FLUORESCENCE STUDIES OF CYTOCHROME $\underline{c}_1$ AND A CYTOCHROME $\underline{c}_1$-CYTOCHROME $\underline{c}$ COMPLEX[†]

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Summary: Cytochrome $\underline{c}_1$ and cytochrome $\underline{c}$ form a complex in aqueous solution. The complex is stable to chromatography on Sephadex but is dissociated in media of high ionic strength. Results from the fluorescence probe technique using 8-anilino-1-naphthaline sulphonic acid strongly suggest that (i) the heme group of cytochrome $\underline{c}_1$ is completely buried in the protein moiety, in contrast to cytochrome $\underline{c}$; (ii) ferricytochrome $\underline{c}_1$ undergoes a conformation change on reduction which produces a more tightly closed structure; and (iii) in the cytochrome $\underline{c}$-cytochrome $\underline{c}_1$ complex the edge of the cytochrome $\underline{c}$ heme group evidently continues to be exposed.

Cytochrome $\underline{c}_1$ plays a key role in mitochondrial bioenergetics by transferring an electron from cytochrome $\underline{b}$ to cytochrome $\underline{c}$. Thus, knowledge of the structure and function of cytochrome $\underline{c}_1$ is essential for understanding the mechanism of electron transport in the mitochondrion. Our recent purification of cytochrome $\underline{c}_1$ (1) has made it possible to obtain samples of modest quantities to initiate such studies. Cytochrome $\underline{c}$ has been the most extensively investigated of the respiratory heme proteins, as a consequence of its ready availability. The spectral similarities and identical prosthetic groups (1) of cytochromes $\underline{c}_1$ and $\underline{c}$ as well as their similar functions of electron transfer enable these previous studies to be used as initiation points for investigations of cytochrome $\underline{c}_1$.

The possible existence of a cytochrome $\underline{c}_1$-cytochrome $\underline{c}$ complex in mitochondria may be deducible from the concept of the respiratory chain. We report here on the preparation of such a complex and on structural studies

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of this complex and cytochrome $\underline{c}_1$ using 8-anilino-1-naphthalene sulphonic acid (ANS) as a fluorescent probe.

Materials and Methods

Horse heart cytochrome $\underline{c}$ (Type III) was purchased from Sigma and beef heart ferrocytochrome $\underline{c}_1$ was prepared according to a method developed in this laboratory (1). ANS was purified and used as its magnesium salt. Water was double distilled and deionized. All other chemicals were of the highest grade commercially available. Ferricytochrome $\underline{c}_1$ was prepared by oxidation of ferrocytochrome $\underline{c}_1$ using potassium ferricyanide and purified by passage through a Sephadex G-25 column. The complex of cytochrome $\underline{c}_1$ and cytochrome $\underline{c}$ was prepared by mixing the two components in aqueous solution in equimolar ratios and isolated by chromatographing on a column of Sephadex G-50. Fluorescence spectra were determined using a Baird-Atomic Fluorispec Model SF-1 fluorescence spectrophotometer with four grating monochrometers. The excitation wavelength was 340 nm and emission spectra were scanned over the range 400-600 nm (2) with slit openings of 32 nm for excitation and for fluorescence. All spectra were determined at room temperature in solutions containing 0.275 M mannitol to enhance fluorescence.

Results and Discussion

When cytochrome $\underline{c}_1$ and cytochrome $\underline{c}$ were mixed in aqueous solution of low ionic strength (10 mM phosphate buffer), a complex formed which was isolated by passage through a Sephadex G-50 column. The formation of the complex was evidently from a protein-protein interaction since neither cytochrome contained lipid. The complex was dissociated to its components in solutions of high ionic strength (0.5 M phosphate buffer, pH 7.4). The stoichiometry of the complex together with details of its properties and physiological implications will be reported elsewhere.

Heme proteins have been demonstrated to quench the fluorescence of molecules such as ANS as a consequence of the exposure of their heme moieties to the solvent (3, 4). Cytochrome $\underline{c}$ has one edge of its heme group protrud-

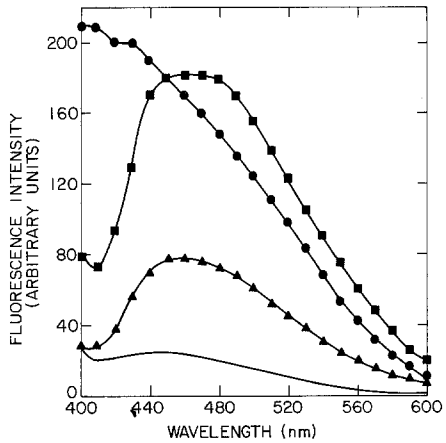

Figure 1. Fluorescence emission spectra of ANS in solutions of: ●——●, sodium cholate, 0.07%; ■——■, ferricytochrome c_1, 13 μM, and sodium cholate, 0.07%; ▲——▲, ferricytochrome c, 13 μM, and sodium cholate, 0.07%; ————, no addition. ANS was 2.8×10^{-4} mM in 10 mM sodium phosphate buffer, pH 7.4, containing 0.275 M mannitol.

