A Complex of Cardiac Cytochrome $c_1$ and Cytochrome $c^*$

(Received for publication, May 27, 1975)

YOUNG-LING CHIANG, LAURENCE S. KAMINSKY,‡ AND TSOO E. KING

From the Department of Chemistry and The Laboratory for Bioenergetics, State University of New York at Albany, Albany, New York 12222

The interactions of cytochrome $c_1$ and cytochrome $c$ from bovine cardiac mitochondria were investigated. Cytochrome $c_1$ and cytochrome $c$ formed a 1:1 molecular complex in aqueous solutions of low ionic strength. The complex was stable to Sephadex G-75 chromatography. The formation and stability of the complex were independent of the oxidation state of the cytochrome components as far as those reactions studied were concerned. The complex was dissociated in solutions of ionic strength higher than 0.07 or pH exceeding 10 and only partially dissociated in 8 M urea. No complexation occurred when cytochrome $c$ was acetylated on 64% of its lysine residues or photooxidized on its 2 methionine residues. Complexes with molecular ratios of less than 1:1 (i.e., more cytochrome $c$) were obtained when polymerized cytochrome $c$, or cytochrome $c$ with all lysine residues guanidinated, or a "1-65 heme peptide" from cyanogen bromide cleavage of cytochrome $c$ was used. These results were interpreted to imply that the complex was predominantly maintained by ionic interactions probably involving some of the lysine residues of cytochrome $c$ but with major stabilization dependent on the native conformations of both cytochromes. The reduced complex was autooxidizable with biphasic kinetics with first order rate constants of $6 \times 10^{-4}$ and $5 \times 10^{-8}$ s$^{-1}$ but did not react with carbon monoxide. The complex reacted with cyanide and was reduced by ascorbate at about 32% and 40%, respectively, of the rates of reaction with cytochrome $c$ alone. The complex was less photoreducible than cytochrome $c_1$ alone. The complex exhibited remarkably different circular dichroic behavior from that of the summation of cytochrome $c_1$ plus cytochrome $c$. We concluded that when cytochromes $c$, and $c$ interacted, they underwent dramatic conformational changes resulting in weakening of their heme crevices. All results available would indicate that in the complex cytochrome $c_1$ was bound at the entrance to the heme crevice of cytochrome $c$ on the methionine-80 side of the heme crevice.

The concept of the respiratory chain as the basis of bioenergetics was actually conceived some 50 years ago by Keilin with his discovery of cytochrome (2) and further defined by Keilin and Hartree (cf. Ref. 3) and other investigators in the field (cf. Ref. 4). The formation of a complex of cytochrome $c_1$ and cytochrome $c$ can be deduced from this concept. However, it was only with the recent availability of a sufficient quantity of cytochrome $c_1$ (5) and cytochrome $c$ and cytochrome $c$ can be deduced from this concept. However, it was only with the recent availability of a sufficient quantity of cytochrome $c_1$ (5, 6) that the opportunity arose to investigate the interaction and complex formation of these two lipid-free components of the chain. We thought that such studies would be useful to bioenergetics, especially the mechanism of mitochondrial electron transfer.

Cytochrome $c_1$ is isolated in solution in the form of a monodisperse pentamer which has resisted all attempts at depolymerization except under very severe denaturing conditions (5, 6). However, the disparity in the molecular weights of cytochrome $c_1$ as a pentamer (5) and cytochrome $c$ (cf. Ref. 7) in solution facilitates their separation on Sephadex gels and thus provides a simple method for investigating complex formation. Likewise, some properties, which distinguish between $c_1$ and $c$ and thus permit studies of the complex, include reaction with cyanide (1), reduction by ascorbate and $N,N',N''$-tetramethyl-$p$-phenylenediamine (7), autooxidation (8), and photoreduction (6) among others. Cyanide reacts with ferricytochrome $c$ and apparently displaces the axial ligand of methionine-80, while it is totally unreactive with cytochrome $c_1$ (1), probably as a consequence of the more deeply buried nature of its heme iron (9). Although both cytochromes $c_1$ and $c$ are reducible by ascorbate the difference in the rates of reduction is large (7) and sufficient to differentiate these two cytochromes in the complex. Both cytochrome $c_1$ (5) and cytochrome $c$ (10, 11) are essentially nonautooxidizable at neutral pH but cytochrome $c_1$ does appear to be more susceptible to denaturants which induce autooxidizability (1). Cytochrome $c_1$ exhibits a much greater susceptibility than cytochrome $c$ to photoreduction under anaerobic conditions in the absence of exogenous reducing agents (6).

