Electron Transfer between Cytochromes c from Horse and *Pseudomonas*

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SUMMARY

The rate of electron transfer between horse cytochrome c and *Pseudomonas* cytochrome c 551 has been examined by stopped flow spectrophotometry. Experiments were conducted at temperatures from 4.5 to 32.5°C and ionic strengths from 0.03 to 0.32. The pH was maintained at 7.0 with 0.02 M potassium phosphate buffer. Comparative data were obtained for the reaction of cytochrome c with potassium ferricyanide under these conditions. Whereas the rate of the ferricyanide reaction varied with the ionic strength, but not the temperature, the reverse held true for the reaction between cytochrome c and cytochrome c 551. The latter process was insensitive to changes in the ionic strength, and exhibited an activation energy of 12 kcal per mole. The equilibrium constant was found to be 1 at all temperatures. At 4.5°C, pH 7.0, and an ionic strength of 0.2, a second order rate constant of $1.57 \pm 0.18 \times 10^4$ M$^{-1}$ sec$^{-1}$ was obtained. Although *Pseudomonas* cytochrome c 551 and horse cytochrome c differ markedly in primary structure, and are oppositely charged at neutral pH, the rate of electron transfer at 20°C corresponds closely to that previously estimated from nuclear magnetic resonance data for the electron exchange reaction between horse ferri- and ferrocytochrome c at this temperature.

A substantial amount of information has become available concerning the main structural features of the cytochromes c. There is much that remains to be clarified, particularly regarding the changes in structure that occur upon oxidation-reduction, but enough is now known to encourage a more detailed examination of the mode of action of these molecules. Among the foremost questions to be resolved are ones pertaining to the manner in which electrons are transferred to and from the heme group. For example, little is known at this time concerning the mechanism of the reaction between cytochrome c and cytochrome c$_2$, or the oxidation of cytochrome c by the cytochrome oxidase system.

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Since the early work of Theorell and Åkesson, it has generally been held that the heme group of the cytochromes c is coordinated to two side chains of the protein, and must thus be partly or wholly enclosed by the latter in a crevice-like arrangement (1-3). X-ray diffraction studies of horse heart ferricytochrome c and *Rhodospirillum rubrum* ferricytochrome c$_2$ have confirmed this view, and have established that the heme group of these molecules is deeply embedded into the protein (4-6). Indeed, the data for the heart compound show that the porphyrin ring does not project beyond the surface of the protein. This type of structure places obvious limitations on the mechanisms by which the prosthetic group can be oxidized and reduced, particularly in the case of reactions involving the transfer of electrons from one embedded heme group to another. Over the years, there have been a number of proposals concerning the ways in which such reactions might proceed, but the experimental evidence is too limited to show whether any of these mechanisms is in fact operative. To distinguish between the various possibilities, additional information must be brought to bear.

It would be helpful, in this connection, to have data concerning the kinetics of the reaction of cytochrome c with cytochrome c$_2$, cytochrome oxidase and other heme-containing systems under conditions which permit the structure of one or both of the reactants to be systematically varied. In undertaking such experiments, we have turned first to studies of the reaction between cytochrome c and *Pseudomonas aeruginosa* cytochrome c 551. Like cytochrome c$_1$, the *Pseudomonas* protein is acidic, and has absorption spectra sufficiently different from those of cytochrome c to allow the reaction to be followed spectrophotometrically. It is, moreover, a small molecule that has been studied in some detail, and for which the properties of a number of potentially useful derivatives have been delineated. We report here the results of experiments conducted at neutral pH with the proteins in the unmodified state, and compare these with observations concerning the oxidation of cytochrome c by potassium ferricyanide.

EXPERIMENTAL PROCEDURE

Materials—Horse heart cytochrome c was obtained from Sigma (type VI). *Pseudomonas* cytochrome c 551 was prepared from a strain kindly given to us by Dr. N. O. Kaplan. This strain has been referred to in previous work as a *Pseudomonas* fluoro-
cultures were grown without aeration at 37°, using the nitrate medium described by Lenhoff and Kaplan (9). The cytochrome c 551 was isolated from an acetone powder according to the procedure of Ambler (7), modified to include steps involving chromatography on DEAE-cellulose.