ing into the solvent (5) and the heme is consequently able to quench (3) the fluorescence of ANS which is induced by binding to cholate (cf. Fig. 1). In contrast, ferricytochrome c_1 did not quench ANS fluorescence in cholate at all although a shift in the wavelength of maximum emission was noted as shown in Fig. 1. This fact strongly suggests that the heme group of cytochrome c_1 is buried in the protein structure and not exposed to the solvent, in contrast to the case of cytochrome c. The buried nature of this heme group might be due to the fact that cytochrome c_1 is most probably isolated as a pentamer (1) and that interactions of the monomeric units with one another could result in the burying of the heme groups. However, isolated cytochrome c_1 is able to rapidly transfer an electron to cytochrome c with a bimolecular rate constant of about 3×10^6 M^{-1} sec^{-1} (6). The above result may suggest that the transfer of electrons between soluble cytochromes c_1 and c is not likely to be through direct heme-heme interactions. A pathway through a limited region of the protein has been proposed for the electron entering the heme iron of cytochrome c (7), but a contrasting theory which requires the electron to enter cytochrome c through the exposed heme edge

has certainly not been excluded (8, 9). A similar exposed edge pathway for electrons in cytochrome $\underline{c}_1$ now appears to be less likely.

These results, which suggest that the heme of cytochrome $\underline{c}_1$ is less accessible to solvent than that of cytochrome $\underline{c}$, are in line with an earlier report that cytochrome $\underline{c}_1$ is less autoxidizable than cytochrome $\underline{c}$ (1).

Attempts to monitor the known conformational change of cytochrome $\underline{c}$ when it undergoes redox changes using hydrophobic fluorescent probes have failed as a consequence of the quenching effect of the heme (2, 3). In the case of cytochrome $\underline{c}_1$, however, the fluorescence of the probe was markedly affected by the redox state of the cytochrome. When bound to reduced cytochrome $\underline{c}_1$, the fluorescence of ANS was diminished relative to when it was bound to the oxidized protein (Fig. 2). Oxidation of the reduced protein

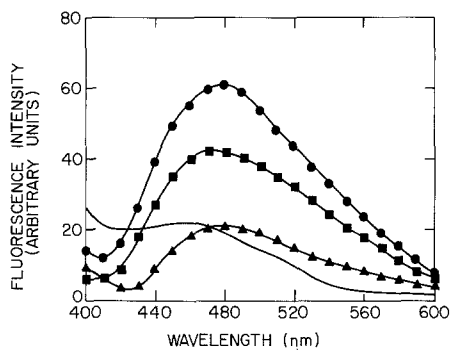

Figure 2. Fluorescence emission spectra of ANS in solutions of: ●——●, ferricytochrome $\underline{c}_1$, 13 μM; ▲——▲, ferrocytochrome $\underline{c}_1$, 13 μM; ■——■, the oxidized form of the cytochrome $\underline{c}_1$-$\underline{c}$ complex 13 μM with respect to $\underline{c}_1$; and ————, no addition. The concentrations of ANS, buffer and mannitol were the same as in Fig. 1.

with ferricyanide produced increases of fluorescence intensity of the bound ANS back to levels obtained with the oxidized cytochrome $\underline{c}_1$.

The diminished fluorescence in the presence of ferrocytochrome $\underline{c}_1$ could arise from a reduction induced conformational change leading to an exposed heme and consequent quenching of fluorescence. We consider that this is unlikely since reduced cytochrome $\underline{c}_1$ did not quench cholate induced

fluorescence of ANS. An alternative and more plausible explanation is that on reduction the conformation of cytochrome $\underline{c}_1$ becomes more tightly closed and that hydrophobic regions of the protein, which in the oxidized state are available to the solvent and thus to ANS, become buried and are no longer able to bind ANS. Such a change would result in a diminished fluorescence intensity for ANS. It is thus apparent that the cytochrome $\underline{c}_1$ is similar to cytochrome $\underline{c}$ in that the conformation of its reduced state is more tightly closed than that of its oxidized state.

The addition of the complex of cytochrome $\underline{c}_1$ and cytochrome $\underline{c}$ to ANS produced a fluorescence intensity lower than that arising from ANS and cytochrome $\underline{c}_1$ alone (Fig. 2). It is clear that the cytochrome $\underline{c}$ in the complex produces a quenching of the fluorescence due to cytochrome $\underline{c}_1$, which indicates that the heme group of cytochrome $\underline{c}$ is still exposed to the solvent. The complexing of the cytochromes thus cannot be at the exposed heme edge of cytochrome $\underline{c}$ since this would be expected to severely restrict the solvent exposure to the heme.

In conclusion, we have demonstrated that cytochrome $\underline{c}_1$ undergoes a conformational change on reduction which results in a more tightly closed structure. The heme group of cytochrome $\underline{c}_1$ appears to be deeply buried in the protein without an exposed edge. In the complex of cytochrome $\underline{c}_1$ with cytochrome $\underline{c}$ the heme group of cytochrome $\underline{c}$ is still exposed to the solvent and thus binding of the cytochromes is most probably not at the exposed heme edge of cytochrome $\underline{c}$.

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