Kinetics of Electron Transfer between Cardiac Cytochrome c₁ and c∗

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SUMMARY

The kinetics and equilibrium of the electron transfer between isolated cytochrome c₁ and cytochrome c and artificial carriers have been studied. The oxidation of ferrocyanochrome c₁ by ferricyanide showed a rate constant of 3.6 × 10⁴ M⁻¹ s⁻¹ in 50 mm phosphate buffer, pH 7.4, 23°C. Under the same condition the rate constant for the reduction of ferricytochrome c₁ by ascorbate was found to be 1.2 M⁻¹ s⁻¹. The second order rate constant of the reduction of ferricytochrome c₁ by N,N',N',N'-tetramethyl-p-phenylenediamine to ferrocytochrome c₁ and Würster’s blue was 2 × 10⁴ M⁻¹ s⁻¹ and that of the reverse reaction was 3.9 × 10⁴ M⁻¹ s⁻¹. Würster’s blue could not be further oxidized by any ferricytochrome c₁ or ferricytochrome c. The oxidation of ferrocyanochrome c₁ by ferricytochrome c to ferrocyanochrome c₁ and ferrocyanochrome c was not a very fast reaction as generally believed and showed a second order rate constant (k₁) of 3.3 × 10⁶ M⁻¹ s⁻¹ at 10°C and the reverse reaction showed a rate constant (k₋₁) of 1.0 × 10⁶ M⁻¹ s⁻¹. These values gave an equilibrium constant (K) of the reaction of 3.3, which was in good agreement with direct spectrophotometric measurements for K. K was independent of the ionic strength and temperature studied, but dependent upon pH. The rate constant, k₁, was, however, a function of temperature and ionic strength as well as pH. The reaction rates toward ferrocyanochrome c were the same whether ferrocyanochrome c₁ was in the soluble form or structured in a particulate succinate cytochrome c reductase.

Since the first solubilization and isolation of cytochrome c by Keilin in 1930 (1) extensive studies on this cytochrome have produced a wealth of information (cf. 2) which has been used in attempts to understand electron transport. On the other hand, cytochrome c₁ has not yet received much attention due partly to the lack of a suitable soluble preparation. In most kinetic studies on mitochondria, or submitochondrial particles, cytochrome c₁ is usually measured together with cytochrome c because of their absorption similarities (cf. 9). Indeed, no precise knowledge is available on the electron transfer rate between cytochrome c₁ and c either in solution or in particles (cf. 4). Since we have succeeded in isolating highly purified cytochrome c₁ (5) with relative ease and have recently crystallized it, the reactions between cytochromes c₁ and c, as well as artificial electron carriers, could be investigated without ambiguity. This paper reports these studies and the comparison of the reaction rates of soluble cytochrome c₁ and bound c₁ with cytochrome c. A part of this work was previously reported (6).

EXPERIMENTAL PROCEDURE

MATERIALS—Crystalline cytochrome c₁, succinate-cytochrome c reductase, and cytochrome c₁ all from bovine heart were prepared according to Margoliash and Walsh (7), Takemori and King (8), and Yu et al. (5), respectively. The purified cytochrome c₁, containing 25 mmoles of heme per mg of protein, was further crystallized from 30% saturated ammonium sulfate at about 4°C. The crystals, in thin plates, were separated by a special washing technique (9). The yield of the crystallization was, however, low. The crystals thus obtained were dissolved in 50 mm phosphate buffer, pH 7.4. Crystalline Würster’s blue was prepared from N,N',N',N'-tetramethyl-p-phenylenediamine (10, 11). All other chemicals in the highest available purity were purchased commercially. The water used was doubly distilled and deionized.