We report here our application of these reactions to the study...
of the complex and the interaction of these two cytochromes in the formation of the complex. It is beyond any doubt that a well defined cytochrome c₁-cytochrome c complex is formed. But whether such a complex indeed exists in situ or is even compatible with the fluid-mosaic concept of the mitochondrial inner membrane cannot be answered at present.

EXPERIMENTAL PROCEDURE

Materials—Cytochrome c₁, from beef heart, was isolated and purified as previously (5, 6). Prior to use, horse heart cytochrome c from Sigma (type III) was purified by passage through a column (25 × 1.5 cm) of super fine Sephadex G-75. All material eluted from the column before the major band which was probably polymerized cytochrome c₁ was discarded. Crystalline cytochrome c was also used and prepared from beef heart (12). Guanidinated cytochrome c and acetylated cytochrome c were made by the modifications (13) of the published methods (14, 15). Polymerized cytochrome c₁ was prepared from cytochrome c₁ with 60% ethanol (16). A 1.68 heme peptide of cytochrome c containing the covalently linked prosthetic group was isolated after the cleavage of cytochrome c by cyanogen bromide (17). Photoxidized cytochrome c in which the methionine residues were oxidized to methionine sulfoxide was prepared by a method recently developed. Sodium ascorbate was purchased from Sigma, and urea, ultra pure grade, from Mann. All other chemicals were used in the purest grade commercially available. Water was double-distilled and deionized.

Methods—The concentrations of cytochrome c₁ and cytochrome c were determined spectrophotometrically using a millimolar extinction coefficient of 19.1 for A₅₅₀ for α₅₃₅ (Ref. 5). The complex of cytochrome c₁ and cytochrome c₁ was routinely prepared by mixing these two cytochromes, either in oxidized or reduced form, with a slight excess of c₁ in 10 mM phosphate buffer, pH 7.4, and passing the mixture through a column (25 × 1.5 cm) of super fine Sephadex G-75 equilibrated with 10 mM phosphate buffer, pH 7.4, at 4°C. The band running in the void volume contained the complex. The same technique was used to investigate the formation of complexes between cytochrome c₁ and various forms of modified cytochrome c. The stoichiometry of the complex was determined by mixing the components with 2- or 3-fold molar excess of cytochrome c₁ and determining the unbound cytochrome c₁ spectrophotometrically after separation on the column. Confirmatory experiments for the ratio of the components were also conducted by measurement of the cytochromes c₁ and c following gel filtration after the dissociation of the complex in a medium of high ionic strength, usually 0.1 M NaCl in 10 mM phosphate buffer, pH 7.4. Instead of gel filtration, ammonium sulfate fractionation at 50% saturation was also used; under this condition, all cytochrome c₁ precipitated but c remained in the supernatant.

The influences of ionic strength, urea, and temperature on the stability of the complex were determined by passing the complex, which had previously been purified on a column of Sephadex G-75, through another column (25 × 1.5 cm) of super fine Sephadex G-75 equilibrated under the required conditions to suit the particular experiment. A clean separation of the complex into its components, cytochromes c₁ and c, was assumed to indicate complete cleavage of the complex while a broadly spread out band of cytochrome c was interpreted as representing partial disruption of the complex. The reactivity of the dithionite-reduced complex with carbon monoxide was investigated spectrophotometrically before and after saturating the system with carbon monoxide gas for as long as 5 min and again after 1 hr.

