On the Electron Transfer Reaction between FerriCytochrome c and Ferrohexacyanide in the pH Range 5 to 7*

(Received for publication, November 5, 1973)

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SUMMARY

The electron transfer kinetics between horse heart ferriCytochrome c and ferrohexacyanide in the pH range 5 to 7 is essentially the same as the corresponding kinetics for the pH range 7 to 10. The equilibrium constant, computed from the ratio of rate constants is slightly under 300 for the range investigated. Spectrophotometrically (5 to 20 min after mixing), a somewhat different result emerged. The value of the equilibrium constant (with ferrohexacyanide in the numerator) increased about 2 times for a pH decrease from 7.0 to 5.0. A small minimum is indicated at pH 6.5. There is some similarity between this equilibrium behavior and the one found for the range above pH 7. If one accepts the previously established apparent pK of 6.0 for ferriCytochrome c, one arrives at a protonic dissociation for ferroCytochrome c of about 5.4. However, both of them should be considered upper limits. Lower limits are 5.0 for ferriCytochrome c and 4 (or less) for ferroCytochrome c. The enthalpy change of the fast electron transfer process is in the range of 14 Cal per mole and shows practically no pH dependence. The differences between the kinetic and the spectrophotometric equilibrium constants are probably due to the fact that slow structural rearrangements are coupled to the electron transfer. Such steps also may be the reason for the fact that the measured rate constants are practically independent of pH. Various control experiments were conducted, to establish the constancy of the spectral characteristics of reduced and oxidized Cytochrome c between pH 5 and 7 and in the wavelength range 520 to 555 nm.

The structure is like that of ferriCytochrome c, but more compact and slightly elongated.

The chemical relaxation experiments of Brandt et al. (5) were apparently never extended below pH 7. Although some rather unusual results were published by Havsteen (6), his experiments cannot be compared in thoroughness to those of Brandt et al. (5). The recent experiments of Czerlinski and Bracokova (7) pointed to the need for the extension of the chemical relaxation experiments to cover the pH range from 5 to 7. The results of these experiments are described here.

EXPERIMENTAL PROCEDURE

Horse heart ferriCytochrome c was obtained from Sigma Chemical Co. (type VI). It was rechromatographed on Sephadex of Sigma Chemical Co., according to Margoliash and Walasek (8). The spectrum of cytochrome c was taken on a Cary 14 recording spectrophotometer before each experimental series to determine its concentration (through absorption at 550 nm (eM = 27.7 X 10^5 M^-1 cm^-1) and to check its extent of oxidation via the absorbance density at 550 nm (eMA = 11.0 X 10^5 M^-1 cm^-1) for the reduced and eM = 9.0 X 10^5 M^-1 cm^-1 for the oxidized form). Only cytochrome c containing more than 99% in its oxidized form was used in the experiments with ferriCytochrome c.

Sodium phosphate and sodium cacodylate were obtained from Fisher Scientific Co.; Tris buffer and sodium sulfate were obtained from Sigma Chemical Co. and Allied Chemicals, respectively. All reagents were of analytical grade. The ionic strength of 8 mM sodium ferrohexacyanide may be computed to 80 mM. As the ionic strength should be close to 100 mM, only 20 mM are left for cytochrome c and the buffer mixture. The contribution by cytochrome c to the ionic strength of the mixture is neglected in this series. In the pH range 5 to 7, Tris buffer has practically constant charge. The cacodylate buffer changes from zero charge to one charge with a pK of about 7. With respect to the buffer mixture, the ionic strength changes from 8 mM at pH 5 to 16 mM at pH 7. To facilitate the experiments, a constant mixture was used containing 0.4 mM dibasic sodium phosphate, 5 mM Tris, and 8 mM cacodylate.