METHODS—Concentrations of cytochrome c₁ and cytochrome c were determined spectrophotometrically. A millimolar extinction coefficient of 17.5 for A₄₅₂S₅ — A₄₄₀S₅ was used for cytochrome c₁ and 19.5 for A₄₃₀S₅ — A₄₄₀S₅ for cytochrome c (5). The spectrophotometric measurements were carried out on Cary spectrophotometers, models 14 or 16, at approximately 25°C, unless otherwise specified. Ferrocyanochrome c₁ solution was prepared by the oxidation of ferrocyanochrome c₁ (as prepared) by a slight excess of potassium ferricyanide and the excess oxidant was removed by passing the mixture through a column of Sephadex G-25 equilibrated with 50 mm phosphate buffer, pH 7.4. A similar method was used for the preparation of ferrocyanochrome c except that the reduction was effected by sodium dithionite, and the excess reductant was removed by a Sephadex G-25 column equilibrated with oxygen-free buffer. The reduced sample was kept under nitrogen to minimize the reoxidation of ferrocyanochrome c after the complete removal of the reductant. The oxidation of N,N',N',N'-tetramethyl-p-phenylenediamine was measured at 612 nm by following the formation of Würster’s blue whose extinction coefficient was taken as 12 cm⁻¹ mm⁻¹ (10, 11). The reduction of cytochrome c₁ in the pres-
For the equilibrium studies of cytochromes $c_1$ and $c$, the concentrations of ferrocytochrome $c_1$ and ferricytochrome $c$ in the reaction system were followed by the difference spectra of the reaction products and reactants using difference millimolar "extinction coefficients" of $-11$ at 554 nm and $-30$ at 420 nm for cytochrome $c_1$ and $+10$ at 548 nm and $+29$ at 409 nm for cytochrome $c$. These extinction coefficients were calculated from the difference spectra obtained from a known concentration of cytochrome $c_1$ and that of $c$ by the following setup. A pair of matched cuvettes containing ferrocytochrome $c_1$ and ferricytochrome $c$ in the same molar concentration were placed in the sample compartment of a spectrophotometer and scanned against a pair of cuvettes containing the same concentration of cytochromes but in oxidation states opposite to those in the reference compartment. The cytochromes in each cuvette were kept fully reduced or oxidized by adding a small amount of ascorbate or potassium ferricyanide. This technique was essentially the same as that reported by Morton et al. (12).

Stopped flow experiments were conducted in a thermostated Durrum-Gibson stopped flow spectrophotometer fitted with a 2 cm cell. The slit opening was adjusted to the same extent as that of the Cary 14 spectrophotometer. The apparatus was calibrated with 0.01 M potassium thiocyanate and ferric nitrate in 0.1 M sulfuric acid.

The experimental error in the kinetic studies was described as smaller than 20%. The direct spectrophotometric measurement for the equilibrium study involved an error not more than 10% under the conditions described.

**RESULTS**

**Oxidation of Ferrocytochrome $c_1$ by Ferricyanide**—Fig. 1 shows an oscilloscope tracing of stopped flow experiments in which the oxidation of ferrocytochrome $c_1$ by potassium ferricyanide (Equation 1) was examined in 50 mM phosphate buffer, pH 7.4.

$$\text{Fe}^{2+} + \text{Fe(CN)}_5^{-} \rightarrow \text{Fe}^{3+} + \text{Fe(CN)}_5^{4-} \quad (1)$$

The concentrations of the cytochrome and ferricyanide as shown in Fig. 1 were 4.8 and 10 $\mu$M, respectively. Five different scan rates were recorded. The oxidation of cytochrome $c_1$ was followed by the increase in transmittance at 552.5 nm. The reproducibility of the results was ensured by the superimposition of the several tracings at each scan rate. A second order rate constant of $3.6 \times 10^4$ M$^{-1}$ s$^{-1}$ was obtained from analyzing the initial rate. This value is lower than the rates of $6.7 \times 10^4$ and $8.1 \times 10^4$ M$^{-1}$ s$^{-1}$ for the oxidation of cytochrome $c$ by ferricyanide reported by Morton et al. (12) and Kowalsky (13) and by Brandt et al. (14), respectively. The differences in the ionic strengths employed by the three laboratories may be responsible, at least in part, for the different rates observed.

