FLUORESCENCE STUDIES OF A CYTOCHROME c MIXED PHOSPHOLIPID COMPLEX[+]

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Summary: The fluorescent probe 8-anilino-1-naphthalene sulphonic acid (ANS) has been used to demonstrate the accessibility of the heme of cytochrome c - mixed mitochondrial phospholipid complexes to the solvent. Contrary to earlier reports fluorescence techniques using ANS do not detect redox induced conformational changes of these complexes.

Cytochrome c *in vivo* is probably complexed with the phospholipids of the mitochondrion (1,2). Consequently cytochrome c - phospholipid complexes have been investigated as a model for endogenous cytochrome c (3,4). In view of the extensive conformational changes that cytochrome c undergoes following changes of oxidation state (5), Azzi *et al*. (6) have recently investigated the occurrence of similar conformational effects in model cytochrome c - phospholipid complexes undergoing redox changes. They reported that the fluorescent intensity of 8-anilino-1-naphthalene sulphonic acid (ANS), a probe for hydrophobic environments, was enhanced by cytochrome c - phospholipid complexes and further increased following reduction of the complexes. We have reinvestigated and extended these fluorescence studies on cytochrome c - mixed mitochondrial phospholipid complexes using the

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heme induced quenching of ANS fluorescence (7).

We have demonstrated the following: The quenching of ANS fluorescence by cytochrome c is identical, irrespective of whether the protein is in the free form or complexed to phospholipids. The fluorescent intensity of ANS in the presence of cytochrome c - phospholipid complexes is not altered following valency change of the heme iron. Previous results (6) on ANS fluorescence changes reported to be due to ascorbate reduction of cytochrome c - phospholipid complexes are, in fact, not a function of the reducing properties of ascorbate and are almost independent of the presence of cytochrome c.

Materials and Methods

Horse heart cytochrome c (Grade I, 95% pure, 0.425% Fe) purchased from Miles Seravac, Maidenhead, England, was oxidised with potassium ferricyanide or reduced with sodium dithionite and purified by passage through a Sephadex G-25 column. Mixed beef heart mitochondrial phospholipids and their complex with cytochrome c were prepared as previously described (Ivanetich, Henderson and Kaminsky, submitted). Both free and complexed phospholipid comprised 9.5% cardiolipin, 39.4% phosphatidylethanolamine and 51.1% phosphatidylcholine. The complex and the phospholipids were ultrasonically dispersed or solubilized in sodium deoxycholate. ANS was recrystallised twice as its magnesium salt. Sodium deoxycholate was from Merck, bacteriology grade. Dicarboxymethylmethionine cytochrome c (8) and succinylated cytochrome c modified at over 16 lysine residues (9) were prepared as described in the literature. Water was distilled and deionized.

Fluorescence spectra were determined with a Perkin Elmer 203 spectrofluorometer. The excitation wavelength was 340 nm and emission spectra were scanned over the range 440-520 nm (6).

All assay mixtures contained 0.275 M mannitol, 0.025 M sucrose, 0.02 M Tris-HCl buffer, pH 7.4 and combinations of 0.025 M sodium deoxycholate, cytochrome c, phospholipids and complex as indicated in the figure legends.

Results and Discussion

Ultrasonically dispersed mixed phospholipids and deoxycholate both markedly enhance the fluorescence intensity band of ANS at 470 nm. A solution of phospholipid in deoxycholate, however, enhances the ANS fluorescence to a lesser extent than does either dispersed phospholipid or deoxycholate alone (Figure 1). Addition of cytochrome c, cytochrome c - phospholipid complex or cytochrome c plus phospholipid to aqueous buffered solutions of ANS in deoxycholate quenches the ANS fluorescence to an identical extent, when corrected for the slight quenching due to the lipid (Figure 1). Dispersions of cytochrome c - phospholipid complex quench the ANS fluorescence relative to that arising from dispersions of phospholipid alone. Since it is the heme moeity of hemoproteins which quenches fluorescence (7) it is clear from these results that the phospholipid sheath around the cytochrome c in the complex does not prevent access of the solvent to the exposed edge of the heme (5) or to some part of the surface of the protein which provides access for electrons to the heme. Thus the presence of the phospholipids would not prevent electron transfer to and from the heme iron, if it occurs via the exposed heme edge, or from the protein surface.

Addition of ascorbate to mixed phospholipids dispersed in solutions of ANS and cytochrome c as described by Azzi et al. (6) enhanced the fluorescence emission of the ANS at 470 nm. The effectiveness of ascorbate in enhancing ANS fluorescence increased with increasing quantities of phospholipid until a plateau was

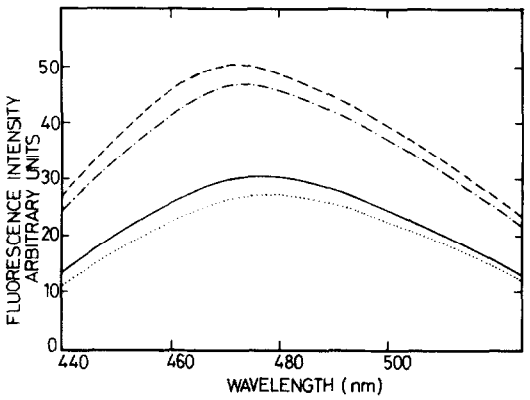

Fig. 1.