A mixture of 200 g of the acetone powder and 650 ml of a 0.1 M ammonium acetate solution of pH 7.0 was ground in a Blender with 15 g of alumina, at a temperature of 45°, for two periods of about 1 min each. The extract thus obtained was cooled to 30°, and treated with a few milligrams of deoxyribonuclease. After centrifugation at 13,000 × g for 20 min, followed by further extraction of the precipitate with 350 ml of the ammonium acetate solution, the combined supernatant fractions were centrifuged for an additional 70 min, and dialyzed against 0.02 M ammonium acetate buffer of pH 6.8. The dialysate thereafter was adjusted to pH 3.9 by the addition of acetic acid, centrifuged to remove precipitate, and diluted with water to a final volume of about 5 liters. The resulting solution was applied to columns of carboxymethyl cellulose equilibrated with 0.05 M ammonium acetate buffer of pH 3.9. The orange fractions obtained upon elution with 0.05 M ammonium acetate buffer of pH 4.5. The fractions containing the product were adjusted with ammonium hydroxide to pH 6.3, and then lyophilized.

The dry powder so obtained was dissolved in about 1 ml of 10−3 M potassium phosphate buffer of pH 6.8, and, after dialysis against this buffer for 18 hours at 4°, was applied to a column of DEAE-cellulose equilibrated with the same buffer at this temperature. The column was developed with potassium phosphate buffer at a concentration gradient of 10−3 to 10−1 M. Azurin, oxidized cytochrome c 551, and reduced cytochrome c 551 emerged as well separated fractions. After dialysis for 18 hours at 4° against 0.02 M potassium phosphate buffer of pH 7.0, the cytochrome fractions were stored at 4° until used. The results of amino acid analysis agreed well with the reported composition (7), and polyacrylamide gel electrophoresis, with a 0.05 M Tris-glycine buffer of pH 8.5, revealed at the most a trace of impurities. All of the experiments reported here were conducted with material from the fraction in which the cytochrome was fully in the oxidized form.

Methods—Cytochrome concentrations were determined spectrophotometrically with the use of published molar absorptivity values (10, 11). Solutions of horse ferrocytochrome c were prepared by reduction of the oxidized form with sodium dithionite, followed by filtration at 4° through Sephadex G-25. The extent of reduction was determined by comparison of the spectra obtained before and after further addition of dithionite. Solutions of Pseudomonas ferricytochrome c 551 were prepared from the fully oxidized fraction obtained upon chromatography on DEAE-cellulose. All solutions were kept at pH 7.0 ± 0.1 with 1.5 × 10−2 M or 2.0 × 10−2 M potassium phosphate buffer.

Stopped flow experiments were conducted with a Durrum-Gibson apparatus, fitted with a 2-cm cell. The data were analyzed graphically and by digital computer. Absorption spectra were recorded with a Cary model 14 spectrophotometer, and a Radiometer pHM4 pH meter was used for pH determinations.

RESULTS

To determine the wave lengths and concentrations at which the reaction between horse cytochrome c and Pseudomonas cytochrome c 551 could be followed most conveniently, difference spectra comparing the components on the left and right sides of Equation 1 were recorded:

$$c_{H}^{II} + c_{P}^{III} \xrightarrow{k_{1}} c_{H}^{III} + c_{P}^{II}$$

Fig. 1. Difference spectrum between the reactants and products of Reaction 1. $\Delta A = \epsilon_{A}^{II} + \epsilon_{B}^{II} - \epsilon_{A}^{III} - \epsilon_{B}^{III}$; temperature, 4.5°; pH 7.0; 0.02 M potassium phosphate buffer.

