Effect of Heme Iron Valence State on the Conformation of Cytochrome c and Its Association with Membrane Interfaces

A CD AND EPR INVESTIGATION

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Recently cytochrome c has been mentioned as an important mediator in the events of cellular oxidative stress and apoptosis. To investigate the influence of charged interfaces on the conformation of cytochrome c, the CD and magnetic circular dichroic behavior of ferric and ferrous cytochrome c in homogeneous medium and in phosphatidylcholine/phosphatidylethanolamine/cardiolipin and dicetylphosphate liposomes was studied in the 300–600 and 200–320 nm wavelength region. EPR spectra demonstrate that the association of cytochrome c with membranes promotes alterations of the crystal field symmetry and spin state of the heme Fe⁺³. The studies also include the effect of Pᵢ, NaCl, and CaCl₂. Magnetic circular dichroism and CD results show that the interaction of both ferrous and ferric cytochrome c with charged interfaces promotes conformational changes in the α-helix content, tertiary structure, and heme iron spin state. Moreover, the association of cytochrome c with different liposomes is sensitive to the heme iron valence state. The more effective association with membranes occurs with ferrous cytochrome c. Dicetylphosphate liposomes, as a negatively charged membrane model, promoted a more pronounced conformational modification in the cytochrome c structure. A decrease in the lipid/protein association is detected in the presence of increasing amounts of CaCl₂, NaCl, and Pᵢ, in response to the increase of the ionic strength.

Cytochrome c is a peripheral membrane protein, and its interaction with negatively charged interfaces is a well known phenomenon (1, 2). The binding of cytochrome c with phospholipid bilayers probably encompasses electrostatic and hydrophobic interactions (3) and induces conformational alterations in the protein (4, 5). In this regard, several works have been concerned with structure-function relationships of cytochrome c (1, 3, 6). Besides its well established role as an electron carrier in the mitochondrial respiratory chain, cytochrome c also exhibits oxidase/peroxidase activity on several substrates, including t-butylhydroperoxide, aldehydes, and β-diketones (7–11).

Cytochrome c associated with the inner mitochondrial membrane or with dicetylphosphate (DCP)¹ liposomes can oxidize diphenylacetaldehyde and methylacetacetone (9, 10). This reaction produces excited species that, in mitochondria, lead to oxidative injury of the organelle. The requirement of Pᵢ for the occurrence of this reaction was attributed exclusively to the increase in the rate of aldehyde enolization catalyzed by this anion (12). Kowaltowski et al. (13) recently have proposed a model for phosphate-stimulated lipid peroxidation. In this model, high phosphate concentration and Ca²⁺ cooperate in reactive oxygen species-mediated mitochondrial damage. The importance of electronically excited triplet states in biological events has been extensively discussed by Cilento and Adam (14).

The recent demonstration that the apoptosis cascade involves cytochrome c-promoted caspase activation (15, 16) shows that cytochrome c plays a broader role in the cells than electron transport in the respiratory chain. In this regard, evidence has been provided for a relationship between apoptosis, the Ca²⁺-induced permeability transition pore (17), and hydrogen peroxide production (18).

In the present work we examine the different types of cytochrome c/membrane association influenced by the heme iron redox state, membrane charge, and ionic strength. For this purpose, CD and magnetic circular dichroism (MCD) have been used to detect the conformational alterations in the protein induced by the interfacial microenvironment. These techniques are sensitive to the secondary and tertiary structures of the protein and also reflect the relative configuration of the prosthetic group within the protein. EPR was employed to refine the results, providing accurate information about the iron valence, spin state, and charge in local symmetry of the iron crystal field around iron.

MATERIALS AND METHODS

Chemicals—Cytochrome c (horse heart, type III), HEPES, phosphatidylcholine (egg yolk), phosphatidylethanolamine (ovine brain, type II-S), cardiolipin (bovine heart), and dicetylphosphate were purchased from Sigma.

Liposome Preparation—DCP liposomes were prepared in HEPES buffer according to Mortara et al. (19). DCP was suspended in HEPES buffer and sonicated for 20 min with a microtip-equipped Cole-Parmer 4710 series ultrasonic processor at an output of 60 watts. In the preparation of phosphatidylcholine/phosphatidylethanolamine/cardiolipin (PC/PE/CL; 15/12/9 molar ratio) liposomes, the lipids were first dis-

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¹ The abbreviations used are: DCP, dicetylphosphate; MCD, magnetic circular dichroism; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CL, cardiolipin; mT, millitelsa.
EPR cavity to obtain the spectra. The MCD spectra (top) and UV-visible absorbance (bottom) of ferric cytochrome c in HEPES buffer, pH 7.4, at room temperature (solid line), PC/PE/CL (dashed line), and DCP liposomes (dotted line) are shown. The magnetic field is 8.6 mT and the optical path is 0.5 cm.