**Reduction of Ferrocytochrome $c$ by Ascorbate**—Fig. 2 shows a protocol of the stopped flow experiment in which ferrocytochrome $c$ was reduced by ascorbate (Equation 2).

$$2\text{Fe}^{3+} + \text{ascorbate} \rightarrow 2\text{Fe}^{2+} + \text{dehydroascorbate} + 2\text{H}^+ \quad (2)$$

The experiment was carried out in 50 mM phosphate buffer, pH 7.4. The concentrations of cytochrome $c_1$ and ascorbate used were 10 $\mu$M and 50 $\mu$M, respectively. The reduction of cytochrome $c_1$ was monitored at 552.5 nm. The rate constant for the reduction calculated from the initial reaction rate was $1.2$ M$^{-1}$ s$^{-1}$. Under the same conditions, the rate of reduction of cytochrome $c$ was found to be $10$ M$^{-1}$ s$^{-1}$. We also found the rate constant of the reaction in Tris buffer, 0.1 M, pH 8.0, to be $140$ M$^{-1}$ s$^{-1}$, as compared to a value of $750$ M$^{-1}$ s$^{-1}$ reported by Greenwood and Palmer (15). The apparent discrepancy could very well be due to a slight difference of the pH employed in the two laboratories, as the reaction was extremely sensitive to pH variations.

**Reduction of Ferrocytochrome $c_1$ and Ferrocytochrome $c$ by Tetramethyl-p-phenylenediamine**—The rate constants for the reductions of ferrocytochromes $c_1$ and $c$ by TMPD to ferrocytochrome $c_1$ and $c$, respectively, (Equations 3 and 4) and Wurster's blue in 50 mM phosphate buffer, pH 7.4 were also determined by the stopped flow technique.

$$\text{Fe}^{2+} + \text{TMPD} \leftrightarrow \text{Fe}^{3+} + \text{WP} + \text{H}^+ \quad (3)$$

$$\text{Fe}^{2+} + \text{TMPD} \rightarrow \text{Fe}^{3+} + \text{WP} + \text{H}^+ \quad (4)$$
The concentrations used for cytochrome $c_1$ and TMPD were in the order of 7 and 200 $\mu M$, respectively. A second order rate constant for the forward reaction of Equation 3 was found to be $2 \times 10^4 M^{-1} s^{-1}$ from the initial slope of the tracing. The stopped flow experiment on the reduction of cytochrome $c$ by TMPD (Equation 4) was carried out under the same conditions. A second order rate constant of $1 \times 10^4 M^{-1} s^{-1}$ was obtained.

Oxidation of Ferrocytochrome $c_1$ and $c$ by Wurster’s Blue—It has been postulated (11, 16) that the semiquinone of 1 ml of 12.9 $M$ Wurster’s blue accepts electrons at the site of either cytochrome $c$ or $c_1$, or both, in the respiratory chain. We thought the comparison of the reactions between cytochromes $c$ and $c_1$ with WB could substantiate or disprove the proposal. The stopped flow experiments on the reduction of WB by cytochrome $c_1$ and $c$ were thus conducted under the same conditions as given in the previous section. Solutions of WB in 50 mM deoxygenated phosphate buffer were freshly prepared from its crystalline preparation. The cytochromes $c_1$ and $c$ solutions were also prepared in deoxygenated buffer. The back reactions of Equations 3 and 4 were followed by the increase in transmittance at 612 nm. The second order rate constant for ferrocytochrome $c_1$ was found to be $3.9 \times 10^4 M^{-1} s^{-1}$ and that for ferrocytochrome $c$ was $5.6 \times 10^4 M^{-1} s^{-1}$.

No reaction whatsoever was observed between WB and ferrocyanochrome $c_1$ or ferrocyanochrome $c$ in 50 mM phosphate buffer, pH 7.4, other than for a small nonenzymatic blank of WB in the absence of cytochrome.

