Kinetics of Electron Transfer between Cardiac Cytochrome $c_1$ and $c^*$

C. A. Yu, L. Yu, and Tsoo E. King

From the Department of Chemistry, State University of New York at Albany, Albany, New York 12222

SUMMARY

The kinetics and equilibrium of the electron transfer between isolated cytochrome $c_1$ and cytochrome $c$ and artificial carriers have been studied. The oxidation of ferrocytochrome $c_1$ by ferricyanide showed a rate constant of $3.6 \times 10^4$ 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 $c_1$ by ascorbate was found to be 1.2 M$^{-1}$ s$^{-1}$. The second order rate constant of the reduction of ferricytochrome $c_1$ by $N,N',N'',N'''$-tetramethyl-p-phenylenediamine to ferrocyanochrome $c_1$ and Wurster’s blue was $2 \times 10^4$ M$^{-1}$ s$^{-1}$ and that of the reverse reaction was $3.9 \times 10^4$ M$^{-1}$ s$^{-1}$. Wurster’s blue could not be further oxidized by ferricytochrome $c_1$ or ferricytochrome $c$. The oxidation of ferrocytochrome $c_1$ by ferrocyanochrome $c$ to ferrocytochrome $c$ and ferrocytochrome $c$ was not a very fast reaction as generally believed and showed a second order rate constant ($k_2$) of $3.3 \times 10^6$ M$^{-1}$ s$^{-1}$ at 10°C and the reverse reaction showed a rate constant ($k_{-1}$) of $1.0 \times 10^4$ 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, $k_2$, was, however, a function of temperature and ionic strength as well as pH. The reaction rates toward ferrocyanochrome $c$ were the same whether ferrocyanochrome $c_1$ 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 $c_1$ 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 $c_1$ is usually measured together with cytochrome $c$ because of their absorption similarities (cf. 3). Indeed, no precise knowledge is available on the electron transfer rate between cytochrome $c_1$ and $c$ either in solution or in particles (cf. 4). Since we have succeeded in isolating highly purified cytochrome $c_1$ (5) with relative ease and have recently crystallized it, the reactions between cytochromes $c_1$ 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 $c_1$ and bound $c_1$ with cytochrome $c$. A part of this work was previously reported (6).

EXPERIMENTAL PROCEDURE

Materials—Crystalline cytochrome $c_1$, cyanoferrochrome $c$ reductase, and cytochrome $c_1$, all from bovine heart were prepared according to Margoliash and Walasek (7), Takemori and King (8), and Yu et al. (5), respectively. The purified cytochrome $c_1$, containing 25 mnoles 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 Wurster’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 $c_1$ and cytochrome $c$ were determined spectrophotometrically. A millimolar extinction coefficient of 17.5 for $A_{532}^{red} - A_{540}^{red}$ was used for cytochrome $c_1$ and 19.5 for $A_{540}^{red} - A_{540}^{red}$ for cytochrome $c$ (5). The spectrophotometric measurements were carried out on Cary spectrophotometers, models 14 or 16, at approximately 23°C, unless otherwise specified. Ferrocyanochrome $c_1$ solution was prepared by the oxidation of ferrocyanochrome $c_1$ (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 Wurster’s blue whose extinction coefficient was taken as 12 cm$^{-1}$ mm$^{-1}$ (cf. 10, 11). The reduction of cytochrome $c_1$ in the pres-
ence of TMPD\(^{1}\) was measured at 552.5 nm, and the difference extinction coefficient was found to be 27.5 cm\(^{-1}\) M\(^{-1}\).

For the equilibrium studies of cytochromes c\(_1\) and e, the concentrations of ferrocytochrome c\(_1\) and ferricytochrome e 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 e. These extinction coefficients were calculated from the difference spectra obtained from a known concentration of cytochrome c\(_1\) and that of e by the following setup. A pair of matched cuvettes containing ferrocytochrome c\(_1\) and ferri-

cytochrome e 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 N sulphuric acid.

The experimental error in the kinetic studies described was 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.

\[
\text{Fe}^{2+} + \text{Fe}^{3+} + \text{Fe}^{2+} + \text{Fe}^{3+} \quad (1)
\]

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 \(3.6 \times 10^5\) M\(^{-1}\) s\(^{-1}\) was obtained from analyzing the initial rate. This value is lower than the rates of \(6.7 \times 10^5\) and \(8.1 \times 10^5\) M\(^{-1}\) s\(^{-1}\) for the oxidation of cytochrome e by ferricya-

nide 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 e by Ascorbate—Fig. 2 shows a protocol of the stopped flow experiment in which ferrocyanochrome e was reduced by ascorbate (Equation 2).