Fig. 2. Data for a representative stopped flow experiment. Tracing of a photograph of the oscilloscope screen; $c_{H}^{III} = 4.06 \times 10^{-3} M$; $c_{H}^{III} = 0.36 \times 10^{-4} M$; $c_{P}^{III} = 2.00 \times 10^{-3} M$; $\lambda = 420 \mu m$; temperature, 4.5°; pH 7.0; 0.02 M potassium phosphate buffer + 0.16 M KCl.
In this procedure, a pair of absorption cells was filled with a solution of horse ferricytochrome c of known concentration, and a second pair with a solution of \textit{Pseudomonas} ferricytochrome c of this concentration. One cell from each pair was positioned in the sample beam of the spectrophotometer, and the other in the reference beam. After temperature equilibration at 4.5°, the cytochrome c solution in the sample beam and the cytochrome c 551 solution in the reference beam were reduced with solid sodium dithionite. The difference spectrum, $A_{551} - A_{Ho}$ was then recorded. Subsequently, the reduced solutions were reoxidized with potassium ferricyanide, and the previously untreated solutions reduced with dithionite, to obtain the difference spectrum in the reverse manner. The results are given in Fig. 1. At 420 nm, a molar absorptivity difference of 1.96 $\times$ 10$^{4}$ M$^{-1}$ cm$^{-1}$ was obtained. This difference, although not large, was adequate for present purposes; for example, if horse ferrocytochrome c and \textit{Pseudomonas} ferri-cytochrome c 551 were mixed in a 2-cm cell at concentrations of 5 $\times$ 10$^{-6}$ M each, the absorbance at 420 nm would change from an initial value of about 1.8 to a final value of approximately 1.7.

Fig. 2 indicates the results of a typical stopped flow experiment, in which the reaction of ferrocytochrome c with ferricytochrome c 551 was examined at 4.5°, pH 7.0, and an ionic strength of 0.2. Three runs at each of three different scan rates are included to show the reproducibility of the results and the lack of an early component in the reaction.

It was found that the data obtained in experiments such as these could be adequately described (Fig. 3) in terms of the first order expression:

$$A_\alpha - A_\alpha = (A_\alpha - A_\alpha) e^{-\alpha t}$$

(2)

where $\alpha = kC_o$; $k = k_1 = k_2$; $C_0 = [cHo]_o + [cP\sigma]_o + [cH\sigma]_o + [cP\tau]_o$, temperature, 4.5°, pH 7.0; 0.02 M potassium phosphate buffer + 0.16 M KCl.

The rate constant was also calculated for these experiments without the use of Equation 2. Two methods were used, each requiring knowledge of $A_\alpha$ (Fig. 1) and the starting concentrations. In one procedure, $k_1$ was determined graphically from the initial rate of the reaction. This approach gave an average value of 1.54 $\pm$ 0.16 $\times$ 10$^{4}$ M$^{-1}$ sec$^{-1}$. In the second procedure, $k_1$ and $k_2$ were calculated from the integrated rate equation with the aid of a computer program. In keeping with the conformity of the data to Equation 2, the results so obtained gave a value of 1 ($\sum k_1/\sum k_2$) for the equilibrium constant. The average value for $k_1$ and $k_2$ obtained by this method was 1.54 $\pm$ 0.16 $\times$ 10$^{4}$ M$^{-1}$ sec$^{-1}$.

Measurements were made at temperatures from 4.5 to 32.5°. Equation 2 satisfactorily describes the data throughout this range. In Fig. 4, the results of experiments at pH 7.0 and $\mu = 0.2$ are compared with those obtained in parallel studies of the process:

$$cHo + K_{Fe(CN)}^6 \rightarrow cHo + K_{Fe(CN)}^6$$

The reaction of $cHo$ with $cP\sigma$ exhibits a temperature dependence which corresponds to an activation energy of approximately 12 kcal per mole. In contrast, the reaction between $cHo$ and ferricyanide was found to be temperature-invariant over this range.

Fig. 5 compares the ionic strength dependence of Reactions 1 and 3. As may be seen, the reaction of $cHo$ with $cP\sigma$ is essentially unaffected by changes in the ionic strength over the range from 0.03 to 0.3, while the reaction of $cHo$ with potassium ferricyanide is influenced very markedly.

The data for the ferricyanide reaction at pH 7.0 and $\mu = 0.18$ were found to yield a rate constant of 6.7 $\times$ 10$^{4}$ M$^{-1}$ sec$^{-1}$. This may be compared with a value of 8.1 $\times$ 10$^{4}$ M$^{-1}$ sec$^{-1}$ obtained.
under generally similar conditions by Brandt et al. (12). In view of the strong ionic strength dependence of the reaction, and the possibility of specific ion effects, this is considered to be a satisfactory degree of agreement.

