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ELECTRON TRANSFER BETWEEN LIPOSOMAL CYTOCHROME c_1 AND CYTOCHROME c: CATALYTIC IMPLICATIONS OF ELECTROSTATIC POTENTIALS

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Kinetics measurements of the electron transfer between ferricytochrome c and liposomal ferrocytochrome c_1 (with and without the hinge protein) were performed. The observed rate constants(k_{obs}) of electron transfer between liposomal ferrocytochrome c_1 and ferricytochrome c at different ionic strengths were measured in cacodylate buffer, pH 7.4, at 2 C. The effect of ionic strength on the rate constant(k_{obs}) of electron transfer between liposomal cytochrome c_1 and cytochrome c is far greater than that in the solution kinetics (Kim, C.H., Balny, C. and King, T.E.(1987) J. Biol. Chem. **262**, 8103-8108). The result demonstrates that the membrane bound cytochrome c_1 creates a polyelectrolytic microenvironment which appears to be involved in the control of electron transfer and can be modulated by the ionic strength. The involvement of electrostatic potentials in the electron transfer between the membrane bound cytochrome c_1 and cytochrome c is discussed in accord with the experimental results and a polyelectrolyte theory.

The biological electron transfer reaction has been extensively studied in a number of systems(1-2 and refs. therein). Nevertheless, the molecular mechanism, the structural pathway and factors controlling the rate of electron transfer between redox partners still remains to be explained. Particularly, there are many questions to be answered regarding the geometrical and physical requirements for the efficient electron transfer between proteins which form the transient complexes(3). One of these extensively studied systems is the electron transfer reaction between bovine heart mitochondrial cytochrome c_1 and cytochrome c. The molecular mechanism for electron transfer between cytochrome c_1 and cytochrome c in

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the cardiac mitochondrial respiratory chain still remains to be clarified, in spite that considerable advances in our knowledge on the electron transfer reaction between purified cardiac cytochrome $\underline{c}_1$ and cytochrome $\underline{c}$ has been made (4-7).

The discovery of the hinge protein (Hp) (8,9), which plays a role in the interaction between solubilized cytochrome $\underline{c}_1$ and cytochrome $\underline{c}$, has given some insight in the electron transfer reaction mechanism between cardiac cytochrome $\underline{c}_1$ and cytochrome $\underline{c}$. Recently, our studies on the electron transfer reaction between cytochrome $\underline{c}_1$ and cytochrome $\underline{c}$, in the presence and absence of the hinge protein (6,7,10) suggest that the hinge protein may be involved in the regulation of electron transfer reaction between these two cytochromes, via the formation of a complex, $\underline{c}_1$-Hp-$\underline{c}$.

In situ, cytochrome $\underline{c}_1$ and the hinge protein exist as part of a large complex which is in turn embedded into a membrane (10). To clarify the molecular mechanism of electron transfer between cytochrome $\underline{c}_1$ and cytochrome $\underline{c}$ in physiological condition, we conducted a model study in that kinetics measurements were performed for the reaction between ferricytochrome $\underline{c}$ and liposomal ferrocytochrome $\underline{c}_1$ (with and without the hinge protein). The results showed that the observed rate constants (k_{obs}) of electron transfer between liposomal cytochrome $\underline{c}_1$ and cytochrome $\underline{c}$ were strongly affected by ionic strength in comparison to those in solution. And this observation is interpreted according to polyelectrolyte theory(11-17). In addition, characteristic difference spectra of $[(c_1^{2+} + \underline{c}^{3+}) - (c_1^{3+} + c^{2+})]$ were detected at higher ionic strength ($I > 0.5$ M) in rapid scanning measurements.

Preliminary investigation described in this communication is the first attempt to demonstrate that the membrane bound cytochrome $\underline{c}_1$, probably by a special structural arrangement, creates a polyelectrolytic micro-environment, which appears to be involved in the electron transfer reaction and modulated by ionic strength. This could be considered as a regulatory model for the *in vivo* reaction, although it remains speculative until a thorough investigation is accomplished.

Material and Methods

Materials -- Sephadex G-75 were purchased from Pharmacia P-L Biochemicals, and cacodylic acid, cytochrome $\underline{c}$ (Type III) and azolectin were purchased from Sigma. All other chemicals were the highest grade commercially available.

