Biochimica et Biophysica Acta, 548 (1979) 417—426

BBA 47744

RATES OF REDUCED CYTOCHROME *c*-FERRICYANIDE BINDING AND ELECTRON TRANSFER

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(Received April 18th, 1979)

Key words: Cytochrome c oxidation; Electron transfer rate; Ferricyanide binding; (Continuous flow apparatus)

Summary

The oxidation of reduced cytochrome *c* by ferricyanide has been studied over a wide range of ferricyanide concentrations using a continuous-flow apparatus. The formation of a ferrocytochrome *c*-ferricyanide complex has been demonstrated and the binding and electron transfer processes separated to give both the oxidation electron transfer rate and the binding rate parameters. The electron transfer rate has been found to be $1.86 \cdot 10^3$ s^{-1} in H_2O buffer and $1.36 \cdot 10^3$ s^{-1} in ^2H_2O demonstrating that a deuterium isotope effect of similar magnitude ($R = 1.37$) to that found in the cytochrome reactions in photosynthetic bacteria [18] is also found in the reaction studied here. The binding association rate parameters also show a similar deuterium isotope effect suggesting that water rotation may be involved in both the binding of ferricyanide to reduced cytochrome *c* and the subsequent oxidation electron transfer.

Introduction

The iron-hexacyanide-cytochrome *c* oxidation-reduction reactions have been studied, beginning in 1961, as a simple model for biological electron transfer. Kinetic studies have been reported on these reactions by many investigators [1—16]. In 1973 Stellwagen and Shulman [5] found from NMR experiments that iron-hexacyanide-cytochrome *c* complexes must be formed before electron transfer takes place. Stellwagen and Cass [17] demonstrated by equilibrium dialysis that the ferricytochrome *c*-ferricyanide, ferrocytochrome *c*-ferrrocyanide, and ferricytochrome *c*-ferrocyanide complexes axist. No data were presented, however, for the existence of a ferrocytochrome *c*-ferricyanide com-

plex. Miller and Cusanovich [8] presented kinetic evidence from stopped-flow experiments on the reduction of ferricytochrome *c* by ferrocyanide that a ferricytochrome *c*-ferrocyanide complex must be formed before electron transfer takes place. We present in this work complimentary kinetic evidence that in the oxidation reaction of ferrocytochrome *c* by ferricyanide a ferrocytochrome *c*-ferricyanide complex exists and we report the results of measuring the association and dissociation kinetic rate constants of this complex; and, in particular, we have been able to make the binding rate large enough so that the intramolecular oxidation electron transfer rate could be measured. To achieve this, we use a continuous flow instrument and observe at a fixed time after mixing while we increase the ferricyanide concentration. The rates at the higher concentrations are too fast to be measured with a conventional stopped-flow apparatus.

The absolute necessity of water for cytochrome *c* oxidation reactions in photosynthetic bacteria and a deuterium isotope effect of value close to $\sqrt{2}$ in the rate ratio was reported by us [18] in 1973. This result was taken as evidence that water rotation might be rate limiting in these cytochrome *c* oxidation-reduction reactions. In 1977 Ilan et al. [12] reported a deuterium isotope effect with $k_{H_2O}/k_{^2H_2O} = 1.25$—$1.85$ for the oxidation of reduced cytochrome *c* by ferricyanide using both pulse radiolysis and temperature-jump methods. The ferricyanide concentrations used in their experiments, however, were too low to be able to separate the binding and oxidation electron transfer, and to study the deuterium isotope effect on both the ferricyanide binding and oxidation electron transfer reaction separately.

Materials and Methods

Sample preparation. Horse heart cytochrome *c*, type VI, in the oxidized form, obtained from Sigma Chemical Company, was first reduced with sodium dithionite in 0.02 M potassium phosphate buffer at pH 7. The residual dithionite was removed by passing the sample through a 2 cm diameter, 20 cm high Sephadex G-25 column. The reduced cytochrome *c* was then lyophilized and dissolved in either H_2O or ^2H_2O buffers. The concentration of the resulting sample was determined with the use of a spectrophotometer and large samples of 5 μmol reduced cytochrome *c* were prepared, either in H_2O/potassium phosphate buffer or ^2H_2O/potassium phosphate buffer. Since the ferricyanide-reduced cytochrome *c* reaction was known to be pH independent [3] in the pH range 7—8 and to be a sensitive function of ionic strength [4], it was necessary to prepare the buffer solutions so that the ^2H_2O/potassium phosphate buffer had the same ionic strength as the H_2O/potassium phosphate buffer. Therefore the buffers were prepared in the following way:

