CYTOCHROME C PHOSPHOLIPID INTERACTION: STRUCTURAL TRANSITIONS ASSOCIATED WITH VALENCY CHANGES

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Cytochrome c is known to combine with phospholipids (1,2) and there is evidence that it also exists in the mitochondrial respiratory chain in close relationship with phospholipids (2,3).

This study is concerned with structural transitions occurring in the cytochrome c-phospholipid complex when the valency of cytochrome c is changed.

The fluorescent probe, 8-anilino-1-naphthalene sulfonic acid (ANS) has a quantum yield and a maximum emission wavelength that are sensitive to the polarity of its environment (4). It has been used to monitor changes of the structure of the cytochrome c-phospholipid complex, reflecting a polarity change at the ANS binding site.

Methods and Materials. Fluorescence intensity and fluorescence spectra were measured in a Hitachi Perkin-Elmer spectrofluorometer. Cytochrome c, type III, was obtained from Boehringer. The phospholipids were prepared according to Fleischer et al. (5) microdispersed in aqueous buffer as described by Fleischer and Klouwen (6). Synthetic dipalmitoyl lecithin was obtained from Calbiochem. ANS (from K and K) was recrystallized twice from hot aqueous solutions of its Mg salt. All other reagents were analytical grade. The excitation wavelength for fluorescence was 340 nm that is an isobestic point in the cytochrome c spectrum.

Results. Valency change and ANS changes. The addition of the cytochrome c-cardiolipin complex to a solution of ANS in water resulted in a fluorescence enhancement (10-20 fold) and in a shift of the maximum emission wavelength of ANS from 520 nm to 470 nm.

The reduction of cytochrome c obtained by addition of 5 mM ascorbate and 50 μM TMPD (or by ascorbate alone) induced a 25% increase in fluorescence at 470 nm and approximately a 5 nm blue shift of the peak.(Fig. 1). The presence of all the phospholipids used in addition to cardiolipin, such as synthetic or natural lecithin, phosphatidylethanolamine, or mitochondrial phospholipids (5) resulted in similar changes in the ANS fluorescence that occurred upon reduction of cytochrome c. Only slight changes of the quantum yield and of the maximum emission wavelength were in fact observed.

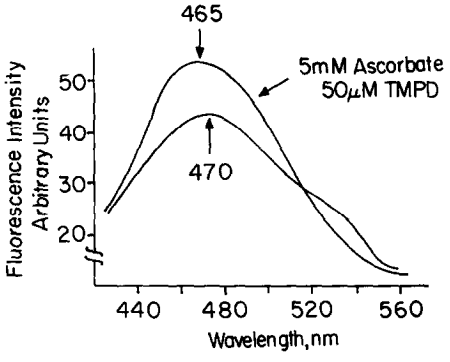

Figure 1. Fluorescence emission spectra of ANS bound to a cytochrome c-phospholipid complex, in the oxidized and in the reduced form. The incubation medium consisted of 0.275 M mannitol, 0.025 M sucrose, 0.02 M Tris-HCl, pH 7.4, 105 μM Cardiolipin, 27 μM Cytochrome c. The excitation wavelength was 340 nm.

Stoichiometry between phospholipid and cytochrome c. In the absence of added phospholipid ,little or no effect on ANS fluorescence was seen upon cytochrome c reduction. On the other hand, in the presence of phospholipid the increase in ANS fluorescence was proportional to the amount of phospholipid up to a fixed value where saturation was obtained.

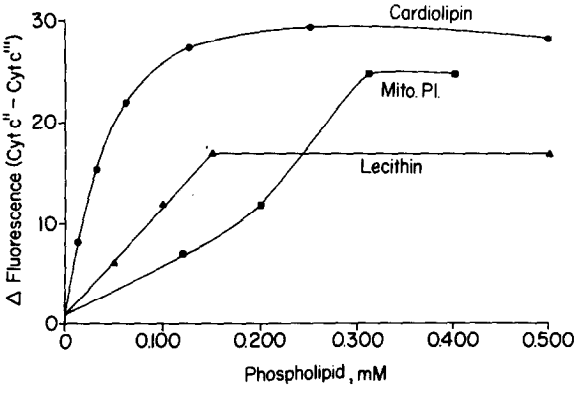

Figure 2. The titration of various phospholipids on the fluorescence changes of the ANS-Cytochrome c-phospholipid complex upon reduction. The experimental conditions were identical to figure 1. The different amounts of phospholipid present is indicated in the figure. Reduction of Cytochrome c was obtained by addition of 5 mM ascorbate. 50 μM TMPD were also present.