The spectra were obtained at room temperature, pH 7.4. For the MCD, the magnetic field was 870 mT and the optical path 5 mm. The spectra were obtained at room temperature, pH 7.4.

EPR Spectrometry—EPR measurements of cytochrome c (300 μM) were obtained by using an X-band Varian E-109 spectrometer and/or an X-band Bruker ELEXSYS E580 spectrometer, equipped with a helium cryostat and temperature controller from Oxford Company under the following conditions: gain, 5 × 10³; modulation amplitude, 1.0 mT; microwave power, 4 milliwatt; temperature, 11 K; and time constant, 0.06 s. After the addition of cytochrome c in different medium, 120 μl of the mixture was quickly introduced into an EPR quartz tube, cooled in liquid nitrogen, and then transferred to the helium cryostat into the EPR cavity to obtain the spectra.

RESULTS

Liposome-induced Structural Modifications in the Cytochrome c Heme Group—In Fig. 1, the MCD spectra (top) and UV-visible absorbance (bottom) of ferric cytochrome c in HEPES buffer (solid line), PC/PE/CL (dashed line), and DCP liposomes (dotted line) are shown. A Cotton effect in the Soret band, with a positive band at 400 nm and a negative band at 420 nm, appears in the 549-nm band that characterizes the ferrous cytochrome c heme iron as indicated by the MCD results. Above the lipid/protein ratio of 24, it is possible to see the appearance of a small quantity of high spin form (g = 4.3, around 160 mT) and g = 2.0, around 330 mT) appear, provided by the reaction of cytochrome c heme iron with cardiolipin peroxide derivatives and a radical product, respectively.

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EPR spectra obtained with increasing amounts of DCP liposomes are in agreement with the MCD spectra obtained under similar conditions (Fig. 6B). Above the lipid/protein ratio of 12, a high spin form \( (g' = 6.0, \text{low field region}) \) and a low spin form \( (g_i = 2.0, \text{high field region}) \) is observed that becomes predominant at higher lipid/protein ratios. A different signal of low spin state superimposed on the signal of the protein in homogeneous medium can be easily identified in the 220 mT region. At the lipid/protein ratio of 24, the concomitant existence of the three species can be clearly identified.

As described above the association of ferrous cytochrome \( c \) with charged interfaces leads to MCD and UV-visible spectral changes very similar to those that occur upon conversion of ferrous cytochrome \( c \) to ferric cytochrome \( c \) (compare Figs. 1 and 2). EPR measurements with liposome-bound ferrous cytochrome \( c \) exclude the possibility of conversion of cytochrome \( c \) from ferrous to ferric after association with membranes because no EPR signal was observed. Moreover, the MCD spectral changes are reversible when liposome-bound cytochrome \( c \) is dissociated from the PC/PE/CL membrane by increasing the ionic strength as shown in Fig. 7A.

As previously mentioned (13), \( \text{Ca}^{2+} \) promotes modifications in the organization of lipid bilayers. However, the results obtained from \( \text{CaCl}_2 \) titration indicate that, in this model, the effect of \( \text{Ca}^{2+} \) is restricted to an increase of the ionic strength, promoting the dissociation of the hemeprotein from the PC/PE/CL membrane (Fig. 7B). In this regard, the addition of 1 mM \( \text{CaCl}_2 \) to 13.5 mM ferrous cytochrome \( c \) bound to 2 mM PC/PE/CL liposomes (lipid/protein ratio, 150:1), a condition in which all cytochrome \( c \) molecules are initially membrane-bound, leads to a partial dissociation of the protein similar to that of a lipid/protein ratio of 60:1.

The Lipid-induced Structural Modifications of Cytochrome \( c \) Extend to the Secondary Structure Content—The MCD spectra in Figs. 1 and 2 reveal that the association of cytochrome \( c \) with lipid bilayers promotes alterations in the structure of the heme group of this protein. To assess whether this effect of cytochrome \( c \)/lipid association extends to the protein fraction, the UV CD spectra of cytochrome \( c \) were obtained in these three different media. Analysis of the spectra (not shown) indicates that the association of cytochrome \( c \) with charged interfaces promotes modifications in the secondary structure. The spectra have been analyzed using the Selcon method (20, 21) to obtain fractions of \( \alpha \) and \( \beta \) and the remainder secondary structure, and these parameters are compared with those obtained from crystallographic data (Tables I and II). In accordance with the data obtained in the Soret and visible region, the UV CD spectra reveal that DCP promotes the most significant modification in both ferrous (Table I) and ferric (Table II) cytochrome \( c \) secondary structure.

