexokinase to Submitochondrial Fractions**—Hexokinase was prepared from mouse heart mitochondria as described by Aubert-Pouchot et al. (12). The binding of hexokinase was assayed according to Ohlendieck et al. (5).

**Chemical Estimations**—Total lipids were extracted from membrane preparations by the method of Folch et al. (13). Separation of neutral lipids and fatty acids from polar lipids was achieved using prepacked silica Sep-Pak columns. Free fatty acids and fatty acids of the total lipid extracts were derivatized as methyl esters and analyzed on a Hewlett-Packard 5880A gas-liquid chromatograph equipped with a flame ionization detector using authentic fatty methyl esters as standards.

Cholesterol and total phospholipids were quantitated as previously reported (14). The phospholipid composition of the extracts was analyzed by thin-layer chromatography (15). Protein was estimated according to Lowry et al. (16).

**Steady-state Fluorescence Measurements**—Steady-state fluorescence polarization studies were performed using a Perkin-Elmer LS 30 spectrofluorometer fitted with an automatic polarizer. The fluorescence anisotropy, r (steady-state measurements) was measured at an excitation wavelength of 360 nm, and emission light was filtered through a high-pass 420 nm filter. r was determined in the usual way,

\[ r = \frac{I_{90} - G L_{90}}{I_{90} + 2G L_{90}} \]  

where \( I_{90} \) and \( I_{90} \) are the vertical and horizontal components of the emission viewed at 90° to the exciting light (the first letter refers to the excitation and the second one, to the emission). The correcting factor \( G = I_{90}/I_{90} \) is the factor which takes into account the different response of the monochromators to the vertically and horizontally polarized light (17). For a fluorophor in an homogeneous environment, the static fluorescence anisotropy \( r \) is related to the fluorescence lifetime \( \tau \) and the correlation time \( \theta \),

\[ \frac{1}{r} = \frac{1}{\tau} \left( 1 + \frac{\theta}{\tau} \right) \]  

Relation 2 indicates that changes in \( r \) can either be due to a change in the correlation time of the dye (i.e., in the local microviscosity referred to in the text under the general term “fluidity”) or to a change of its fluorescence lifetime. This is why the fluorescence decay measurements described below were performed. A mean correlation time, \( \langle \theta \rangle \), has been approximated from Relation 2, in which \( \tau \) has been replaced by its mean value of the first order, \( \tau \),

\[ \langle r \rangle = \sum_i c_i \tau_i \]  

in order to take into account the inhomogeneity of the environment and to reveal changes in fluidity. The direct physical determination of the fluorescence anisotropy decays will be performed later.

**Fluorescence Decay Measurements**—The fluorescence decay experiments were carried out using a single photoelectron-counting apparatus. The excitation light pulse was generated by a free-running flash lamp operating in nitrogen/helium mixtures at a frequency of 10 kHz. The excitation wavelength was selected by an interference filter at 360 nm (bandwidth, 6 nm; transmission, 40%), and the fluorescence was observed through a short wavelength cut-off filter with 50% transmission at 412 nm. The photons were detected by means of a Radiotechnique XP2020 photomultiplier. The counts were accumulated at a counting rate of 1-2% of the flash frequency in order to remain in the single photoelectron condition. Experiments were stopped after 2-3 h when the total counts under the fluorescence curve were about 10^6. The excitation function, \( G(t) \), which characterizes the true distribution of the flash intensity and the apparatus response function at the emission wavelength, was obtained with a mixture of tetraphenylbutadiene in deaerated cyclohexane (\( \tau = 1.76 \) ns) (18). This procedure takes into account the wavelength dependence of the photomultiplier response (19). The measurements of \( G(t) \) were performed before and after each observation on membrane solutions in order to test the stability of the response function over the long periods of the data accumulation. The contribution of stray light was estimated, with solutions of small unilamellar vesicles having a light-scattering intensity equal to that of membrane samples. The amount of stray light was systematically subtracted from the fluorescence curves.