The stock solution of sodium ferrohexacyanide contained 10 times the amount of the final solution, was prepared immediately before use, and was kept in the dark. The ferriCytochrome c solution was prepared in 2 to 3 mM concentrations, containing the buffer mixture of low ionic strength. The final mixtures were prepared from these stock solutions and the buffer of low ionic strength. The temperature jump apparatus was described previously (9). The original apparatus was modified to contain a switching network developed recently (10). The voltage signals from the temperature jump...
apparatus were branched so that they were fed into a Tektronix 549 storage oscilloscope and also into a Biomation 802 transient recorder. The data from the Biomation 802 were subsequently transferred onto paper tape. This paper tape was submitted onto the disk of a CDC 6400 computer for further evaluation, using a nonlinear least squares program.

Parallel to the temperature jump experiments, the spectrum of the reaction mixture was taken on the Cary 14 recording spectrophotometer, using a cell of 10-mm path length, thermostatted at 25°. Experimental points were taken at wavelengths 550 and 541 nm. The equilibrium data were evaluated, as described earlier (5).

Results

The composition of the five solutions, used at pH 7, are summarized in Table I, which also contains the observed difference extinctions and relaxation time constants. Similar experiments were carried out at pH 6.5, 6.0, 5.5, and 5.0.

Fig. 1 shows the quotient of the inverse observed relaxation time over the analytical ferrohexacyanide concentration versus the quotient of the sum of the determined equilibrium concentrations of ferrocytochrome c and of ferrihexacyanide over the analytical concentration of ferrohexacyanide. The presentation becomes thereby directly comparable with the presentation of earlier data for the pH range above 7.0 (Fig. 1 of Ref. 5). Only data for two pH values are shown; data for other pH values coincide with those for pH 6.5. The intercepts and slopes lead directly to the two bimolecular rate constants defined by:

$$\frac{1}{r} = k_1 + k_2 ([C^{II}] + [Fe^{III}]/[Fe^{II}])$$

The symbols Fe^{II}, Fe^{III}, and C^{II} refer to ferro- and ferrihexacyanide and ferrocytochrome c; brackets around symbols denote equilibrium concentrations. If Fe^{II} and C^{II} represent the analytical concentrations of ferrohexacyanide and ferrocytochrome c, originally mixed together, it is

$$Fe^{II} = [Fe^{III}] + [Fe^{II}]/[Fe^{II}]$$

$$C^{II} = [C^{II}] + [C^{I}]$$

As under all experimental conditions:

$$C^{III} \ll Fe^{II}$$

the original expression for the inverse relaxation time,

$$r^{-1} = k_1([Fe^{III}]) + [C^{II}] + k_2([Fe^{III}] + [C^{III}])$$

Table I

| pH | Experiments at pH | 7 |
|---|---|---|
| 5.0 | 26.7 | 25.5 |
| 6.0 | 25.3 | 25.5 |
| 7.0 | 25.3 | 25.5 |

The concentration of ferricytochrome c is determined as a difference in the amount of horse heart ferricytochrome c at 25°.

Fig. 1. Evaluation of data from temperature jump experiments. Only the results associated with two pH values are shown. The data for pH 5.5, 6.0, and 7.0 practically coincide with those at pH 6.5. The expression used for the independent variable in this figure contains the concentration of ferrihexacyanide separately from that of ferrohexacyanide. This separation is due to the fact that small amounts of ferrihexacyanide are produced in our system through the (indirect?) action of molecular oxygen upon ferrohexacyanide at these pH values. Successive experiments on the temperature jump apparatus with various solutions took time and made it necessary to correct for the additional amount of ferrihexacyanide which can no longer be neglected at low concentrations of initial cytochrome c concentrations. The chemical mixture consisted (initially) of 8 mM ferrohexacyanide, 8 mM cacodylate, 0.4 mM phosphate, 8 mM Tris, and varying amounts of horse heart ferrihexacyanide at 25°.
TABLE II
Values of molar difference extinction coefficients at 660 nm for horse heart ferro- and ferricytochrome c