It has been shown that inhibition of the electron transport chain by antimycin A can be bypassed through TMPD (17, 18). Since the introduction of WB in the studies of intracellular respiration, we have been able to show that the antimycin bypass (17, 18) is actually only one of the very general characteristics of TMPD-WB coupled interactions with respiratory components. The above results substantiate our earlier conclusions, as summarized in the scheme presented for these interactions (cf. Fig. 8 of Reference 11). The above rate constants yield an equilibrium constant of 2 for cytochrome $c_1$ as illustrated in Equation 3 and 0.5 for cytochrome $c_1$ in Equation 4. These values would give a midpoint potential ($E_m$) of 245 mvolts for the TMPD-WB half-cell, in good agreement with the $E_m$ of 260 mvolts as the midpoint potential ($E_m$) for the isolated soluble cytochrome $c_1$, then $E_m$ of cytochrome $c_1$ is 228 mvolts, or 32 mvolts more negative than that of cytochrome $c$. This value is very close to that reported recently by Dutton et al. (21).

Reaction of Cytochrome $c_1$ and Cytochrome $c$—The kinetics of the oxidation of ferrocytochrome $c_1$ by ferrocytochrome $c$ (cf. Equation 5) was studied by the stopped flow technique. Low concentrations of the cytochromes were employed because of the high reaction rate. Fig. 4 shows a protocol of an experi-

$$c_1^{2+} + c_3^{2+} \rightarrow k_{-1} \rightarrow c_1^{3+} + c_2^{3+}$$

For the forward reaction, a representative experiment was conducted as follows. One milliliter of 12.9 $\mu M$ ferrocytochrome $c_1$ and 1 ml of 13.6 $\mu M$ ferrocytochrome $c$ were placed separately into two pairs of 0.5-cm light path cuvettes. One cuvette from each pair was used for reference and the other was placed in the sample beam. After measuring the base-line, the solutions in the sample beam were mixed and the spectra were recorded again. A difference spectrum is shown in Fig. 3. The final concentrations of these cytochromes in the sample cuvette calculated from the figure were $c_1^{2+}$, 8.6; $c_3^{2+}$, 4.3; $c_1^{3+}$, 5.0, and $c_2^{3+}$, 8.6. The equilibrium constant, $K$, was thus found to be 3.5. Similar results were obtained when reactions were started with ferrocytochrome $c$ and ferrocytochrome $c_1$ and different concentrations of these components. The equilibrium experiments were also carried out at various temperatures, ionic strengths, and pH values. As indicated in Table I, changing the temperature from 4$^\circ$ to 23$^\circ$, and addition of 0.5 M KCl had practically no effect on the equilibrium constant. In contrast to temperature and ionic strength, a large effect of pH on the equilibrium constant was observed. The equilibrium constant decreased rapidly with increases in pH.

Oxidation-Reduction Potential of Cytochrome $c_1$—The oxidation-reduction potential of cytochrome $c$ has been well documented and is dependent upon its energy state (20). If we use 260 mvolts as the midpoint potential ($E_m$) for the isolated soluble cytochrome $c_1$, then $E_m$ of cytochrome $c_1$ is 228 mvolts, or 32 mvolts more negative than that of cytochrome $c$. This value is very close to that reported recently by Dutton et al. (21).

Reaction of Cytochrome $c_1$ and Cytochrome $c$—The kinetics of the oxidation of ferrocytochrome $c_1$ by ferrocytochrome $c$ (cf. Equation 5) was studied by the stopped flow technique. Low concentrations of the cytochromes were employed because of the high reaction rate. Fig. 4 shows a protocol of an experi-

<table>
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**Table 1**

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</tr>
<tr>
<td>0.5 M</td>
<td>3.5</td>
</tr>
<tr>
<td>Temperature</td>
<td></td>
</tr>
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<td>23$^\circ$</td>
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A tracing of the forward reaction of $c_{1}^{+} + e^{*} \xrightleftharpoons[k_{-1}]{k_{1}} c_{1}^{+} + e^{*}$. Concentrations of $c_{1}^{+}$ and $e^{*}$ were 4.3 and 5.65 mM, respectively, in 50 mM phosphate buffer, pH 7.4. Wave length used was 420 nm, temperature, 10°.