**Fluorescence Decay Analysis**—The true fluorescence decay, \( i(t) \), is related to the observed fluorescence decay, \( i(t) \), by the convolution product,

\[ i(t) = \int_0^t G(T) \delta(t - T) dT \]  

where \( G(T) \) was measured as described above. We assumed that \( G(T) \) is a sum of exponential terms.

\[ i(t) = \sum_i \omega_i \exp(-t/\tau_i) \]  

The parameters of Relation 5 were determined by a program based on the nonlinear least squares method of Marquardt. The short component (\( r < 0.2 \) ns) due to the stray light was eliminated by the appropriate subtraction described above. The quality of the fit between the calculated and experimental curves was estimated by examining the deviation curve \( DV^2 \) and the \( \chi^2 \) test of distribution defined by the following expressions,

\[ DV^2 = \sum (i(t) - \bar{i}(t))/(\tau(t)) \]  

\[ \chi^2 = \sum_i^n \left( i(t_i^b) - i(t_i^a) \right)^2/(\tau(t_i^a)) \]  

where \( \bar{i}_i^a \) and \( \bar{i}_i^b \) are the number of counts in the kth channel of the experimental and calculated curves, respectively, and \( n \) is the total number of channels.

**Statistics**—All results are expressed as mean values ± S.E., and a t test was used for all statistical analysis. A p value < 0.05 was considered significant.

**RESULTS**

**Characterization of the Submitochondrial Fractions**—The membranes obtained from the first swelling procedure (total outer membranes) were fractionated into two populations. The activity profiles of specific marker enzymes in the gradient (Fig. 1a) revealed an outer membrane fraction (C1), characterized by a high activity of monoamine oxidase, at a density of 1.14 g/ml and a second one (C2), characterized by the presence of both monoamine oxidase and cytochrome c oxidase activities, at a density of 1.19 g/ml. The membranes obtained from the second swelling (mitoplasts) were also fractionated into two populations. According to the specific marker enzyme content (Fig. 1b), we were able to separate an inner membrane fraction (C4), characterized by a high activity of cytochrome c oxidase, at a density of 1.25 g/ml and another fraction (C3), characterized by the presence of both monoamine oxidase and cytochrome c oxidase activities, at a density of 1.21 g/ml.

If one assumes that the two mitochondrial boundary membranes are tightly connected in fractions C2 and C3 (contact site fractions), the inner and the outer membrane parts in these fractions should not be separable. Therefore, fractions C2 and C3 were recentrifuged on a discontinuous sucrose gradient. Fig. 2, a and b, shows that these fractions could not be further fractionated on a second gradient. Moreover, since it could not be excluded that the presumptive contact site fractions may contain free outer and inner membranes of the same density, the protein distribution in the two density gradients (Fig. 2, a and b) revealed only one protein peak at intermediate density (i.e. 1.19-1.21 g/ml) between free outer and free inner mitochondrial membranes. These results demonstrate that fractions C2 and C3 are homogeneous and can be considered to be “contact site”-enriched fractions. In addition, according to previous findings (6, 20) that reported the ability of contact sites to bind hexokinase, fractions C2 and C3 were effectively able to bind hexokinase (0.170 and 0.190 unit/mg, respectively).

**Lipid Composition Studies**—Clarification of the nature of
FIG. 1. Separation of mitochondrial fragments by density gradient centrifugation. a, after exposure of liver mitochondria to a swelling procedure. C1, outer membrane (gradient fractions 10-14); C2, outer membrane contact sites (gradient fractions 17-26). b, after exposure of mitoplasts to a swelling procedure. C3, inner membrane contact sites (gradient fractions 16-28); C4, inner membrane (gradient fractions 30-35). The activity of marker enzymes (expressed in units: min⁻¹ . mg⁻¹) was determined in the different fractions of the gradient. ▲, NADPH-cytochrome c reductase; ●, monoamine oxidase (MAO); ◊, cytochrome c oxidase; □, protein content.

proteins and lipids involved in the formation of contacts between mitochondrial membranes would provide much information about their function. While numerous studies (5-7) report a preferential location of proteins or enzymes in the contact sites of mitochondria, very limited information is available on the lipid composition of these particular regions. So, we examined the lipid composition of the submitochondrial fractions obtained after gradient density centrifugation. While the results obtained for outer and inner membranes were very close to those reported by Colbeau et al. (21), 'outer membrane contact sites' were found to possess a greater molar ratio of cholesterol/phospholipid (20 times more, p < 0.05) together with more saturated fatty acyl chains compared with 'inner membrane contact sites' (Tables I and II). The other parameters, particularly the protein/lipid (w/w) ratio, were not significantly different in these two fractions.