Solids of KH_2PO_4 (1.06 g) and K_2HPO_4 (2.13 g) dissolved in H_2O to 1 l gave a 0.02 M buffer solution at pH 7.0. The same total amount of solids in ^2H_2O gave a p^{2}H of 7.6 (pH meter reading of 7.16 plus 0.44 correction factor [19]). The equations below show that the ionic strength of the two phosphate buffer systems was the same.

$$\mathrm{pH} = \mathrm{p}K_a^{H} + \log \frac{[B^{H}]}{[A^{H}]}\,; \quad \mathrm{p}^2\mathrm{H} = \mathrm{p}K_a^{^2H} + \log \frac{[B^{^2H}]}{[A^{^2H}]}$$

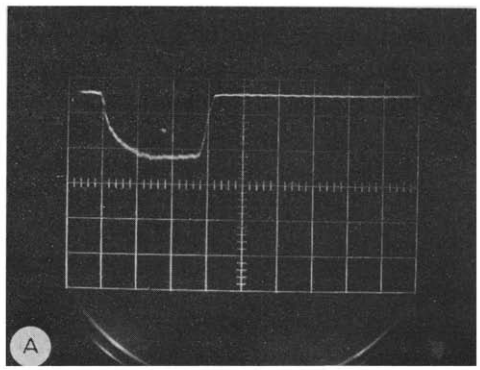
By substituting pH = 7.0, $p^2H = 7.6$, $pK_a^H = 7.2$ and $pK_a^{^2H} = 7.8$ [19], one obtains $7.0 = 7.2 + \log [B^H]/[A^H]$; $7.6 = 7.8 + \log [B^{^2H}]/A^{^2H}]$, thus $([B^H]/[A^H] = [B^{^2H}]/[A^{^2H}])$.

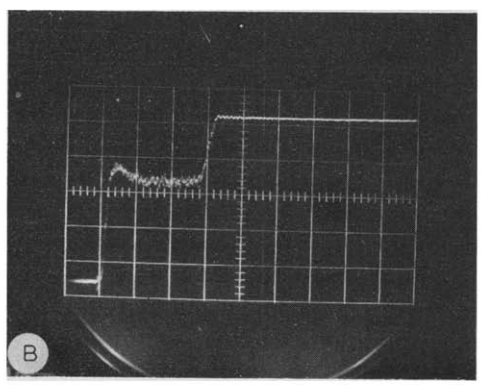
Since the total amount of phosphate was the same in both the H_2O and ^2H_2O cases and the ratio of the dibasic to monobasic species was the same, then the concentrations of monobasic and dibasic species were the same in both the H_2O and ^2H_2O systems. This means then that the ionic strength $\mu = 0.5\Sigma C_i Z_i^2 = 0.07$ was the same. The other ionic species present in the reaction mixture other than the buffer ions have such low concentrations that they make negligible contributions to the overall ionic strength. ^2H_2O was obtained from ICN and the H_2O used was glass distilled. Ferricyanide was obtained from J.T. Baker Chemical Co.

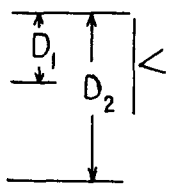
Apparatus and procedures. The mixing instrument used in the experiment was a Chance-Johnson Research Foundation type B continuous-flow apparatus [20]. With this instrument we have looked at fixed times after mixing and then varied the concentration of ferricyanide over a wide range of values. The distance between the point of mixing and the center of the observation window was 1.05 cm and the limiting flow velocity of the solution in the observation tube was either 7.5 or 15 m/s. When nitrogen pressure is applied to the main syringe flow starts and terminal velocity is quickly reached. Near the end of the stroke, the main syringe trips a microswitch so that the pressure differential is released and the syringe movement is then slowed down and stops. The flow velocity was monitored with a circular variable slide wire potentiometer and the resulting variable voltage, due to the variable resistance, was differentiated electronically to give a voltage proportional to the velocity of the large main syringe, which had a diameter of 4 cm. A typical flow trace is shown in Fig. 1A. The ratio of the areas of the observation tube and the main syringe then give the fluid flow velocity. The side syringe had a diameter of 2.38 mm so the resulting dilution factor was 1/283.