The effect of the complexing of cytochrome c₁ and cytochrome c on the 695 nm region of the absorption spectra of the components was determined by difference spectrophotometry. Solutions of ferricytochrome c₁ and ferricytochrome c in separate cuvettes were together scanned against the complex at the same concentration of the hemeproteins. All spectrophotometric experiments were performed with Cary spectrophotometers model 14 or 16. Circular dichroic measurements were made with a Cary spectropolarimeter, model 60, with CD attachment model 6002. Ellipticities are expressed in degrees-cm² per dmol of heme. The resultant circular dichroism spectrum of cytochrome c₁ and c was obtained by using two separate cells placed in series in the beam of the spectropolarimeter. After determination of this spectrum the cytochrome c₁ and c solutions were thoroughly mixed and the resultant complex was returned to the two cells, which were again scanned in series to obtain the CD spectrum of the complex.

Amino acid analysis was performed as previously described (19).

Sedimentation velocity experiments were performed at 20°C using a Beckman model E analytical ultracentrifuge. Red-sensitive Eastman Kodak 1N plates and a Wratten No. 25 filter were employed. The partial specific volume of cytochrome c₁ was estimated from its amino acid composition to be 0.732 cm³/g.

RESULTS

Complex Formation with Cytochrome c₁ and Various Forms of Cytochrome c—Only a single band at the position of the void volume was obtained when equimolar quantities of beef heart cytochrome c₁ and horse or beef heart cytochrome c were mixed in 10 mM phosphate buffer and passed through a Sephadex G-75 column (Fig. 1). When a 2- or 3-fold molar excess of cytochrome c₁ was used, the complex with a 1:1 molar ratio was also obtained and the excess cytochrome c₁ was eluted in the elution volume corresponding to that of free cytochrome c₁ (Fig. 1). The same ratios were formed when ferro- or ferricytochrome c₁ was mixed with ferro- or ferricytochrome c.

1 Results to be published.
2 The abbreviation used is: TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine.
Ultracentrifugation of cytochrome c₁ revealed that the sample was homogeneous with a sedimentation coefficient, $s_{20, w} = 13.5 \text{ S}$, which is equivalent to a molecular weight of 220,000. The complex also behaved as a homogeneous preparation on ultracentrifugation with $s_{20, w} = 16.2 \text{ S}$ equivalent to a molecular weight of 590,000. These figures further substantiate the unity ratio of cytochrome c₁ to cytochrome c in terms of heme in the complex.

Table I is a summary of the complexing powers of cytochrome c₁ with various forms of cytochrome c under a variety of conditions.

The polymerized cytochrome c (16) was purified on Sephadex G-50 and was a mixture of dimers, trimers, and higher polymers, these polymers exhibited no absorbance maximum at 695 nm. The guanidinated cytochrome c (13) migrated as a single band on cellulose-acetate strip electrophoresis in Tris-glycine buffer, pH 8.0. Amino acid analysis of the purified preparation revealed that all the lysine residues of cytochrome c were guanidinated. The acetylated cytochrome c (13) also migrated as a single band, but to the anode, on cellulose acetate strip electrophoresis. A ninhydrin assay (20), using native cytochrome c as a standard, indicated that 64% of the lysine residues were acetylated. Thus, approximately 7 lysine residues remained unacylated in the modified cytochrome c.

The photooxidized cytochrome c was oxidized for 1 hour which was sufficient to completely eliminate the 695 nm absorbance band of cytochrome c₁.

In experiments on the complex formation between cytochrome c₁, and guanidinated cytochrome c, addition of the latter in excess of cytochrome c₁ resulted in precipitation of a complex under conditions where both components in the free form were completely soluble. Such a precipitation was not observed when native cytochrome c or its other derivatives were used in excess. It was apparent that the precipitation resulted from the binding of cytochrome c₁ by the modified cytochrome c less than the 1:1 ratio and is probably similar to the precipitation obtained by adding polylysine solution to cytochrome c₁.