Expressed as a percentage of weight of total lipids (Table I) and, apart from cholesterol, the principal neutral lipids were triacylglycerols in outer membrane contact sites, whereas a higher content of diacylglycerols and of free fatty acids was found in inner membrane contact sites.

The phospholipid content of each submitochondrial fraction was further determined by the use of thin-layer chromatography. While the phospholipid contents of outer and inner mitochondrial membranes are in good agreement with prior studies (21), the most striking result obtained was the high concentration of cardiolipin (up to 24% of the lipid content) in both contact site-enriched fractions, whereas phosphatidylcholine (26%) and phosphatidylethanolamine (22%) represented the remainder of the phospholipid content.

FIG. 2. Centrifugation of contact site-enriched fractions on a discontinuous sucrose gradient. a, outer membrane contact sites (fraction C2); b, inner membrane contact sites (fraction C3). The activity of marker enzymes (expressed in units: min⁻¹ . mg⁻¹) was determined in the different fractions of the gradient. ▲, monoamine oxidase (MAO); ●, cytochrome c oxidase; □, protein content.

In addition, cardiolipin was found to be fully unsaturated in both contact site fractions, whereas phosphatidylethanolamine was only 50% unsaturated (data not shown).

Fluorescence Studies—Since differences in membrane composition are expected to underlie differences in membrane dynamics, the lipid phase of the submitochondrial fractions has been further characterized by steady-state and pulse fluorimetry after insertion of DPH¹ into the membrane bilayers (Table III).

Previous works on oriented planar bilayers have suggested that DPH molecules are distributed according to two populations, one aligned parallel to the bilayer and the other orthogonally oriented (24). The fluorescence decays reported in Table III reflect this complicated situation since they could only be analyzed in terms of at least two exponential terms in order to reduce the χ² factor characterizing the fit between computed and experimental decays (see "Experimental Procedures"). As these two lifetimes probably represent the mean values of at least two distributions of lifetimes (25), a much more complicated analysis should be undertaken in order to get a complete picture of the chemical environment reflected by DPH in the membranes. For the present purpose, this study has been limited to the mean lifetime of the first order of each decay curve.

The values reported in Table III indicate rather short fluorescence lifetimes (~6 ns) as compared, for example, with the one observed for DPH in small unilamellar vesicles of pure dimyristoylphosphatidylcholine at a temperature above that of the transition point of gel to the liquid/crystalline phase of fatty acid chains (7.6 ns (26)). This effect can partly be related to the possible dynamic fluorescence quenching induced by intrinsic proteins. As the quenching is more pro-

¹ The abbreviation used is: DPH, 1,6-diphenyl-1,3,5-hexatriene.
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Compositional analysis of lipid extracts prepared from mitochondrial membranes and mitochondrial contact sites
Values are means of five separate preparations in percent by weight of total lipids. OM, outer membrane; IM, inner membrane.