Cytochromes $\underline{c}_1$ (with and without the hinge protein) were prepared by the method published (18). Liposomal cytochrome $\underline{c}_1$ was prepared by incorporating purified cytochrome $\underline{c}_1$ into azolectin vesicles using the method of Bonfils et al (19) with a slight modification. Incorporation of cytochrome $\underline{c}_1$ into lipid vesicles was achieved by chromatography on Sephanex G-75 of a detergent solubilized mixture of enzyme and lipids(20). 50 mg of purified azolectin by the reported method (21), were sonicated in 1.5 ml of 50 mM cacodylate buffer, pH 7.4 containing 2% cholate to clearness in ice bath. 0.2 umol of one-band $\underline{c}_1$ or two-band $\underline{c}_1$ were mixed into 15 mg of azolectin in a total volume of 0.7 ml of 50 mM cacodylate buffer, pH 7.4 containing 1.5% cholate and incubated for 30-40 min in an ice bucket. The mixture of cytochrome $\underline{c}_1$ and azolectin were then passed through Sephadex G-75 column preequilibrated with the 50 mM cacodylate buffer, pH 7.4. Liposomal cytochrome $\underline{c}_1$ was eluted in the void volume of the column.

Fast kinetics and rapid-scanning measurements -- Stopped-flow and rapid-scan measurements were performed using a special stopped-flow mixing device, designed by Dr. Balny and built by the Institut National de la Sante et de la Recherche Medicale (INSERM), Montpellier, adapted to a Union Giken (Model RA 415 and 401) fast response spectrophotometer, or to the cell compartment of the Aminco DW2 spectrophotometer. The temperature of syringes and mixing chamber of the stopped-flow were maintained at 2 $\pm$ 0.1°C with a circulating Haake bath model f3-Q. Rapid-scan absorption spectra were measured with a multichannel photodiode (maximum speed, 95 x 10^3 nm sec^{-1}). The spectra were stored in a memory unit and then analyzed with a digital computer system. The observed rate constants were determined from a nonlinear squares analysis using a computer fitting program as described before (6,7).

Results and Discussion

The reactions of liposomal $\underline{c}_1{}^{2+}$, with or without the hinge protein, and $\underline{c}^{3+}$ were measured after stopped-flow rapid mixing of the two cytochromes in sodium cacodylate buffer, pH 7.4, at 2 ° C as a function of ionic strength. The concentrations were 0.75 μM and 2.5 μM for cytochromes $\underline{c}_1{}^{2+}$ and $\underline{c}^{3+}$, respectively. The recorded wavelength for these kinetics were 423 minus 415 nm or 409 minus 415 nm in the dual mode of Aminco DW2 spectrophotometer. As shown in Fig. 1, optical difference spectra yielded a characteristic spectrum of (liposomal $\underline{c}_1{}^{2+}$ - $\underline{c}^{3+}$) - (liposomal $\underline{c}_1{}^{3+}$ - c^{2+}) giving an absorbance decrease at 423 nm and increase at 409 nm as seen in the case of liposome free soluble cytochrome $\underline{c}_1$ in presence or absence of the hinge protein (6.7). The data indicate that the reaction occurs via the formation of transient intermediates as we reported previously in solution kinetics:

$$c_1{}^{2+} + \underline{c}^{3+} \rightleftarrows (c_1{}^{2+} \cdot \underline{c}^{3+}) \rightleftarrows (c_1{}^{3+} \cdot c^{2+}) \rightleftarrows c_1{}^{3+} + c^{2+}.$$

However, The k_{obs} at either 423 minus 415 or 409 minus 415 nm was found to be ionic strength dependent as shown in Table 1. These results are