The oxidation reaction was monitored with light from a water-cooled tungsten-iodide lamp with a heat filter placed before the observation window. After the observation window a 50/50 beam splitter directed the light to two different 9592 B EMI photomultipliers which were masked with either a 550 nm interference filter or a 525 nm interference filter. (This is the wavelength pair used to monitor oxidation of membrane-bound cytochrome *c* and 525 nm is an isosbestic point for the oxidized and reduced spectra.) The voltages from the two photomultipliers were then subtracted in a difference amplifier.

For a single experiment both the side syringe and main syringe were first filled with the corresponding buffer solution (H_2O or ^2H_2O) and the mixing machine was then repeatedly operated until all bubbles in the observation tube and main syringe were removed. The side syringe was emptied, removed, dried and replaced. Then the side syringe was filled with a solution of ferricyanide, for example 40 mM, and several mixes were performed into buffer to assure that the side syringe mixing solution was uniform. The main syringe was then filled from the top, allowing the buffer in the main syringe to exit at the bottom of the syringe, until an undiluted volume of 100 ml of reduced cytochrome *c* was present in the main syringe. At this time top and bottom syringe valves were closed. The type B instrument is a 'regenerative' type instrument so







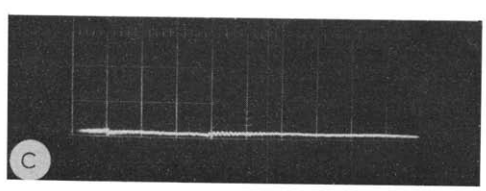


Fig. 1. (A) Typical flow trace 50 mV/vertical division and 50 ms/horizontal division. (B) Corresponding transmission changes for continuous flow mixing of 5 μM reduced cytochrome *c* in the main syringe and 40 mM ferricyanide in H_2O phosphate buffer (0.02 M) in the side syringe. Observation time was 700 μs. (200 mV/vertical division and 50 ms/horizontal division.) (C) Corresponding trace for second mixing indicated all of reduced cytochrome *c* was oxidized in first experiment. Same conditions as above except vertical scale is 100 mV/division.

the main syringe plunger was moved down with mixing valve closed, without the side plunger in, so that no mixing occurred but reduced cytochrome *c* filled the observation tube. Then the voltages corresponding to the transmission of the sample at the two different wavelengths were both adjusted to be 8 V. The sensitivity of the difference amplifier was then increased to, for example, 200 mV/division on the oscilloscope. The main syringe plunger was brought back up into its original position and the side syringe plunger replaced. Then an actual mixing trace was obtained as shown in Fig. 1B. At the beginning of

the trace, the transmission due to the reduced cytochrome *c* sample is being monitored at the observation window. Then the slower velocity fluid reaches the observation window so the transmission seen is that through material which has had a longer time to react. When the limiting or terminal velocity is reached the transmission seen is that due to faster moving fluid and hence material which has had a shorter time to react. The time delay in this picture was 700 μs. The transmission remains then at a constant value until the microswitch is tripped and the pistons decelerate. By the time the pistons come to rest, the reaction has gone to completion and the final transmission is that due to the fully oxidized sample. The plungers were then brought back to their original positions and a second mixing was performed with the result shown in Fig. 1C. The complete absence of a transmission change is consistent with the conclusion that all of the reduced cytochrome *c* is oxidized in the first mixing experiment.

The relative concentrations of ferricyanide and reduced cytochrome *c* were adjusted so that a pseudo first-order reaction should result such that the ferricyanide concentration changes little over the extent of the measured reaction. For example, the value of the reduced cytochrome *c* concentration was 1.5 μM for the 8.8 and 17.7 μM ferrocyanide experiments, 5--6 μM for the 35.3 and 70.7 μM ferricyanide experiments and 20 μM for the 141.3 μM and higher concentration ferricyanide experiments. Since the transmission changes are small compared with the initial sample transmission, the absorption changes will be directly proportional to the transmission changes. These absorption changes are then proportional to the concentrations of reduced cytochrome *c* at time $t = d/v$ = (delay distance/flow velocity) and to the initial amount of reduced cytochrome *c*. The corresponding transmission changes are designated on Fig. 1B as D_1 and D_2, respectively. For a simple pseudo first-order bimolecular reaction one would expect $D_1 = D_2 e^{-ktc}$ where *c* is the concentration of ferricyanide, and *k* is the apparent second-order rate constant. A plot of $\ln D_2/D_1$ as a function of ferricyanide concentration *c* would yield a straight line if the binding of ferricyanide to reduced cytochrome *c* dominated over the subsequent electron transfer.