In order to obtain a reliable measurement from the oscilloscope tracings, the scan rate was chosen at the fastest limit at which the apparatus could still function properly. Although a certain amount of noise was encountered, the reproducibility of the results was still satisfactory. An average second order rate constant ($k_{1}$ of Equation 5) of $3.3 \times 10^{5} \text{ M}^{-1} \text{s}^{-1}$ was obtained from the initial rate. Under the same conditions the rate constant for the reverse reaction ($k_{-1}$) was also determined. An average rate constant of $1.0 \times 10^{5} \text{ M}^{-1} \text{s}^{-1}$ was obtained.

The equilibrium constant for the reaction calculated from these rate constants was 3.3, in remarkably good agreement with that obtained by direct spectrophotometric measurements.

Effect of pH on Rate Constant, $k_{1}$ for Oxidation of Ferrocytochrome $c_{1}$ by Ferricytochrome $c$—A plot of log $k_{1}$ (Equation 5) against reaction pH is shown in Fig. 5. Higher rate constants were observed at neutral pH and gradually decreased as the acidity or basicity increased. A sharp drop in the rate constant was observed at higher pH. At pH 10 the rate constant was only $0.8 \times 10^{5} \text{ M}^{-1} \text{s}^{-1}$.

Effect of Ionic Strength on Rate Constant $k_{1}$—The effect of ionic strength on the rate constant $k_{1}$ was followed by increasing the potassium chloride concentration of the reaction system. The concentrations of added KCl ranged up to 0.5 M. The results are summarized in Fig. 6. Increase of the ionic strength caused a decrease of the rate constant in the oxidation of ferrocytochrome $c_{1}$ by ferricytochrome $c$. Under the same conditions the ionic strength showed much less effect when ferricyanide was used as oxidant. Morton et al. (12) observed that in a similar system with cytochrome $c$, ionic strength greatly affects the rate.

Effect of Temperature on Rate Constant $k_{1}$—Fig. 7 shows the effect of temperature on the rate constant, $k_{1}$. The rate constant increased with the increase of temperature up to 10°, while further increases of temperature decreased the rate. The activation energy of the reaction below 10° was found from the Arrhenius plot to be approximately 9.2 Cal per mole. This value is similar to those of many other electron transfer reactions. Since the equilibrium of the electron transfer between cytochromes $c_{1}$ and $c$ is insensitive to temperature but the forward reaction of Equation 5 is temperature dependent, the back reaction must be also affected by temperature and, more importantly, to the same extent.

Since the temperature at which $k_{1}$ begins to decrease (Fig. 7) is much lower than physiological temperature and since both cytochromes are stable at these temperatures, it is unlikely that the decrease in rate is due to even partial "denaturation" of the reactants. Moreover, samples which has been exposed at higher temperature showed results similar to those never exposed. One possible reason for such biphasic behavior towards temperature is that the components involved in the reaction may undergo some subtle conformational change.

Comparison of Rate Constants for Oxidation of Bound and
cytochrome cl of good purity became available. They could be obtained only when soluble cytochromes in particles. These cytochromes are useful in spectrophotometric studies of these cytochromes c and cl in the presence of each other. As reported under "Experimental Procedure," difference extinction coefficients of cytochromes c and cl in the presence of each other are useful in spectrophotometric studies of these cytochromes in particles. They could be obtained only when soluble cytochrome c1 of good purity became available.

From the data relating to the equilibrium constant for the reaction of cytochrome c1 and cytochrome c at pH 7.4, we have calculated the oxidation-reduction potential of cytochrome c1 to be 32 mvolts less positive than that of cytochrome c. However, the kinetic studies described show clearly that electron transfer between c1 and c is not instantaneous and must involve more than a mere redistribution of electrons.