\[
2\text{Fe}^{2+} + \text{ascorbate} \rightarrow 2\text{Fe}^{3+} + \text{ascorbate} \rightarrow 2\text{Fe}^{3+} \quad (2)
\]

The experiment was carried out in 50 mM phosphate buffer, pH 7.4. The concentrations of cytochrome e and ascorbate used were 10 \(\mu\)M and 50 \(\mu\)M, respectively. The reduction of cyto-

\(^{1}\) The abbreviations used are: \(\text{Fe}^{2+}\), ferrocyanochrome c; \(\text{Fe}^{3+}\), ferrocyanochrome e; \(\text{Fe}^{2+}\), ferrocyanochrome c; \(\text{Fe}^{3+}\), ferrocyanochrome e; TMPD, \(N,N',N''\)-tetramethyl-p-phenylenediamine; WB, Wurster's blue.
The concentrations used for cytochrome c1 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 c1 and c by Wurster’s Blue**—It has been postulated (11, 16) that the semiquinone of TMPD or Wurster’s blue accepts electrons at the site of either cytochrome c or c1, or both, in the respiratory chain. We thought the comparison of the reactions between cytochromes c and c1 with WB could substantiate or disprove the proposal. The stopped flow experiments on the reduction of WB by cytochromes c1 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 c1 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 c1 was found to be \( 3.9 \times 10^4 M^{-1} s^{-1} \) and that for ferrocytochrome c was \( 5 \times 10^3 M^{-1} s^{-1} \).

No reaction whatsoever was observed between WB and ferri-cytochrome c1 or ferri-cytochrome 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 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 as illustrated in Equation 3 and 0.5 for cytochrome c1 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 at pH 7.4 reported by Michaelis and Hill (19).

**Equilibrium Constant for Reaction of Ferrocyanochrome c1 and Ferrocyanochrome c**—The equilibrium of Equation 5 was studied by direct spectrophotometric measurements as well as by the kinetics of the forward and backward reactions.

\[
K_c = \frac{k_{-1}}{k_1} \quad \text{for reaction} \quad c^+ + c' \rightleftharpoons c^+ + c'^+.
\] (5)

For the forward reaction, a representative experiment was conducted as follows. One milliliter of 12.9 \( \mu M \) ferrocyanochrome c1 and 1 ml of 13.6 \( \mu M \) ferrocyanochrome 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 \( c^{+1} = 8.6, c^+ = 4.3, c'^+ = 5.0, \) and \( c' = 8.6 \mu M \). The equilibrium constant, \( K_c \), was thus found to be 3.5. Similar results were obtained when reactions were started with ferrocyanochrome c and ferrocyanochrome c1 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° to 23°, 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 c1**—The oxidation-reduction potential of cytochrome c1 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 c1, then \( E_m \) of cytochrome c1 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).

**Table I**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>( K )</th>
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<tr>
<td>pH 7.4</td>
<td>3.5</td>
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<tr>
<td>7.4</td>
<td>3.5</td>
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<tr>
<td>8.2</td>
<td>1.7</td>
</tr>
<tr>
<td>8.6</td>
<td>0.7</td>
</tr>
<tr>
<td>KCl added 0.5 m</td>
<td>3.5</td>
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<tr>
<td>Temperature 4°</td>
<td>3.5</td>
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<tr>
<td>23°</td>
<td>3.5</td>
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FIG. 4. A tracing of the forward reaction of $c^+ + c^+ \rightarrow k_{-1} c^+ + c^-$. Concentrations of $c^+$ and $c^+$ were 4.3 and 5.65 mM, respectively, in 50 mM phosphate buffer, pH 7.4. Wave length used was 420 nm, temperature, 10°.

ment at 10°, in which concentrations of 4.3 and 5.5 mM of cytochrome $c_1$ and cytochrome $c$, respectively, were used in 50 mM phosphate buffer, pH 7.4. 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 $3.3 \times 10^5$ M$^{-1}$ 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 $1.0 \times 10^5$ M$^{-1}$ 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 $0.8 \times 10^5$ M$^{-1}$ 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 ferrocytochrome $c$. Under the same condition 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 $c_1$; 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 temperatures 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 c of good purity became available. They could be obtained only when soluble 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 microbial cytochrome c.

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.

**REFERENCES**


13. KOWALSKY, A. (1968) Biochemistry 6, 2362


19. MICHAELIS, L., AND HILL, E. S. (1933) J. Amer. Chem. Soc. 55, 1481


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, \( \times 10^5 \) \( \text{M}^{-1} \text{s}^{-1} \) should read:

\( \times 10^6 \) \( \text{M}^{-1} \text{s}^{-1} \). 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. TOPPER. 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.

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