The listed results for the molar difference extinction coefficients (of ferro- versus ferricytochrome c), $\Delta \varepsilon_{660}$, are based on triplicate spectrophotometric measurements on the Cary 14, using 0.032 mM ferricytochrome c. Dithionite was added as powder, to cause full reduction of the heme protein; light path length was 1.0 cm. The molar extinction coefficient for ferricytochrome c at 650 nm, $\varepsilon_{650}$, was added for reference (in the same units, $\text{M}^{-1} \text{cm}^{-1}$; the error is close to 1%).

| pH  | $\Delta \varepsilon_{660}$ | $\varepsilon_{650}$ |
|-----|--------------------------|---------------------|
| 5.0 | $2.09 \pm 0.03 \times 10^4$ | $8.38 \times 10^4$ |
| 5.5 | $2.04 \pm 0.007 \times 10^4$ | $8.39 \times 10^4$ |
| 6.0 | $2.10 \pm 0.02 \times 10^4$ | $8.47 \times 10^4$ |
| 6.5 | $2.05 \pm 0.03 \times 10^4$ | $8.43 \times 10^4$ |
| 7.0 | $2.09 \pm 0.03 \times 10^4$ | $8.40 \times 10^4$ |
| Average | $2.07 \times 10^4$ | $8.41 \times 10^4$ |

TABLE III
Rate constants of electron transfer

The data are derived from intercepts and slopes of plots of the type shown in Fig. 1 for two pH values.

| pH  | $k_1$ | $k_2$ |
|-----|-------|-------|
| 7.0 | $0.0239 \pm 0.0027$ | $6.52 \pm 0.45$ |
| 6.5 | $0.0248 \pm 0.0018$ | $5.85 \pm 0.22$ |
| 6.0 | $0.0243 \pm 0.0023$ | $4.92 \pm 1.05$ |
| 5.5 | $0.0237 \pm 0.0029$ | $4.67 \pm 0.40$ |
| 5.0 | $0.0005 \pm 0.0006$ | $6.61 \pm 0.54$ |
| Mean | $0.0266 \pm 0.0049$ | $6.07 \pm 0.61$ |

Fig. 2. Results of spectrophotometric titrations shown for three pH values. The data from the remaining two pH values (6.0 and 5.0) lie between the data shown for pH 5.5 and 6.5. They are not included in this graph to maintain clarity.

Fig. 3. Summarizing graph showing the equilibrium constant as a function of pH. $\circ$, equilibrium constant derived from spectrophotometric titrations; $\bullet$, equilibrium constant derived from the kinetic data. The conditions are otherwise those indicated in Fig. 1.
constant $\tau$ (or $\tau_i$), the factor $\Delta S_1$ in front of the exponential term, and a constant $\Delta S_0$. Values of $\Delta S_0$ and $\Delta S_1$ carry the unit millivolt and represent equilibrium signal changes observed at the output of the temperature jump apparatus. The parameter values $\Delta S_1$ may be algebraically connected to the enthalpy of the electron transfer step, utilizing

$$\frac{d\ln K}{d\tau} = \frac{\Delta S_1}{RT^2}$$

A variety of specific cases for evaluating $d\ln K = \Delta\ln K = \Delta K/K$ were discussed in a monograph (11). A more condensed relation may be obtained by applying DeDonder’s “extent of a reaction,” as introduced by Eigen and DeMaeyer (12) and used for specific reactions by Thusius (13).

In zero approximation, one may consider the following relation between $\Delta S$ and $\Delta\ln K_{1;i}$:

$$\Delta S_1 = \Delta\ln K_{1;i}$$

with the definitions:

$$\Delta \ln = \sum_i n_i \ln \frac{1}{n_i} = 38/38$$

$$G = \left( \sum \frac{1}{c_i} \right)^{-1}$$

In the last two equations it is $c_i$, the equilibrium concentration of the i-th component; $n_i$ becomes then the photometric conversion constant for the i-th component. In the system under consideration, summation proceeds from 1 to 4. Equation 14 becomes then for the system under consideration:

$$G = \left( \frac{1}{[C^{\pm}] + \left[\text{Fe}^{\pm}\right] + \left[\text{H}^{\pm}\right] + \left[\text{Fe}^{\pm}\right]} \right)^{-1}$$