Results and Analysis

Examples of the type of data obtained at 1.4 ms for ferricyanide plus reduced cytochrome *c* in H_2O and in ^2H_2O buffers are shown in Fig. 2 where $\ln D_2/D_1$ is plotted as a function of ferricyanide concentration *c*. The data were taken at 23°C and pH 7.0 and p^{2}H 7.6. As can be seen, the result for a particular curve is not just a single exponential, with straight line behavior on a logarithmic plot, but instead the curve tends to saturate at high concentrations of ferricyanide. This behavior is interpreted according to the model introduced by Stellwagen and Shulman [5]. We assume that ferricyanide first binds to reduced cytochrome *c*, with possible thermal dissociation, and then an intramolecular electron transfer takes place.

$$Fe^{III} + C^{II} \underset{k_{+3}}{\overset{k_{-3}}{\rightleftharpoons}} C^{II} \cdot Fe^{III} \overset{k_{-2}}{\longrightarrow} C^{III} \cdot Fe^{II}$$

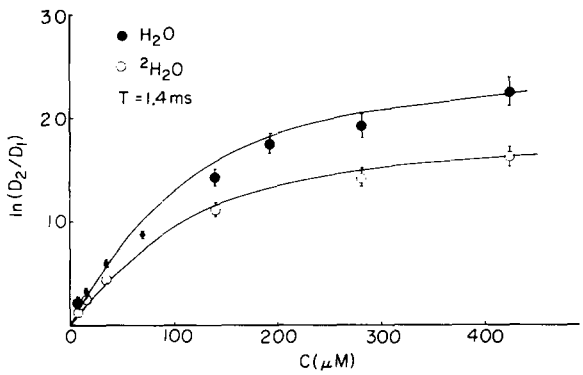


Fig. 2. ●, ferricyanide-reduced cytochrome *c* reaction as function of ferricyanide concentration. The data were taken in H_2O/potassium phosphate buffer with ionic strength 0.07 at 1.4 ms after mixing and pH 7 at 23°C. ○, the corresponding reaction under the same conditions as those above but in ^2H_2O/potassium phosphate buffer at the same ionic strength 0.07 and p^{2}H 7.6.

On the time scale of our experiments the backward electron transfer reaction may be assumed negligible [3,18]. We interpret this result in the following way: at low concentrations of ferricyanide the binding of ferricyanide to reduced cytochrome *c* is the slower process and consequently a limiting straight line behavior is observed. At high ferricyanide concentrations the binding rate has become faster than the subsequent electron transfer so that in the limit of high ferricyanide concentrations a constant ferricyanide concentration-independent rate is observed.

The differential rate equations for the decrease of reduced hemes were set up and solved by Laplace transforms (under pseudo first-order conditions for ferricyanide concentration c much greater than the cytochrome *c* concentration). The expected fractional decrease of reduced cytochrome *c* molecules, D_1/D_2, at time t, as a function of ferricyanide concentration, c, was found to be

$$D_1/D_2 = e^{-at}\left[\cosh(\sqrt{a^2 - b^2}t) + \frac{a}{\sqrt{a^2 - b^2}}\sinh(\sqrt{a^2 - b^2}t)\right]$$

where $2a = k_{-3}c + k_{+3} + k_{-2}$, and $b^2 = k_{-2}k_{-3}c$.