A complex was separated by Sephadex G-75 chromatography from the interaction of cytochrome c₁, and the ferric heme peptide from cyanogen bromide-cleaved cytochrome c (17) (i.e. the so-called 1-65 heme peptide) or polymerized cytochrome c. These complexes showed a single band on the column with a molar ratio of 1:1. However, when the cytochrome c derivatives were used in excess, the complexes formed showed molar ratios less than unity and dependent upon the cytochrome c derivatives in the initial mixture.

**Dissociation of Cytochrome c₁-Cytochrome c Complex**—The cytochrome c₁-cytochrome c complex was susceptible to disruption to elevated ionic strength. The addition of 0.05 M sodium chloride to the complex in 10 mM phosphate buffer caused dissociation as demonstrated by Sephadex chromatography (Fig. 2). Although the complex was stable at pH 9.5, it was not formed at pH 10 (cf. Table I). Urea, however, was less effective and 8 M urea could apparently only effect a partial disruption of the complex (Fig. 2).

**Circular Dichroic Behavior of Cytochrome c₁-Cytochrome c Complex**—The circular dichroic spectra of the cytochrome c₁-cytochrome c complex and the two cytochromes together but in the unmixed state are presented in Fig. 3. It can be...
Cytochrome $c_1$, $c$ Complex

Circular dichroic spectra (Fig. 3) in the Soret region when $c_1$ and $c$ were mixed together to form the complex. The variations in the Soret region arise from the heme and its ligands. Similar increases in the Soret ellipticity are observed when cytochrome $c$ interacts with denaturants (21). The failure of the reduced complex to react with carbon monoxide and the absence of any spectral change in the 695 nm region for the complexes from the components indicate that any such crevice weakening, if indeed it has occurred, is only to a very limited extent (22, 23).

The biphasic nature of the autoxidation rates of the complex may indicate that both cytochromes become autooxidizable but to different degrees in the complex.

Cytochrome $c_1$, Cytochrome $c$ Complex

Cytochrome $c_1$ does not react with cyanide at neutral pH as evidenced by the absence of any spectral change upon the addition of cyanide to ferrocytochrome $c_1$ (1). In contrast, cytochrome $c$ reacts with cyanide under the same conditions to produce a red spectral shift of the Soret absorbance band of 4 nm and the formation of an a band maximum near 550 nm resembling the reduced $c$. The red shift gives rise to a difference spectrum having a peak at 417 nm and trough at 402 nm as shown in Fig. 5. The maximum at 417 nm was used to study the kinetics of cyanide reaction with cytochrome $c$ and the cytochrome $c_1$, cytochrome $c$ complex. The results of these experiments are summarized in Fig. 6. While cyanide still reacted with the complex at neutral pH its rate of the reaction was slower than that with cytochrome $c$ alone. The extent of reaction (or spectral change) was also equivalent to that of cytochrome $c$ although the total concentration of cytochrome $c_1$ and cytochrome $c$ in terms of heme was double that of cytochrome $c$ alone.

The rate of reaction of cyanide with cytochrome $c$ was somewhat diminished when the reaction took place at higher ionic strength (i.e., upon the addition of 0.1 M NaCl) (Fig. 6). The reaction of the complex with cyanide at high ionic strength, however, was markedly altered as the rate of the reaction became faster but the reaction proceeded to only about 70% of that in low ionic strength (Fig. 6). These reactions were all clean pseudo-first order except for the one for the complex at low ionic strength which showed a small portion (about 5%) of a slightly faster initial phase (Fig. 7). The apparent pseudo-first order rate constants for the reaction with cyanide determined from these plots are: (a) for cytochrome $c$, $k_1 = 4.59 \pm 0.04 \times 10^{-3}$ s$^{-1}$ at low ionic strength and $2.70 \pm 0.07 \times 10^{-3}$ s$^{-1}$ at high ionic strength; (b) for the complex, $k_1 = 1.49 \pm 0.04 \times 10^{-3}$ s$^{-1}$ at low ionic strength and $3.58 \pm 0.15 \times 10^{-3}$ s$^{-1}$ at high ionic strength.