<table>
<thead>
<tr>
<th>Components and parameters</th>
<th>OM</th>
<th>OM contact sites</th>
<th>IM contact sites</th>
<th>IM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty acids</td>
<td>8.2%</td>
<td>3.4%</td>
<td>5.6%</td>
<td>7.6%</td>
</tr>
<tr>
<td>Diacylglycerol</td>
<td>0.3%</td>
<td>1.7%</td>
<td>5.8%</td>
<td>2.8%</td>
</tr>
<tr>
<td>Triacylglycerol</td>
<td>0.7%</td>
<td>5.8%</td>
<td>5.1%</td>
<td>0.6%</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>7.1%</td>
<td>9.4%</td>
<td>0.4%</td>
<td>2.3%</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>40.9%</td>
<td>25.9%</td>
<td>25.8%</td>
<td>35.1%</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>1.8%</td>
<td>3.1%</td>
<td>2.2%</td>
<td>0.7%</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>9.1%</td>
<td>7.7%</td>
<td>7.7%</td>
<td>5.0%</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>&lt;0.1%</td>
<td>0.1%</td>
<td>0.1%</td>
<td>0.1%</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>20.6%</td>
<td>21.5%</td>
<td>21.7%</td>
<td>20.5%</td>
</tr>
<tr>
<td>Cardiolipin</td>
<td>4.0%</td>
<td>20.2%</td>
<td>24.4%</td>
<td>18.0%</td>
</tr>
<tr>
<td>Lysophosphatidylcholine</td>
<td>0.4%</td>
<td>0.1%</td>
<td>0.1%</td>
<td>0.2%</td>
</tr>
<tr>
<td>Lysophosphatidylinositol</td>
<td>0.1%</td>
<td>&lt;0.1%</td>
<td>&lt;0.1%</td>
<td>0.1%</td>
</tr>
<tr>
<td>Phospholipid/protein (w/w)</td>
<td>0.59 ± 0.06</td>
<td>0.37 ± 0.03</td>
<td>0.36 ± 0.03</td>
<td>0.30 ± 0.02</td>
</tr>
<tr>
<td>Protein/lipid (w/w)</td>
<td>1.66 ± 0.05</td>
<td>2.12 ± 0.09</td>
<td>2.25 ± 0.1</td>
<td>2.85 ± 0.2</td>
</tr>
<tr>
<td>Cholesterol/phospholipid (mol/mol)</td>
<td>0.092 ± 0.007</td>
<td>0.099 ± 0.009</td>
<td>0.004 ± 0.001b</td>
<td>0.025 ± 0.002</td>
</tr>
<tr>
<td>Saturation indexb</td>
<td>0.699 ± 0.03</td>
<td>0.505 ± 0.05</td>
<td>0.374 ± 0.04</td>
<td>0.368 ± 0.03</td>
</tr>
</tbody>
</table>

TABLE II
Compositional analysis of total fatty acids of mitochondrial membranes and mitochondrial contact sites
Values are means ± S.E. for lipid extracts from five distinct preparations of each fraction. OM, outer membrane; IM, inner membrane.

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>OM</th>
<th>OM contact sites</th>
<th>IM contact sites</th>
<th>IM</th>
</tr>
</thead>
<tbody>
<tr>
<td>C12:0</td>
<td>0.1 ± 0.01</td>
<td>&lt;0.1%</td>
<td>&lt;0.1%</td>
<td>&lt;0.1%</td>
</tr>
<tr>
<td>C14:0</td>
<td>0.2 ± 0.01</td>
<td>0.2 ± 0.05</td>
<td>0.4 ± 0.06</td>
<td>0.1 ± 0.01</td>
</tr>
<tr>
<td>C16:0</td>
<td>36.3 ± 1.1</td>
<td>20.3 ± 0.90</td>
<td>29.8 ± 1.4</td>
<td>98.3 ± 1.9</td>
</tr>
<tr>
<td>C18:1</td>
<td>0.6 ± 0.05</td>
<td>3.0 ± 0.08</td>
<td>6.3 ± 0.1</td>
<td>21.1 ± 0.05</td>
</tr>
<tr>
<td>C16:1</td>
<td>0.5 ± 0.05</td>
<td>18.7 ± 1.0</td>
<td>14.0 ± 0.9</td>
<td>15.6 ± 1.0</td>
</tr>
<tr>
<td>C18:1</td>
<td>18.0 ± 1.3</td>
<td>17.8 ± 1.2</td>
<td>13.7 ± 1.0</td>
<td>16.8 ± 1.3</td>
</tr>
<tr>
<td>C16:2</td>
<td>16.6 ± 0.9</td>
<td>21.5 ± 1.3</td>
<td>21.8 ± 0.7</td>
<td>23.5 ± 0.7</td>
</tr>
<tr>
<td>C18:3</td>
<td>&lt;0.1%</td>
<td>&lt;0.1%</td>
<td>0.2 ± 0.05</td>
<td>0.1 ± 0.05</td>
</tr>
<tr>
<td>C20:0</td>
<td>0.5 ± 0.02</td>
<td>0.1 ± 0.01</td>
<td>1.0 ± 0.05</td>
<td>1.2 ± 0.05</td>
</tr>
<tr>
<td>C20:4</td>
<td>7.1 ± 0.2</td>
<td>8.3 ± 0.5</td>
<td>12.9 ± 0.2</td>
<td>12.4 ± 0.9</td>
</tr>
<tr>
<td>Ratio, saturated/unsaturated</td>
<td>0.33</td>
<td>0.97</td>
<td>0.8</td>
<td>0.78</td>
</tr>
</tbody>
</table>