The effect of pH on the rate constant k1 for the oxidation of ferrocytochrome c1 by ferricytochrome c, particularly at higher pH, may be explained as a change in the charge characteristics of the molecules. In the neutral pH range, the change in charge on both molecules as the pH is changed is probably small, and therefore, the rate constant is expected to be rather pH insensitive. On the contrary, at high pH, especially when the pH approaches the isoelectric point of cytochrome c, the highly charged cytochrome c molecule is expected to have a drastic reduction in its positive charge and change to a molecule whose net charge is almost zero. This effect may be related to the decrease of the rate constant. This pH dependence is in line with the x-ray crystallographic molecular model of cytochrome c proposed by Dickerson et al. (22). According to these workers, the molecule possesses two positive regions which are separated by a cluster of nine negatively charged acidic groups. These positively charged regions may be involved in the binding of other macromolecular complexes, such as the reductase or oxidase.

The effect of ionic strength on the rate constant for the oxidation of ferrocytochrome c1 is more prominent when cytochrome c is used as an oxidant than with ferricyanide. The oxidation of horse ferrocytochrome c by Pseudomonas ferricytochrome c showed opposite effects, as reported by Morton et al. (12). They have found that ionic strength has a large effect on the oxidation of mammalian cytochrome c by the microbially oxidized horse cytochrome c1.

It is significant to note that the rate constant of the electron transfer between cytochromes c and c1 is practically the same whether c1 is soluble in solution or structured in particles. This observation may substantiate the claim (5) that the isolated cytochrome c1 is native and has not undergone degradation. Perhaps equally importantly, it supports the validity (at least as far as cytochromes c and c1 are concerned) of those extensive kinetic studies on mitochondrial particles pioneered by Chance (3, 23), whose most basic assumption involves the treatment of the heterogenous system of mitochondria or submitochondrial particles by the classical solution kinetics.

**Acknowledgment**—Technical assistance by Michael Seaman is gratefully acknowledged.

**TABLE II**

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<th>Cytochrome c1 preparations</th>
<th>Rate constant (10⁻⁶)</th>
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<tr>
<td>Purified, 25 nmoles/mg protein</td>
<td>3.3 × 10⁻⁶</td>
</tr>
<tr>
<td>In succinate-cytochrome c reductase, 1.4 nmoles/mg protein</td>
<td>2.8 × 10⁻⁶</td>
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**DISCUSSION**

Unlike many other components in the respiratory chain, kinetic studies of the reactions between cytochrome c1 and cytochrome c in the intact chain or segments of the chain are rather difficult because of the similarity of their spectral properties. No suitable reductant or oxidant or specific inhibitor has been found to achieve the selective oxidation-reduction of only one of the two cytochromes. Consequently, the study of electron transfer reactions of the two cytochromes must be dependent upon the isolation of these cytochromes in a sufficient quantity. As reported under “Experimental Procedure,” difference extinction coefficients of cytochromes c and c1 in the presence of each other are useful in spectrophotometric studies of these cytochromes in particles. They could be obtained only when soluble cytochrome c1 of good purity became available.

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<th>REFERENCES</th>
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Additions and Corrections

Vol. 247 (1972) 7743-7749

In SARKAR, Pranab K., DONALD A. FISCHMAN, Eugene Goldwasser, and ARON A. MOSCONA. Isolation and Characterization of Glutamine Synthetase from Chicken Neural Retina.

Page 7745, Paragraph 1 under “Molecular Weight of Native Enzyme,” Line 4 should read:

(0.375, 0.55, and 0.75 mg per ml) in 0.1 M phosphate buffer,

Vol. 248 (1973) 528-533

In YU, C. A., L. Yu, and Tsoo E. King. Kinetics of Electron Transfer between Cardiac Cytochrome c1 and c.

Page 531, left-hand column, Lines 9, 11, and 22, $\times 10^5$ M$^{-1}$ s$^{-1}$ should read:

$10^6$ M$^{-1}$ s$^{-1}$. In all other places, including the Summary, the data are correctly printed.

Vol. 248 (1973) 472-477

In OWENS, Ida S., Barbara K. Vonderhaar, and Yale J. TURRISI. Concerning the Necessary Coupling of Development to Proliferation of Mouse Mammary Epithelial Cells.

Page 475, Table VI, Column 4, the heading should read:

$\Delta$ Casein per $10^4$ cells.

Due to a mishap in printing the $\Delta$ was omitted.

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.
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