The reaction rate of cytochrome $c$ with cyanide, which results in the displacement of the methionine-80 as an axial ligand by the cyanide, is markedly diminished when cytochrome $c$ is complexed with cytochrome $c_1$. The extent of the difference spectral changes produced by the reaction of cyanide with the complex and that with cytochrome $c$ alone are the same. This fact suggests that of these two cytochromes in the complex only cytochrome $c_1$ is reactive with cyanide. The diminished rate of reaction of cyanide with the cytochrome $c$ in the complex could be a direct consequence of conformational changes, which restrict access of the cyanide to the heme iron, or from the binding of cytochrome $c_1$ at, or close to, the exposed heme edge of cytochrome $c$. Such a binding would inhibit the cyanide reaction. The former explanation is unlikely in view of the previously discussed autoxidation of the complex which requires a loosening of the heme crevice. It is also unlikely that

Fig. 3. Circular dichroic spectra of the cytochrome $c$, cytochrome $c$ complex and the unmixed cytochrome $c_1$ plus cytochrome $c$. The systems were in 10 mM phosphate buffer, pH 7.4. Cytochrome concentrations in the complex and in the free form (i.e., unmixed in two separate cuvettes) were 7 µM each for $c_1$ and $c$. Noted from the figure that there is a remarkable increase in the ellipticity in the Soret region when the two cytochromes are in the form of the complex.

Autooxidation and Lack of Carbon Monoxide Reactivity of Cytochrome $c_1$, Cytochrome $c$ Complex—At pH 7.4, ferrocytochrome $c_1$ displays virtually no autoxidation while ferrocytochrome $c$ is only very slowly autooxidizable. In 3 hours subsequent to the complex formation both cytochrome components became more than 50% oxidized. The autoxidation did not exactly follow either first or second order kinetics but appears to consist of two biphasic consecutive first order reactions with rate constants of $6.2 \times 10^{-3}$ s$^{-1}$ and $5.0 \times 10^{-3}$ s$^{-1}$ (cf. Fig. 4).

The reduced complex, however, did not react with carbon monoxide as no spectral change was observed even after an hour subsequent to saturation of the solution with carbon monoxide. Since the autooxidizability of the complex could arise from displacement of the axial ligands of the heme iron of the cytochrome $c$ or $c_1$, component of the complex, the 695 nm absorbance band of the complex was examined. No detectable change was observed in the difference spectrum (i.e., the complex versus the components of the complex in separate cuvettes) in the 695 nm region on formation of the complex.

The observed autoxidation of the reduced cytochrome $c_1$, cytochrome $c$ complex probably arises from conformational changes in either or both cytochromes induced by interaction with one another. Since both ferrocytochrome $c$ (11) and ferrocytochrome $c_1$ (8) autoxidize when their heme crevices are weakened or opened by hydrophobic denaturants, it may be assumed that similar crevice weakening occurs in the complex. This is borne out by the change observed in the

1 It must be pointed out that rigorous analysis of the kinetics for the autoxidation was not done; the quantitative values of the rate constants given must be considered approximate. However, these are beyond the central theme of the present work. Our main endeavor here is to show the change of the behavior from the components to the complex.
Cytochrome c₁–c Complex

Cytochrome c₁ completely covers the exposed heme edge of cytochrome c in the complex, thus burying the heme, in view of our earlier report (9) that the heme of cytochrome c quenches the fluorescence of 8-anilino-1-naphthalene sulfonic acid bound to cytochrome c₁. Thus from all the results, it is not unreasonable to consider that cytochrome c₁ binds to cytochrome c at the methionine-80 side of its heme crevice and partially covers that side of the entrance to the crevice.

The rates of reaction of cyanide with the complex and with cytochrome c alone in media of high ionic strength, sufficient to disrupt the complex, would be expected to be similar. However, they are not. Because of the considerable difference it appears likely that a weak interaction between cytochrome c₁ and cytochrome c exists at high ionic strength.