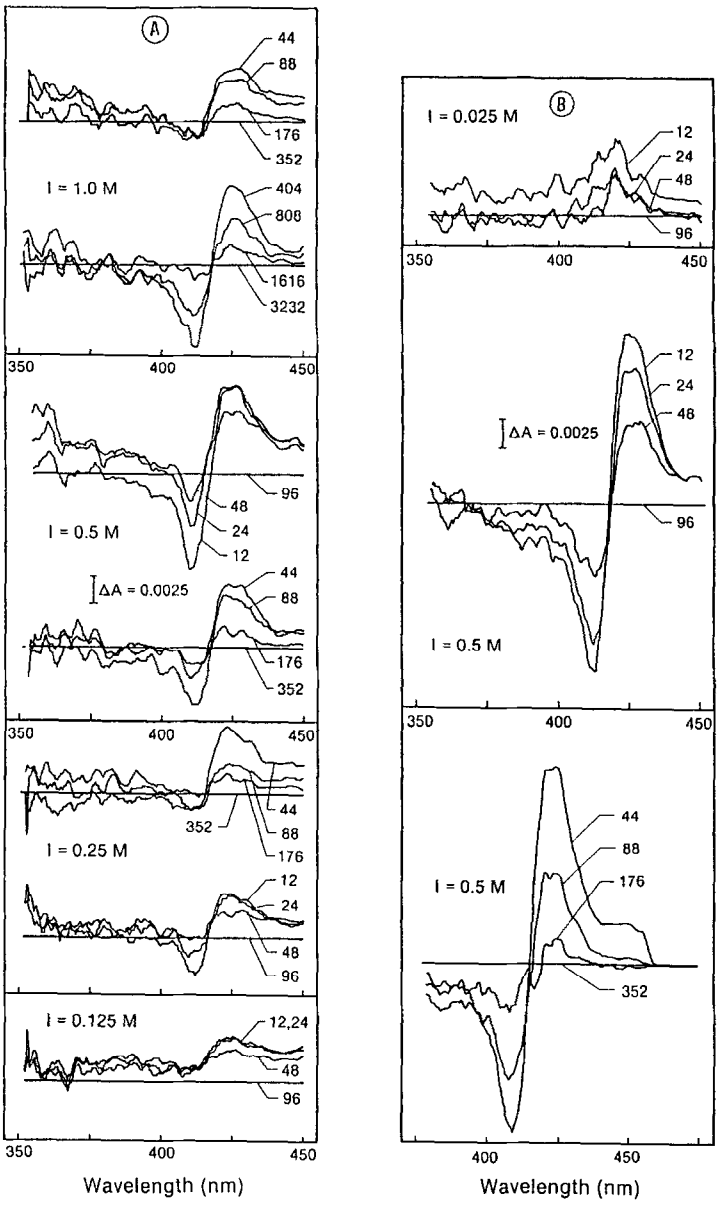

Figure 1. Rapid-scan spectrophotometric measurements of the reaction between liposomal ferrocytochrome $\underline{c}_1$ (with the hinge protein) and ferricytochrome $\underline{c}$ at various ionic strengths. The spectra are the computer-calculated difference spectra against the reference to the final spectrum (flat lines are final spectra). Scanning time was 2 msec for each spectrum. The numbers on each spectrum indicate the elapsed time after mixing, in msec. The concentrations of liposomal $\underline{c}_1^{2+}$ and $\underline{c}^{3+}$ are 0.75 μM and 2.5 μM, respectively in A , and 5 μM and 2.5 μM, respectively, in B . I, ionic strength in cadodylate buffer, pH 7.4, at 2°C.

different from the data found in the case of soluble cytochromes. In the case of reaction between cytochrome $\underline{c}_1$ without the hinge protein and cytochrome $\underline{c}$ in solution, both the amplitude of the spectra and the rate

Table 1. Ionic strength dependence of the k_{obs} measured for oxidation of liposomal ferrocytochrome $\underline{c}_1$(without the hinge protein) by ferricytochrome $\underline{c}$ in cacodylate buffer, pH 7.4, 2°C

I (ionic strength)	k_{obs},(s^{-1}) at 423 - 415 nm	k_{obs},(s^{-1}) at 409 - 415 nm
0.05 M	too fast to be recorded	too fast to be recorded
0.125 M	11.4 ± 2	12.1 ± 2
0.25 M	6.6 ± 0.8	7.1 ± 1
0.5 M	1.06 ± 0.05	1.3 ± 0.2
0.75 M	0.81 ± 0.05	0.86 ± 0.06

$\underline{c}_1^{2+}$ and $\underline{c}^{3+}$ concentrations are 0.75 μM and 2.5 μM, respectively.

constants were found to be independent of the ionic strength of cacodylate buffer used from 0.02 to 0.5 M with k_{obs} = 48 ± 9 sec^{-1} (6).

The strong ionic strength dependence observed in the rate of reaction between liposomal cytochrome $\underline{c}_1$ and cytochrome $\underline{c}$ can be interpreted according to the polyelectrolyte theory. This theory has been developed in various papers (11-17). And the polyelectrolyte theory states that the microenvironment of the polyelectrolyte, to which the enzyme is bound or which is created by enzymes themselves, is exposed to the electrostatic field developed by the permanent electric charges. In these conditions, the local concentration of any charged molecules (protein, enzyme, ion, etc.) are modified by the local electrostatic potential according to the polyelectrolyte theory. As the ionic strength can modify the electrostatic potential by a screening effect, the pH and ionic strength are tightly interdependent in this process. In the present experiments, the ionic strength is varied at constant pH, and the electrostatic potential is decreased as ionic strength is increased. Therefore, the measured potential is decreased as the logarithm of ionic strength is increased. According to the Debye-Huckel theory, the electrostatic portential depends on the ionic strength through the electrostatic screening of the mobile charges, and in the first approximation, $\psi = A - B \log I$, where A and B are constants (16). When k_{obs} depends on log I, it can be concluded that the reaction is affected by the local electrostatic potential ψ (16). The opposite effects would be observed depending on whether the reaction is carried out in the presence of polyanion or polycation.