In order to examine the possibility that cHo and cPs might readily form a complex with one another, sedimentation equilibrium and velocity experiments were performed with the assistance of Dr. Marvin Cassman. In the sedimentation equilibrium experiment, cHo and cPs were added at concentrations of $1.0 \times 10^{-5}$ M each. In the sedimentation velocity run, the concentrations were $6.4 \times 10^{-4}$ and $5.2 \times 10^{-4}$ M, respectively. Both studies were performed at 7.8°, pH 7.0, and $\mu = 0.2$. In neither case was there any evidence of an association reaction. It is estimated that had 10% of the protein been present in the form of a complex this would readily have been detected.

**DISCUSSION**

The data for the reaction between horse cytochrome c and Pseudomonas cytochrome c 551 can be accounted for most simply on the basis of the second order mechanism of Equation 1, with $k_i/k_{-1} = 1$ under the conditions of the present experiments. At a temperature of 4.5°, pH 7.0, and $\mu = 0.2$, a rate constant of $1.57 \pm 0.18 \times 10^4$ M$^{-1}$ sec$^{-1}$ was obtained. The observation that $K = 1$ is consistent with the fact that essentially equal oxidation-reduction potentials have been found for the two proteins at pH 7: +0.254 volt for cytochrome c (13), and +0.25 volt for Pseudomonas cytochrome c 551 (14).

The rate of the electron transfer reaction is sufficiently rapid to explain the observations of Yamanka and Okumuki (15) concerning the effects of cytochrome c and cytochrome c 551 on reactions such as the following:

\[
\text{Cytochrome } c^\text{II} (\text{eukaryotic}) \rightarrow \text{cytochrome oxidase (Pseudomonas)} \rightarrow \text{cytochrome } c^\text{III}
\]

\[
\text{Cytochrome } c^\text{III} (\text{Pseudomonas}) \rightarrow \text{cytochrome oxidase (eukaryotic)} \rightarrow \text{cytochrome } c^\text{II}
\]

Reaction 4 can be accelerated by the addition of catalytic amounts of cytochrome c 551 to the reaction mixture, while Reaction 5 proceeds more rapidly upon the addition of cytochrome c. The basis for this is undoubtedly to be found in the introduction of intermediate steps of the type illustrated by Equation 1.

In addition to the simple scheme of Equation 1, there are, of course, more involved mechanisms that can account for the data obtained in the present experiments. For example, the reaction between cHo and cPs could be written as:

\[
cHo^\text{II} + cPs^\text{II} \xrightarrow{k_1} cHo^\text{II} - cPs^\text{II} \xrightarrow{k_2} cHo^\text{II} + cPs^\text{II}
\]

A mechanism of this sort would yield a rate relationship similar to that of Equation 2, provided that (a) the intermediate complexes are present at steady state concentrations which are negligible with respect to the concentrations of the other species, and (b) certain of the rate constants are approximately equal to one another. For instance, if $K = k_1/k_{-1} = k_2/k_3$ and $k_3 = k_2$, the expression for the reaction rate would assume the form:

\[
A_n - A_t = (A_n - A_0) e^{-k_c t + K_C t}
\]

In this equation, the second order rate constant of Expression 2 has become the product of an equilibrium constant and a first order rate constant indicative of the rate of electron exchange within a complex of cytochrome c and cytochrome c 551. Since the results of the ultracentrifugation experiments suggest that the concentration of such a complex could have amounted to at most 10% of the total protein used in those studies, the value of $K$ can be assigned an upper limit of $-10^4$ M$^{-1}$, and the value for $k_3/k_2$ a lower limit of $-1$ sec$^{-1}$.