Reduction of Cytochrome c₁–c Complex—The reducibility of the complex as compared to that of its components was investigated using ascorbate and TMPD. TMPD reduced ferricytochrome c₁ and ferricytochrome c at similar rates with second order rate constants of $8 \times 10^4 \, \text{M}^{-1} \, \text{s}^{-1}$ and $4 \times 10^4 \, \text{M}^{-1} \, \text{s}^{-1}$, respectively, confirming previous findings (7).

The cytochrome c₁–cytochrome c complex was reduced by TMPD with a second order rate constant of $7 \times 10^4 \, \text{M}^{-1} \, \text{s}^{-1}$.
The reducibility of the complex by ascorbate, however, differed from that of its components. At 10 mM ascorbate, which was sufficient to produce pseudo-first-order reactions but was not high enough to decompose the complex, cytochrome c reduction was a monophasic pseudo-first order with a rate constant $k_1 = 5.0 \times 10^{-1} \text{s}^{-1}$; cytochrome c$_1$ reduction was also a monophasic pseudo-first order with $k_1 = 0.25 \times 10^{-1} \text{s}^{-1}$. The reduction of the complex was found, however, to be biphasic with $k_1 = 2.0 \times 10^{-1} \text{s}^{-1}$ (corrected for contribution of slower rate) and $0.5 \times 10^{-1} \text{s}^{-1}$ for the complex. The rates of reduction of the complex were the same whether measured at 550 nm or 552.5 nm. When ascorbate concentration was increased to 100 mM, "sufficient" to dissociate the complex as a consequence of the elevated ionic strength, the rates of reduction of the complex still differed from the sum of the rates of reduction of the components. The pseudo-first order rate constants were now $k_1 = 3.4 \text{ s}^{-1}$ for cytochrome c, $1.4 \times 10^{-1} \text{ s}^{-1}$ for cytochrome c$_1$, and $4.1 \text{ s}^{-1}$ and $1.0 \text{ s}^{-1}$ for the complex which was biphasic.

**Photoreduction of Cytochrome c$_1$.Cytochrome c Complex**—Both cytochromes c$_1$ and c are photoreduced but to different extents (6) under anaerobic conditions, in the absence of exogenous reducing agent. When the complex was photoreduced under conditions identical with those used for reduction of the components, the rate of reduction was the same as for cytochrome c alone and was much slower than the rate of reduction of cytochrome c$_1$ (Fig. 9).

**Nonreduction of Ferrocytochrome c$_1$ in Cytochrome c$_1$.Cytochrome c Complex**—The heme peptide of cytochrome c$_1$-polymerized cytochrome c$_1$.Cytochrome c Complex. The cytochrome c$_1$, in the complex, which had been formed from ferrocytochrome c$_1$, and the 1-65 heme peptide of cytochrome c cleaved by cyanogen bromide, remained in the reduced state and was not oxidized by the ferricytochrome c heme peptide. The same phenomenon was observed in the cytochrome c$_1$.Cytochrome c$_1$ complexes with polymerized cytochrome c; reduced c$_1$ was not oxidized in these complexes. Similarly, a mixture of ferrocytochrome c$_1$ and ferricytochrome c, whose methionine-80 residue had been oxidized to methionine sulfoxide, did not show any electron transport reaction between the components as determined by stopped-flow spectrophotometry using the technique described previously (7).

**DISCUSSION**

From the results presented, it is beyond reasonable doubt that a well defined cytochrome c$_1$.cytochrome c complex is formed. The rate of formation of the complex from its components is fast and cannot be estimated by the available instrumentation. The complex possesses a definite stoichiometry with a ratio of 1. The formation and stability of the complex is apparently independent of the oxidation state of the components. There is no dissociation of the complex subsequent to electron transfer from ferrocytochrome c$_1$ to ferricytochrome c, or vice versa. If the complex is physiologically relevant then this result may suggest the apparent absence of any in vivo migration of cytochrome c from cytochrome c$_1$ to cytochrome oxidase; the possibility of such a migration has been speculated on (e.g., Ref. 24) although an equilibrium association and dissociation must exist: c$_1$ + c = complex. Regarding the equilibrium, it can be inferred that the association constant is very high. Considerably higher than $10^7 \text{ M}^{-1}$ because of the failure of the Sephadex column to effect any separation of the components or dissociation of the complex. The association constant of a cytochrome c-cytochrome c peroxidase complex, for example, which is partially dissociated on Sephadex chromatography, has been reported to lie between $10^4$ and $10^7 \text{ M}^{-1}$ (25).