In Fig. 2 the phospholipid concentrations are plotted versus the fluorescence enhancement of ANS which results from reduction of cytochrome c in the presence of phospholipid. The half maximal effect of cardiolipin is obtained at a concentration of 30 μM, while lecithin and a total phospholipid extract from mitochondria have half maximum effects at 70 and 200 μM respectively. At saturation the molar ratios of cytochrome c-phospholipid present are 4:1 for cardiolipin, 5:1 for lecithin and 10:1 for total mitochondrial phospholipid.

In the presence of excess lecithin (not shown) the change of ANS fluorescence upon reduction of cytochrome c was found linearly proportional to the cytochrome c concentration up to 24 μM cytochrome c.

Discussion.

Binding of ANS to cytochrome c. The binding of ANS to either ferri- or ferro-cytochrome c is small, as judged by the maximum emission wavelength of the fluorescence and the lack of fluorescence enhancement. On the other hand, binding to a highly polar site could not be distinguished by using these parameters from the lack of binding.

ANS binding to the cytochrome c in the presence of phospholipid is apparent from both a blue shift from 520 nm to 470 nm and from the increase in quantum yield.

The bulk phase dielectric constant measured by the peak shift of ANS fluorescence to 470 nm corresponds to a value of about 25 and the more correct Z value (7) according to Turner and Brand (8) is of about 85. It is, of course, very difficult without making some assumptions to consider these values the corrected D or Z estimates of the ANS microenvironment in the complex, but it is instead very safe to conclude that the polarity of the ANS sites in the complex is lower than the environment of ANS in water, and therefore that the complex has regions to which ANS, but not water, have access.

Structural changes in the ferri- to ferro-cytochrome c transition. While no changes of ANS fluorescence are observable in the transition of ferri- to ferro-cytochrome c in the absence of lipids, changes in the fluorescence characteristics of bound ANS during such a transition in the presence of phospholipids are clearly observed. In particular the change in λ max and the increase in quantum yield of ANS parallel to reduction of cytochrome c are an indication that some of the ANS binding sites become more shielded from water in the reduced than in the oxidized form or alternatively, that water at the ANS binding site becomes more structured in the reduced state. In particular it can be speculated that such changes in the relative hydrophobicity of the ANS environment reflect changes in the structure of the protein during the transition from oxidized to reduced. The role of lipid may be in this case to create the possibility for ANS to interact with the hydrophobic regions of the cytochrome c molecules. But it can also be speculated that the structural transitions of the complex may be different from that of the free cytochrome c, and that these transitions are recorded by ANS. In this case the conformational

change can be located in either the lipid or the protein moieties of the complex or in both.

Stoichiometries between cytochrome c and lipids. The primary interaction of phospholipid microdispersions in water with cytochrome c is electrostatic in nature. The number of phospholipid molecules which can be associated with cytochrome c are 4 for cardiolipin (contains two phosphorus atoms), 8 for phosphatidyl inositol, about 80 for phosphatidyl ethanolamine and greater than 500 for lecithin. Cytochrome c has a net excess of 8 positive charges per molecule at pH 8. Thus interaction with cardiolipin and phosphatidylinositol are stoichiometric in terms of charge neutralization; lecithin a zwitterion combines to a negligible extent with cytochrome c (9,10). On the other hand, the transition in ANS fluorescence upon reduction of cytochrome c is maximal when 4 cardiolipin, 5 lecithin, and 10 total mitochondrial lipid molecules are added. This may suggest at least two hypotheses: a) only the first sites occupied by phospholipid are necessary for seeing the ANS response: or, b) that the effect of phospholipid in this case is different in nature and involves an other type of binding onto other protein sites. We favor the latter interpretation.

In conclusion, evidence that structural changes occur during the transition ferri- to ferro-cytochrome c in a cytochrome c-lipid complex has been presented. The system described can be considered a model system of protein-lipid interaction, in which the reactivity of the single components is different from the reactivity of the complex. It can also be considered a system that is closer in its characteristics to the cytochrome c in its natural environment. In this case, a change in the structure of the complex during redox transitions may play an important role in the mechanism of electron transport and also of energy conservation in mitochondria.

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