**DISCUSSION**

The Association of Cytochrome \( c \) with Liposomes Promotes Conformational Changes in the Protein—The MCD spectra of ferric and ferrous cytochrome \( c \) reveal that the association of this hemeprotein with vesicles promotes conformational changes in the heme group and protein fraction (Figs. 1 and 2; Tables I and II). However, the comparison of Figs. 1 and 2 reveals that only the association with DCP liposomes promotes
significant conformational alterations in both ferric and ferrous cytochrome c. The Association of Cytochrome c with an Inner Mitochondrial Membrane Model Is a Cooperative Process—The plot of molar ellipticity at 415 nm for ferric cytochrome c (Fig. 3, inset), or at 400 and 550 nm for ferrous cytochrome c, versus DCP concentration reveals a hyperbolic curve. The same curves obtained for PC/PE/CL-bound ferrous cytochrome c are sigmoidal, suggesting that binding of this hemeprotein to an inner mitochondrial mimetic membrane is a cooperative process. As a result, significant conformational alterations in both ferric and ferrous cytochrome c.

The Structure of Cytochrome c Associated with Membranes
the association of cytochrome c to PC/PE/CL might alter the membrane structure, favoring the attachment of other cytochrome c molecules.

The Association of Cytochrome c with Charged Interfaces Is Modulated by the Heme Iron Oxidation State—Whereas the MCD and EPR spectra of ferric cytochrome c in PC/PE/CL liposomes (Figs. 1 and 6A) suggest a lipid/protein interaction with little protein modification, Fig. 2 indicates an interaction with strong modification in the case of PC/PE/CL liposomes, when the heme iron of cytochrome c is in oxidation state II. In the case of DCP liposomes, the MCD and EPR spectra suggest a strong modification in both ferric and ferrous cytochrome c (Figs. 3, 5, and 6B). The titration of ferrous cytochrome c with DCP liposomes exhibits protein partitioning between the aqueous and liposomal media (Fig. 5). Increasing the DCP/protein ratio up to 320:1 does not modify the spectra (not shown) from those obtained with a 120:1 lipid/protein ratio of 120:1 (Fig. 5). This partitioning suggests a weak lipid-protein interaction.

The Association of Ferric and Ferrous Cytochrome c with Charged Interfaces Promotes Spin State Changes in the Heme Iron—The comparison of the MCD and absorbance spectra of ferric cytochrome c in homogeneous medium (Fig. 1, top and bottom, respectively, solid lines) with those of ferrous cytochrome c in PC/PE/CL liposomes (Fig. 2, top and bottom, respectively, dashed lines) might suggest, at first glance, that the association of cytochrome c with biological mimetic membranes leads to the oxidation of the heme iron. Iwase et al. (22) propose a reaction of ferrous cytochrome c and cardiolipin with production of the monoepoxide of linoleic acid and oxidation of the heme iron. The authors suggest a change of the heme iron redox state based only on the absorbance spectral changes (loss of the Soret band) observed after the addition of cytochrome c to a cardiolipin-containing medium. However, our results show that the detachment of the protein from DCP or PC/PE/CL liposomes induced by increasing the ionic strength with NaCl (Fig. 7A) recovers the ferrous cytochrome c spectra. This clearly indicates the absence of oxidation of heme iron by phospholipids. This result was corroborated by titration with CaCl₂ (Fig. 7B), which concomitantly reverts the ferrous cytochrome c MCD spectra in the presence of lipid membranes. This spectral modification must therefore be explained by environmental modifications in the heme iron crevice. One possibility is a change in the heme iron spin state modulated by conformational changes controlling the heme iron crystal field. In homogeneous medium the absorbance spectrum of ferric cytochrome c exhibits a 695-nm charge transfer band, indicative of the heme iron sixth coordination position with methine nitrogen (7) that disappears in the presence of charged interfaces (10). This charged interface-promoted loss of the cytochrome c sixth coordination position significantly enhances the peroxidase/oxidase activity of this protein (8–11). The comparison of the results obtained with ferric cytochrome c bound to DCP and PC/PE/CL liposomes provides the following information. (i) The secondary structure modifications are more significant in DCP vesicles than PC/PE/CL vesicles. (ii) Con-
moting its detachment from the inner mitochondrial membrane (25). On the other hand, there is evidence pointing to ferric cytochrome c as the caspase activator in the cytosol (25, 30). In any event, a sustained steady state of cytochrome c in the ferric form seems to favor its detachment from the inner mitochondrial membrane and the caspase activation in the cytosol. Thus, the ability of cytochrome c to participate in the caspase activation might be dependent on the association of this protein with membranes and/or protein complexes, as suggested by recent literature (29). In this context, it is important to verify whether the association of cytochrome c with protein complexes, such as apoptosomes (29), is dependent on the heme iron valence state and association with membranes.

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