In current experiments, cytochrome $\underline{c}_1$ has a hydrophobic cluster in the COOH-terminal region which can be buried in liposome and cytochrome $\underline{c}_1$ being negative (pI = 5.8) at pH 7.4, a simplified model of the cytochrome

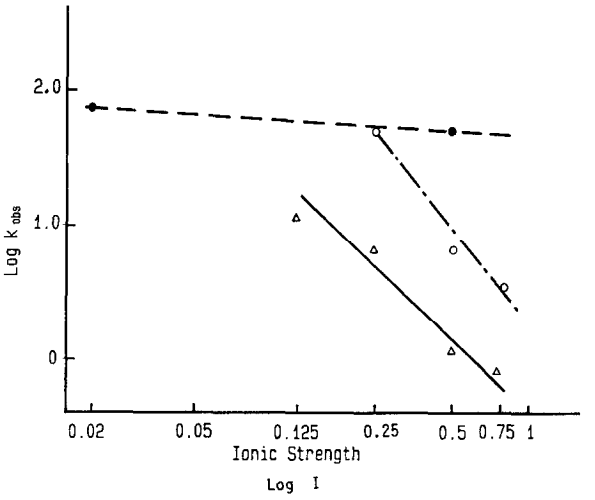

Figure 2. Dependence of the ionic strength of cacodylate buffer on the k_{obs} of the electron transfer reaction of liposomal ferrocytochrome $\underline{c}_1$ and ferricytochrome $\underline{c}$. Solution conditions; pH 7.4, 2°C. (●---●---●), cytochrome $\underline{c}_1$ with the hinge protein (2.5 μM) and cytochrome $\underline{c}$(2.5 μM) in solution (from ref.7). (○———○), liposomal $\underline{c}_1$ with the hinge protein (0.75 μM) and cytochrome $\underline{c}$(2.5 μM). (△———△), liposomal $\underline{c}_1$ (0.75 μM) and cytochrome $\underline{c}$(2.5 μM).

$\underline{c}_1$-liposome complex can be postulated. The cytochrome $\underline{c}_1$-liposome complex can be considered like a polyanionic system generating the electrostatic potential ψ which depends on ionic strength. This dependence is very well verified for k_{obs} as shown in Fig. 2.

At lower ionic strength (I < 0.125), the electron transfer reaction between liposomal cytochrome $\underline{c}_1$ and soluble cytochrome $\underline{c}$ was too fast to measure k_{obs} as shown in Table 1. Certainly, the reaction between cytochrome $\underline{c}$ and liposomal cytochrome $\underline{c}_1$ is much faster than that of two cytochromes in solution. This can be explained that cytochrome $\underline{c}_1$ may undergo a conformational rearrangement when its solution form is incorporated into liposome. Probably the conformation of cytochrome $\underline{c}_1$ in liposome is in a favorable orientation to interact with cytochrome $\underline{c}$. When ionic strength increased, the screening effect occurs and lowers the electrostatic potential of the reaction between liposomal cytochrome $\underline{c}_1$ and cytochrome $\underline{c}$. As a result, the rate of electron transfer become slower.

The present data suggest that the control of the electron transfer of this system, by slight changes in ionic strength, might be mediated through "external modulations" of the electrostatic potential promoted by the spacial architecture of liposomal cytochrome $\underline{c}_1$.

The result presented here is particularly important as it is compared to the other in vitro investigations. A large number of enzyme systems in a particular molecular structure (membrane, cell walls, mitochondria) present a similar, but not thoroughly understood behavior(11-13). The modulation of activity (k_{cat}) and affinity (K_m) of these systems as a function of both pH and ionic strength has been interpreted according to the polyelectrolyte theory. One of these examples is the enzyme, cytochrome c oxidase which contains a large amount of phospholipids (22). Similar observations have been reported for the hydrolysis of RNA by ribonuclease (23,24), as well as for the reaction involving small molecules as substrate (25). Recently, Cusanovich's group also reported that the effect of ionic strength on the redox potential of a variety of c type cytochromes can be explained by a simple Debye-Huckel model combined with consideration of ion binding(26).

The model studied above provides the insight and clues necessary to understand the in situ behavior of electron transfer reaction of membrane bound enzymes. Further experiments are needed to clarify the phenomena. However, the present data suggest that the control of electron transfer by modulations of electrostatic potentials might be an important regulatory mechanism. Moreover, with these preliminary results, it is clear that the membrane indeed has an important effect on the electron transfer process, and the role of the hinge protein may be in part to mediate the electrostatic effects. These are further to be investigated by altering the pH and the ionic strength of the reaction medium.

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