In order to determine the electron transfer rate k_{-2} from the high ferricyaide concentration data it was necessary to develop an asymptotic expansion so that a proper extrapolation could be made. To this end the function $\ln D_1/D_2$, with D_1/D_2 given as above, was expanded in a power series with the independent variable being $1/c$ and only terms up to first order in $1/c$ retained. The resulting equation is then that for a straight line with a corresponding slope and intercept.

$$\ln D_1/D_2 = -k_{-2}t + \alpha\left(\frac{1}{c}\right)$$

where the slope is given by

$$\alpha = \frac{k_{-2}}{k_{-3}} + \frac{t}{16}\left(\frac{3k_{+3}^2 + 10k_{+3}k_{-2} + 3k_{-2}^2}{k_{-3}}\right)$$

Fig. 3 shows the experimental data of Fig. 2 replotted in this fashion and illustrate that at high concentrations straight line behavior of $\ln D_1/D_2$ versus $1/c$ does ensue for both the H_2O data and the ^2H_2O data. From the above equation for $\ln D_1/D_2$ it is seen that the electron transfer rate may be obtained directly from the intercept $-k_{-2}t$. The values obtained by least-squares fitting to a straight line are $k_{-2}^{H_2O} = (1.86 \pm 0.06) \cdot 10^3\ s^{-1}$ and $k_{-2}^{^2H_2O} = (1.36 \pm 0.06) \cdot 10^3\ s^{-1}$ from Fig. 3. Uncertainties were estimated using the calculated standard deviation of the intercepts. For low ferricyanide concentration values it is appropriate to obtain $\ln D_2/D_1$ up to first order in c. An expansion of $\ln D_2/D_1$ in powers of c gives

$$\ln D_2/D_1 = \beta c$$

where

$$\beta = \frac{k_{-2}k_{-3}}{k_{+3} + k_{-2}}\left[t - \frac{(1 - e^{-(k_{+3}+k_{-2})t})}{k_{+3} + k_{-2}}\right]$$

is the limiting slope. Fig. 4 illustrates the limiting straight line behavior for both the H_2O and ^2H_2O data at low ferricyanide concentrations. The straight lines shown were obtained by least-squares fitting. From the α and β slope equations we see that after k_{-2} has been determined we may determine k_{-3} and k_{+3} graphically from the experimental slopes α and β.

$$k_{-3} = F_\alpha(k_{+3}) = \frac{1}{\alpha}\left[k_{-2} + \frac{(3k_{+3}^2 + 10k_{+3}k_{-2} + 3k_{-2}^2)t}{16}\right]$$

and

$$k_{-3} = F_\beta(k_{+3}) = \frac{\beta}{\dfrac{k_{-2}}{k_{+3} + k_{-2}}\left[t - \dfrac{(1 - e^{-(k_{+3}+k_{-2})t})}{k_{+3} + k_{-2}}\right]}$$

Computer solutions for $[k_{-3} = F_\alpha(k_{+3}) = F_\beta(k_{+3})]$ were obtained with computer-generated curves which are shown graphically in Fig. 5 where curves of $\log_{10}F$

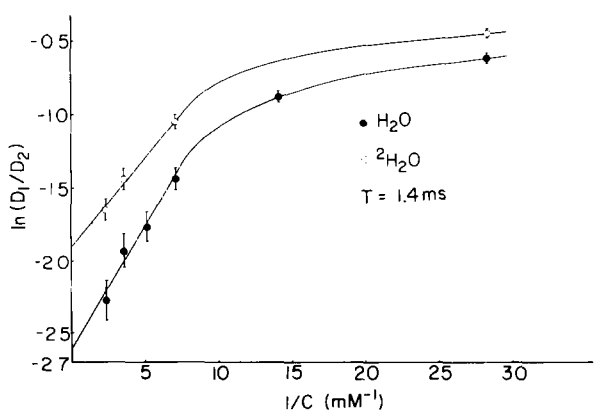


Fig. 3. Asymptotic plot of H_2O and ^2H_2O data given in Fig. 2 in the high ferricyanide concentration region.