TABLE III
Fluorescence studies of the submitochondrial fractions using 1,6-diphenyl-1,3,5-hexatriene as a probe

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Steady-state fluorescence anisotropy at 25 °C r</th>
<th>Mean anisotropy parameter (((r/\langle r \rangle \rangle - 1))</th>
<th>Mean lifetime (&lt;\tau&gt;) ns</th>
<th>Mean correlation time (&lt;\xi&gt;) ns</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer membrane</td>
<td>0.185 ± 0.005</td>
<td>1.027</td>
<td>5.9</td>
<td>6.0</td>
</tr>
<tr>
<td>Outer membrane contact sites</td>
<td>0.190 ± 0.003</td>
<td>1.085</td>
<td>5.1</td>
<td>5.5</td>
</tr>
<tr>
<td>Inner membrane contact sites</td>
<td>0.109 ± 0.003b</td>
<td>0.771</td>
<td>6.4</td>
<td>4.9</td>
</tr>
<tr>
<td>Inner membrane</td>
<td>0.161 ± 0.002</td>
<td>0.789</td>
<td>6.0</td>
<td>4.7</td>
</tr>
</tbody>
</table>

* p < 0.05 compared with outer membrane contact sites.

nounced, however, for outer membrane contact sites that do not exhibit the highest protein content (Table I), it is possible that another factor such as a change in the DPH location or a change in the solvent permeability of the membranes may play an additional important part.

It is clear that changes in the steady-state anisotropy of DPH in the different membranes cannot be exclusively attributed to the changes in fluorescence lifetimes. They also reflect the mean correlation time of the dye (i.e., the local microviscosity), which can be deduced from Relation 2 (see "Experimental Procedures"). (\(\theta\)) is in the range 4.7–6 ns. This is much longer than what is observed for DPH in dimyristoylphosphatidylcholine taken as reference membranes (\(\theta\) = 1.7 ns at 37 °C) but comparable with what is obtained when a protein such as apolipoprotein C-1 is complexed with the same reference membrane (\(\langle \theta \rangle = 5 \text{ ns} (27)\)). The values observed here are not only due to the general properties of the lipidic phases studied but also to the constraints applied to them by the intrinsic proteins. It can be noticed that since (\(\langle \theta \rangle\)) is a mean value, much longer correlation times arising from the contribution of highly restricted motions cannot be a priori excluded (at least 30 ns, as in Ref. 27) even if this was not detected by the EPR signal of spin-labeled (1, 14) stearic acid incorporated into the inner mitochondrial membranes (\(\theta = 1 \text{ ns} \)) (28). The fluorescent probe studied here and the paramagnetic probe may have completely different
locations since one is nonanchored and the other is anchored to the fatty acid side chain.

From Table III, it can also be deduced that the motions of DPH are faster (and/or have a greater amplitude) in the inner membranes and in contact sites derived from them when compared with outer membranes and contact sites.

Taken all together, the spectroscopic measurements gathered in Table III lead to the idea that the two populations of contact sites have different physicochemical properties that are probably mainly determined by the membrane from which they are derived.

**DISCUSSION**

Contacts between the two mitochondrial membranes in freeze-fractured mitochondria have been found to be related to the degree of coupling (3). Separation of osmotically lysed mitochondria and mitoplasts led us to separate two membranous fractions containing outer and inner membrane fragments (Figs. 1 and 2). These two fractions, which cannot be further fractionated on a second sucrose gradient, contain both monoamine oxidase and cytochrome c oxidase activities and bind hexokinase. Recently, we were able to distinguish these two fractions with regard to their cholesterol content (11).