The data for the variation of the rate with the temperature indicate that, at 20°, pH 7.0, and $\mu = 0.2$, the second order rate constant for Reaction 1 assumes a value of $4.9 \times 10^4$ M$^{-1}$ sec$^{-1}$. This figure agrees well with the value of $5 \pm 3 \times 10^4$ M$^{-1}$ sec$^{-1}$ obtained by Kowalsky (16) in a nuclear magnetic resonance study of the electron exchange reaction:

\[
cHo^\text{II} + cHo^\text{III} \rightarrow cHo^\text{II} + cHo^\text{III}
\]

Although the latter investigation was conducted under conditions somewhat different from those of the present work, it would appear that the rates of electron transfer between ferri- and ferro-cytochrome c and between cytochrome c and cytochrome c 551 are essentially identical. Since cytochrome c and cytochrome c 551 are oppositely charged at pH 7, it follows that the rate of electron transfer is unaffected by the net charge of the reactants, a conclusion which is consistent with the further finding that the reaction of cytochrome c with cytochrome c 551 is insensitive to...
changes in the ionic strength. In marked contrast, the reaction between cytochrome c and cytochrome oxidase is known from a variety of studies to be very strongly charge-dependent.

The *Pseudomonas* and horse heart molecules differ not only in net charge, but also with regard to important uncharged residues and in terms of amino acid sequence (7, 17, 18). For example, whereas the eukaryotic cytochromes c contain 4 or more tyrosine residues, 3 of which are invariant, *Pseudomonas* cytochrome c 551 contains but 1 such residue. Moreover, the *Pseudomonas* molecule lacks the characteristic sequence of amino acids found in positions 70 to 80 of all of the cytochromes c of eukaryotic origin thus far examined. The x-ray diffraction analysis of horse ferricytochrome c (4, 5) has shown that this invariant segment contains but 1 such residue. Moreover, the net charge, but also with regard to important uncharged residues and in terms of amino acid sequence (7, 17, 18). For example, residues, 3 of which are invariant,

between cytochrome c and cytochrome oxidase is known from a number of studies. The x-ray diffraction analysis of horse ferricytochrome c (4, 5) has shown that this invariant segment provides the sixth ligand about the central iron atom (8, 19, 20). In *Pseudomonas* cytochrome c 551, the sixth ligand is provided by methionine residue 61 (8, 19, 20), which occurs in a segment of the peptide chain that differs strikingly from the corresponding segment of the eukaryotic proteins (17, 18):

Horse:  
Asn. Pro. Lys. Tyr. Ile. Pro. Gly. Thr. Lys. Met. Ile. Phe. Ala

*Pseudomonas*:  
Gly. Ser. Gln. Gly. Val. Trp. Gly. Pro. Ile. Pro. Met. Pro. Asn  
61

There are, therefore, significant differences in the nature of the protein side chains deployed in close proximity to the heme groups of the two systems (21). However, these as well as other differences in the structures of cytochrome c and cytochrome c 551 appear not to affect the rates of electron transfer observed under solution conditions; i.e., Reaction 1 proceeds at the same rate as Reaction 8. It would seem that the rate-determining step is dependent only on structural features common to both cytochrome c and cytochrome c 551, or, less likely, involves only the cytochrome c component of the two reaction systems.

A number of mechanisms have been proposed for the transfer of electrons to and from the heme group of the cytochromes c under conditions comparable to those prevailing in the reaction with cytochrome c 551. These include: (a) transfer via π-orbital overlap of the heme group with that of the electron donor or acceptor molecule; (b) transfer via the protein groups occupying coordination positions 5 and 6 about the heme iron; (c) transfer via the thioether groups linking the protein to porphyrin side chains 2 and 4; (d) transfer via one or more aromatic side chains of the protein in π-orbital overlap with one another and with the heme group; (e) transfer via segments of the peptide chain; and (f) transfer via bound solvent or small solute molecules. Some of these proposals seem unlikely in the light of present structural data. For example, the dimensions of the crevice and the arrangement of the heme group within it (4, 5) render it improbable that Mechanism a prevails. Similarly, the structural arrangement in the vicinity of methionine 80 makes it unlikely that reduction of ferrocyanochrome c would proceed via direct interaction of the electron donor with the coordinated sulfur atom. However, it is evident that a detailed analysis of the situation must await information not currently available. In part, this information can be gained from further structural investigations, such as crystallographic comparisons of ferric and ferrocyanochrome c, but much of it will have to come from studies focusing on the relationship between structure and kinetics. In this connection, rate experiments are being conducted with cytochrome preparations of selectively varied structure, obtained by the use of chemical modification procedures.

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