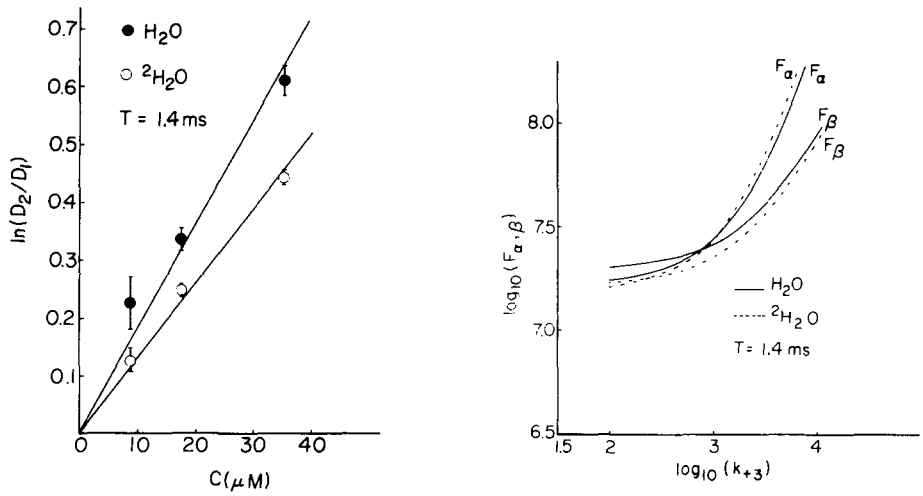


Fig. 4. Low ferricyanide concentration plots for H_2O and ^2H_2O data taken under the conditions given in Fig. 2.

Fig. 5. Graphical solutions to find k_{-3} and k_{+3} for H_2O and ^2H_2O buffer data.

versus $\log_{10}k_{+3}$ are plotted. The corresponding rate constants obtained by this procedure are

$$k_{-3}^{H_2O} = (2.47\ ^{+0.48}_{-0.05}) \cdot 10^7\ M^{-1} \cdot s^{-1}$$

$$k_{-3}^{^2H_2O} = (1.84\ ^{+0.56}_{-0.18}) \cdot 10^7\ M^{-1} \cdot s^{-1}$$

$$k_{+3}^{H_2O} = (7.6 \pm 5.5) \cdot 10^2\ s^{-1}$$

$$k_{+3}^{^2H_2O} = (3\ ^{+3}_{-2}) \cdot 10^2\ s^{-1}$$

The deuterium isotope effect ratios are

$$R_{-2} = k_{-2}^{H_2O}/k_{-2}^{^2H_2O} = 1.37 \pm 0.07$$

and

$$R_{-3} = k_{-3}^{H_2O}/k_{-3}^{^2H_2O} = (1.34\ ^{+0.44}_{-0.34})$$

$$R_{+3} = k_{+3}^{H_2O}/k_{+3}^{^2H_2O} = (2.6\ ^{+10}_{-\ 2.2})$$

Because of the insensitivity of the expression given above for D_1/D_2 to k_{+3} the values for $k_{+3}^{H_2O}$ and $k_{+3}^{^2H_2O}$ cannot be obtained accurately. Therefore, the ratio R_{+3} is so uncertain that no conclusion can be made as to whether or not an isotope effect exists for the ferricyanide-ferrocytochrome *c* complex dissociation process. The dissociation constants for the binding of ferricyanide to

reduced cytochrome *c* can now be estimated and were found to be

$K_3^{H_2O} = (3 \pm 2.3) \cdot 10^{-5}$ M

and

$K_3^{^2H_2O} = (1.6\ ^{+2.1}_{-1.2}) \cdot 10^{-5}$ M

The rate constants found above were then substituted into the equation given at the beginning of this section for D_1/D_2 and the results of these calculations are shown by the solid line curves in Fig. 2.