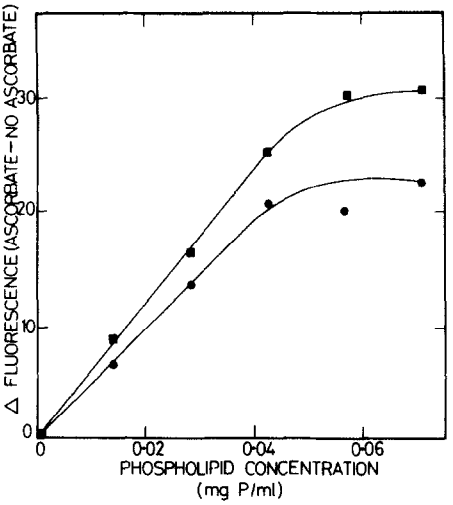

Fig. 2.

Figure 1. Fluorescence emission spectra of ANS in solutions of: (— — —) 0.025 M sodium deoxycholate; (—·—·—) 0.03 mg P/ml (0.75 mg phospholipid/ml) mixed mitochondrial phospholipids in 0.025 M sodium deoxycholate; (——) 13 μM ferricytochrome c in 0.025 M sodium deoxycholate; (·····) cytochrome c - phospholipid complex (13 μM cytochrome c and 0.03 mg P/ml phospholipids) in 0.025 M sodium deoxycholate. 7.2 x 10^{-5} mM ANS, 50 mM ascorbate.

Figure 2. The influence of mixed phospholipid concentration on the enhancing effect of ascorbate on ANS fluorescence in the presence of: (●) 13 μM cytochrome c and ultrasonically dispersed phospholipid; (■) ultrasonically dispersed phospholipid alone. 7.2 x 10^{-5} mM ANS, 50 mM ascorbate.

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reached (Figure 2). We have now shown that this effect of ascorbate is not due to its reducing properties since other carbohydrates such as glucose and glucuronic acid elicit similar responses, perhaps by altering the micellar properties of the lipid to provide a more hydrophobic environment for the ANS. Attempts to mimic reduction of the cytochrome c by ascorbate using dithionite apparently also resulted in reduction of the ANS with a consequent complete disappearance of fluorescence.

Dicarboxymethylmethionine cytochrome c, in which the environment of the heme is significantly altered from cytochrome c (10,11), behaved identically with cytochrome c in this system. Consequent-

ly structural features of the heme environment of cytochrome c are not likely to play a role in these effects. The enhanced fluorescence intensity with increasing lipid concentration (Figure 2) is not dependent on the formation of a cytochrome c-phospholipid complex since succinylated cytochrome c, which does not complex with mixed phospholipids (Ivanetich, Henderson and Kaminsky, submitted), yields identical results.

When the system was constituted with reduced cytochrome c, from which all traces of reducing agent were removed and the cytochrome c permitted to autoxidise, no difference in the fluorescence emission was observable. It is thus clear that cytochrome c - phospholipid complexes enhance ANS fluorescence to the same extent irrespective of the oxidation state of the cytochrome c and that previous reports of redox induced changes (6) are only the result of non-specific effects of ascorbate on the phospholipid ANS interaction. Finally when cytochrome c is omitted completely from the reaction mixture the results are not significantly altered (Figure 2). The slightly diminished effectiveness of ascorbate in enhancing ANS fluorescence in the presence of cytochrome c possibly results from alterations in the micellar properties of the phospholipids due to the presence of the protein.

In conclusion it is apparent that the structural changes known to occur when cytochrome c undergoes redox changes cannot be detected using this fluorescent technique and that the previous report concerning the use of this technique to detect such conformational changes for cytochrome c - phospholipid complexes and the relevance of such changes for the mechanism of electron transport cannot be substantiated.

References

1. D.E. Green and S. Fleischer, Biochim.Biophys.Acta, 70, 554 (1963).

2. K.S. Ambe and F.L. Crane, Science, 129, 98 (1959).
3. M. Reich and W.W. Wainio, J.Biol.Chem., 236, 3058 (1961).
4. H.K. Kimelberg, C.P. Lee, A. Claude and E. Mrena, J.Membrane Biol., 2, 235 (1970).
5. T. Takano, R. Swanson, O.B. Kallai and R.E. Dickerson, Cold Spring Harbour Symposium on Quantitative Biology, 36, 397 (1971).
6. A. Azzi, S. Fleischer and B. Chance, Biochem.Biophys.Res.Commun., 36, 322 (1969).
7. C-G. Rosén, FEBS Lett., 6, 158 (1970).
8. A. Schejter and I. Aviram, J.Biol.Chem., 245, 1552 (1970).
9. K. Wada and K. Okunuki, J.Biochem., 64, 667 (1968).
10. L.S. Kaminsky, V. Miller and K. Ivanetich, Biochem.Biophys. Res.Commun., 48, 1609 (1972).
11. R.M. Keller, I. Aviram, A. Schejter and K. Wüthrich, FEBS Lett., 20, 90 (1972).