Results on the dissociation of the complex in media of high ionic strength or high pH as well as others (cf. Table I), suggest that ionic interactions play a role in maintaining the integrity or stability of the complex. These interactions probably arise from the lysine residues of cytochrome c, which confer net positive charges on cytochrome c (24), and the glutamic and aspartic acid residues of cytochrome c$_1$ for negative charges. The requirement of net positive charges on cytochrome c for complex formation is further supported by two lines of evidence: (a) the failure of partially acetylated cytochrome c which confer net negative charges on cytochrome c$_1$ (24), and the glutamic and aspartic acid residues of cytochrome c$_1$ for negative charges. The requirement of net positive charges on cytochrome c for complex formation is further supported by two lines of evidence: (a) the failure of partially acetylated cytochrome c which confer net negative charges on cytochrome c$_1$ (24), and the glutamic and aspartic acid residues of cytochrome c$_1$ for negative charges. The requirement of net positive charges on cytochrome c for complex formation is further supported by two lines of evidence: (a) the failure of partially acetylated cytochrome c which confer net negative charges on cytochrome c$_1$ (24), and the glutamic and aspartic acid residues of cytochrome c$_1$ for negative charges. The requirement of net positive charges on cytochrome c for complex formation is further supported by two lines of evidence: (a) the failure of partially acetylated cytochrome c which confer net negative charges on cytochrome c$_1$ (24), and the glutamic and aspartic acid residues of cytochrome c$_1$ for negative charges. The requirement of net positive charges on cytochrome c for complex formation is further supported by two lines of evidence: (a) the failure of partially acetylated cytochrome c which confer net negative charges on cytochrome c$_1$ (24), and the glutamic and aspartic acid residues of cytochrome c$_1$ for negative charges. The requirement of net positive charges on cytochrome c for complex formation is further supported by two lines of evidence: (a) the failure of partially acetylated cytochrome c which confer net negative charges on cytochrome c$_1$ (24), and the glutamic and aspartic acid residues of cytochrome c$_1$ for negative charges.
with guanidinated cytochrome c, polymerized cytochrome c, or the 1-65 heme peptide of cytochrome c (which contains 10 lysine residues) probably arises from nonspecific ionic interactions which may differ from those in the complex of native cytochrome c1 and cytochrome c. This result may be related to the precipitation of the cytochrome c1 even at very low concentrations in the presence of excess guanidinated c.

Ionic attraction alone is not the only requirement for the complex formation and the maintenance of the integrity of the complex; hydrophobic interaction also plays an important role in the process, as evidenced by the partial dissociation of the complex in 8 M urea. The fact that methionine sulfoxide cytochrome c does not complex with cytochrome c1 (Table I) suggests a further role for hydrophobic interactions in formation of the complex. Although this modified cytochrome c has no charge alterations, with 2 methionine residues, including the methionine-80, being oxidized to sulfoxide, unpublished results have shown the existence of considerable conformational change, particularly in the region of the heme crevice. The contribution of hydrophobic interactions to the complex formation may thus arise directly from the action of hydrophobic residues of the two cytochromes or indirectly by controlling the conformational requirements for complexing of the protein moieties.