Discussion

If the data shown in Fig. 4 for low ferricyanide concentrations is interpreted in terms of a simple bimolecular reaction, as has previously been done by various investigators (other than Stellwagen and Shulman [5] and Miller and Cusanovich [8]), then an apparent second-order rate constant for the oxidation of reduced cytochrome *c* by ferricyanide in H_2O buffer of value $k_{app}^{H_2O} = (1.29 \pm 0.06) \cdot 10^7\ M^{-1} \cdot s^{-1}$ is found at 23°C; pH 7 and ionic strength 0.07. This value is to be compared with the value found at comparable ionic strength (0.1) by Sutin and Christman [1] which was $1.6 \cdot 10^7\ M^{-1} \cdot s^{-1}$. The corresponding value of the apparent oxidation rate constant in ^2H_2O buffer would be $k_{app}^{^2H_2O} = (9.23 \pm 0.26) \cdot 10^6\ M^{-1} \cdot s^{-1}$. These values then give a deuterium isotope rate ratio of $R = 1.40 \pm 0.08$, which is to be compared with that found by Ilan et al. [12] which was $R = 1.5$ at pH 7.3 and ionic strength 0.1. Our experimental results are thus consistent with previously measured values. However, when the possibility of a ferrocytochrome *c*-ferricyanide complex is allowed with both complex dissociation and electron transfer then the data must be reanalyzed. When this is done using experimental slope measurements at both high and low ferricyanide concentrations, as shown in Results and Analysis, higher values are obtained and identified as the association rate constants for complex formation: $k_{-3}^{H_2O} = 2.47 \cdot 10^7\ M^{-1} \cdot s^{-1}$ and $k_{-3}^{^2H_2O} = 1.84 \cdot 10^7\ M^{-1} \cdot s^{-1}$ with deuterium isotope rate ratio $R_{-3} = 1.34$. The presence of this effect suggests that solvent (water) reorientation must take place before binding can occur.

The saturation effects observed at high ferricyanide concentration in Fig. 2 show that a complex is being formed by the ferricyanide and reduced cytochrome *c* molecules before electron transfer takes place, thus supporting the model put forth by Stellwagen and Shulman [5]. The existence of the other kinetically important complex of ferrocyanide with oxidized cytochrome *c* has previously been reported by Miller and Cusanovich [8]. The value we have found for the electron transfer rates in the ferrocytochrome *c*-ferricyanide complex, $1.36 \cdot 10^3\ s^{-1}$ in ^2H_2O, may be compared with the value estimated by Stellwagen and Shulman [5] from NMR line-broadening experiments which was $2 \cdot 10^4\ s^{-1}$. However, in their analysis they had to arbitrarily choose a value for K_3. They chose a value similar to the values found for the other complexes. If the lower value for K_3 estimated by us in this work is used in their calculation then they would obtain a lower value for k_{-2} closer to that which we are reporting. Recently Cusanovich [22] has recalculated the value of k_{-2} from the

data of Stellwagen and Shulman [5] using their methods and has estimated a value of K_3 of $4.7 \cdot 10^{-6}$ M from available kinetic data. The result which he finds for ^2H_2O is $k_{-2} = 2.8 \cdot 10^3\ s^{-1}$ which is in quite good agreement with our value. Using a form of excitation modulation spectroscopy Potasek and Hopfield [21] have detected a charge transfer band in the cytochrome *c*-$Fe(CN)_6$ system from which, with the use of the vibronically coupled electron-tunneling theory, they have calculated a value for $k_{-2}^{^2H_2O} = (3.3 \pm 2.7) \cdot 10^4\ s^{-1}$.

It has also been possible to estimate the dissociation rate constant of the transient intermediate complex, as indicated in Results and Analysis, giving values comparable to the electron transfer values, of $k_{+3}^{^2H_2O} = 7.6 \cdot 10^2\ s^{-1}$ in H_2O buffer and $k_{+3}^{^2H_2O} = 3 \cdot 10^2\ s^{-1}$ in ^2H_2O buffer. There may be a deuterium isotope effect here but it cannot be considered accurate because of the insensitivity of the expression for D_1/D_2 to k_{+3}. The measured association and estimated dissociation binding rate constants have, however, been combined to give equilibrium dissociation constants $K_3^{H_2O} = 3 \cdot 10^{-5}$ M and $K_3^{^2H_2O} = 1.6 \cdot 10^{-5}$ M.

From the extrapolated intercepts of the curves shown in Fig. 3, we have been able to measure the oxidation electron transfer rates separately from the effects of ferricyanide binding and find values of $k_{-2}^{H_2O} = 1.86 \cdot 10^3\ s^{-1}$ in H_2O buffer and $k_{-2}^{^2H_2O} = 1.36 \cdot 10^3\ s^{-1}$ in ^2H_2O buffer giving a deuterium isotope rate ratio of value $R_{-2} = 1.37$. Thus, it appears that a deuterium isotope effect of similar magnitude to that found in photosynthetic bacteria [18] also is present in this intramolecular electron transfer process.

Acknowledgements

The authors would like to thank Professor Britton Chance for use of a type B mixing instrument, Professor John J. Hopfield for useful discussions and Herbert Roy for his help with analysis of the data.

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