The present study reports for the first time a detailed analysis of the lipid composition of mitochondrial contact site-enriched fractions. As shown under "Results," the major phospholipid in these fractions was phosphatidylethanolamine, phosphatidylethanolamine (half-unsaturated), and cardiolipin (fully unsaturated) as a major component. Cardiolipin, as well as phosphatidylethanolamine, is known to adopt the hexagonal (H II) phase in the presence of divalent cations (29). These results lead to the remarkable possibility that over 45% of the endogenous phospholipids of the contact site-enriched fractions would be able to adopt nonbilayer arrangements. The occurrence of such structures in mitochondrial contact sites would be of great interest, since it has been previously suggested (2) that lipid particles interpreted as inverted intratubule micelles might be involved in the formation of semilattice between the two membranes. This joining site model, in which lipids can easily diffuse from the outer to the inner mitochondrial membrane, could explain the import of cytoplasmically synthesized phospholipids previously reported (9, 30). As a matter of fact, evidence has been presented (9) for a vectorial translocation of newly synthesized phospholipids from the outer to the inner mitochondrial membrane and in the opposite direction. Since there is no evidence for the existence of phospholipid transfer proteins in the mitochondrial intermembrane space (9), it is conceivable that the migration of phospholipids between the two membranes occurs via specific contact sites in a "quasi-one-step" transfer from their site of synthesis to the inner membrane. Such a mechanism would be independent of a "classic" flipase and, thus, consistent with the absence of such an enzyme in the outer mitochondrial membrane (31).

The steady-state fluorescence anisotropy provides a sensitive technique for examining the lipid fluidity of artificial and natural membranes (32, 33) when fluorescence decay experiments are conducted in parallel. The two sets of experiments are necessary in order to check if changes in anisotropy are not due to changes in fluorescence lifetimes. From the results presented above, we have deduced a mean correlation time of DPH in the membranes which is greater in the outer membrane contact sites than in the inner membrane contact sites. This clearly indicates that the rate of motion of DPH molecules is greater in inner membranes and in contact sites to which they are derived.

The effect of proteins on the membrane fluidity is not only determined by protein-lipid but also by protein-protein interactions. Although the nature of these interactions depends mainly on the particular molecules involved, the number of interactions may be related to the total protein content of the membranes. As there were no significant differences in the protein/lipid ratio (w/w) of the two contact site-enriched fractions, these results suggest that each membrane’s fluidity is influenced approximately to the same extent by the intrinsic membrane lipids and proteins. For this reason, the lipid content itself may constitute the major contribution to the membrane fluidity. One can consider a number of terms that have been previously defined by extensive studies on model membranes as well as biological membranes (22, 23). In that way, the lipid composition of the two contact site-enriched fractions suggests that the molecular bases for the decrease of the lipid order in the inner membrane contact sites may be the increase in unsaturation of fatty acyl chains, together with the decrease of the cholesterol/lipid ratio. The factors controlling the establishment and maintenance of the nonhomogeneous distribution of cholesterol among subcellular membranes are intriguing and still poorly understood. Although cholesterol can spontaneously desorb from membranes and migrate, recent studies have presented evidence that cholesterol may also exist in more than one pool in model membranes (34, 35). Moreover, in binary phospholipid mixtures, Bar et al. (36) proposed that cholesterol-rich and cholesterol-free domains coexist in these bilayer systems. Under their experimental conditions, there would be two phases: one, liquid/crystalline, composed of a mixture of phospholipids without cholesterol and the other, a gel-phase, composed of phospholipid mixture and cholesterol. Despite the use of spectroscopic methods such as steady-state fluorescence anisotropy (37, 38) to investigate cholesterol-phospholipid interactions (37, 38), no data are actually available to determine the partitioning of cholesterol inside biological membranes whose composition is much more complex than model membranes.

The data reported in this paper provide further evidence that the idea of bulk or average membrane fluidity is not valid for biological membranes and particularly not for the mitochondrial contact site-enriched fractions. The use of intrinsic fluorescent (or spin-labeled) probes and a systematic study of lipid lateral and transverse distribution in connection with the membrane lipid composition should be of great help in better understanding the involvement of contact sites in the regulation of the mitochondrial structure and function. However, there are still a number of potential methodological and technical difficulties, since the membrane domains might be very small and changes of physical properties in the domains might certainly be difficult to analyze.

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**REFERENCES**

Mitochondrial Contact Sites