The correlation of the lack of complexation between acetylated cytochrome c or methionine sulfoxide cytochrome c with cytochrome c1 and the failure of these modified cytochromes to be reduced by succinate-cytochrome c reductase (15) suggest the requirement of prior complex formation for electron transfer to cytochrome c in the complex (16). The electron transfer between c1 and c in this reference employ a basal medium with an ionic strength higher than 50 mM phosphate buffer plus other dissociable components). The converse is not true, since the polymerized cytochrome c or the 1-65 heme peptide binds to cytochrome c1 but does not ensure electron transfer reaction. Other factors, such as the oxidation-reduction potentials of the modified cytochrome c could, of course, control the transfer reactions too.

The kinetics of the ascorbate reduction of the complex are complicated since both component cytochromes are reducible by ascorbate and there is also rapid electron transfer between c1 and c (2). It is, however, clear from the pseudo-first order rate constants (Fig. 8) that the fastest component of the rates of reduction of the complex is only about 40% of the rate of reduction of the cytochrome c alone. The rate of ascorbate reduction of the cytochrome c in the complex is thus considerably slower than that of unbound cytochrome c. Furthermore, when the complex is reduced by ascorbate under dissociating conditions of ionic strength, the rates obtained differ from those predicted from the individual reductions of cytochrome c and cytochrome c1 under the same conditions. This result together with the previously discussed reaction of cyanide with the complex indicates that some interaction remains between c1 and c at high ionic strength. The binding strength for this association is apparently too weak to withstand Sephadex G-75 chromatography.

In a cytochrome c-cytochrome oxidase complex the rates of the cyanide reaction remain the same whether the cytochrome c is free or complexed with the oxidase (25). Comparison of these results with the results presented here may indicate that cytochrome c1 and oxidase bind to cytochrome c at different sites but that both molecules interact with or effect the ascorbate binding site on cytochrome c. This difference in binding sites is consistent with the proposal (26) that succinate-cytochrome c reductase (cytochrome c1 is a component of the reductase) and cytochrome oxidase have different binding sites on cytochrome c molecules.

It is difficult to visualize the molecular structure of the complex taking into account that isolated cytochrome c1 in solution is in the pentameric form (5), that there is rapid electron transfer from cytochrome c1 to cytochrome c (7), and that a definite 1:1 stoichiometry exists in the complex. A reasonable arrangement would be one with the five cytochrome c1 molecules polymerized together probably through hydrophobic regions of the protein thus creating a very hydrophobic center. The predominantly hydrophilic portions of the proteins could then radiate out from this binding center like spokes from the hub of a wheel with the buried heme groups situated toward the ends of the exposed hydrophilic regions. One cytochrome c molecule can then complex with each cytochrome c1 in the region of the c1 heme group. Such a scheme is compatible with our ultracentrifugation studies which reveal that five cytochrome c molecules are bound to a pentameric aggregate of cytochrome c1. It is unknown whether cytochrome c1 is similarly polymerized in vivo but in the respiratory chain on the oxygen side of cytochrome c is cytochrome oxidase which may be legitimately considered as a polymer (cf. for example, Ref. 27).

Finally, there is no evidence to indicate whether the cytochrome c1-cytochrome c complex exists in situ or possesses physiological significance or not. The formation of the cytochrome c1-c complex presented in this paper, the cytochrome c-cytochrome oxidase complex described previously (28, 29), and a tertiary complex of cytochrome c1-cytochrome c-cytochrome oxidase to be reported elsewhere seems to imply, at least superficially, a rigidity of the respiratory chain or segments. This very rigidity would pose a serious difficulty as to how an electron can transfer in such a system (29, 30). But it can also be viewed that the rapid association and dissociation occur dependent on the microenvironment, as suggested in connection with description of these complexes. Nevertheless, the formation of these complexes may have opened the door to think about theories alternative to, or modification of, the fluid mosaic concept of membranes (31) as well as to seriously ponder the possibility of the fluidity of respiratory enzymes in groups but not in individual components in order to effectuate electron transfer in the mitochondrial inner membrane.

REFERENCES
15. Wada, K., and Okunuki, J. (1968) J. Biochem. 64, 667-681
A complex of cardiac cytochrome c1 and cytochrome c.
Y L Chiang, L S Kaminsky and T E King