A plot of $\Delta S_1$ versus $G$ should give $\Delta\ln K$. However, proportionality is only valid for small deviations of transmission from 100%. As this is not the case in the presented experiments, $n_i$ becomes dependent upon concentration and one has to employ:

$$I = I_0 \left[ \exp(-c_1 c_{1}) + \exp(-c_1 c_{1}) \right]$$

$I_0$ is the signal for 100% transmission, $c_i$ is the extinction coefficient for the i-th component; for simplicity $c_i = [C^{\pm}]$ and $c_i = [C^{\pm}]$ with $c_{1} = c_{1} > 0$ for 520 cm$^{-1} \leq \lambda \leq 655$ cm$^{-1}$. One obtains from the last equation for $S = 1$:

$$\frac{38c_1}{38c_2} = -c_1 c_{1} \left[ \exp(-c_1 c_{1}) + \exp(-c_1 c_{1}) \right]$$

$$\frac{2c_1}{38c_2} = -c_2 c_{2}$$

Equation 13 becomes then:

$$\Delta \ln = -I \ln \left[ \exp(-c_1 c_{1}) + \exp(-c_1 c_{1}) \right]$$

If

$$c_1 = c_1 c_{1} \left( -c_1 c_{1} \right) + c_2 \exp(-c_1 c_{1})$$

one obtains

$$\Delta S_1 = -I \ln \left[ \exp(-c_1 c_{1}) + \exp(-c_1 c_{1}) \right]$$

A plot of $\Delta S_1$ versus the concentration-dependent $c_1$ should give the concentration independent factor, from which one may compute the enthalpy change $\Delta H$. The experimental conditions provided $I_0 = 1.5$ volts, $l = 10$ mm, $\Delta T = 2.5^\circ$, and $T = 300^\circ$.

The $\Delta H$ values varied between 15.3 Cal per mole (at pH 6) and 11.0 Cal per mole (at pH 5) with standard errors varying between 10% (at pH 6) and 40% (at pH 6.5). Although the average is 12.45 Cal per mole, this value considers equal weighting of the individual $\Delta H$ values. If the error limits are properly considered, one arrives at a weighted average of 14.0 Cal per mole.

**DISCUSSION**

While the kinetic results from the chemical relaxation experiments were anticipated, the associated equilibrium results from spectrophotometry were not. If we assume in very crude approximation that $K_{eq}$ changes with pH like a sigmoidal curve, one could get along with two apparent protonic dissociation constants, one each on the side of reduced and oxidized cytochrome $c$. The associated schematic is shown in Fig. 4A. The various protonated forms of cytochrome $c$ are labeled, as introduced previously (7). The schematic leads directly to an expression for the pH-dependent equilibrium constant:

$$K_{eq} = \frac{k_1}{k_2} \left[ \left[\text{Fe}^{\pm}\right] \left[\text{H}^{\pm}\right] \right]$$

The protonic concentration in this equation is written $\tilde{c}_2$, which refers to buffered proton concentrations. However, the buffering is weak enough so that addition of strong base or strong acid could produce a stepwise pH change. Let us temporarily assume that the inflection point of a sigmoidal curve is reached at pH 5.0. The sigmoidal change then should flatten out near pH 4.0 with $K_{eq}$ increasing 3-fold over the value at pH 6.5. If one assumes $K'_{eq} = 300, K''_{eq} = 6.0$, as derived earlier (7), one obtains for $K'_{eq}$ a value of 5.4. However, it is obvious from the experimental data that $K''_{eq} < 6.0$. Furthermore, the data show no evidence for sigmoidal shape, meaning $pK''_{eq} < 5.4$ (and most likely $pK''_{eq} < 5.0$).

Aside from the upper limit for $pK''_{eq}$ (being 6.0), a lower limit could be derived from the data. The lower limit is directly obtained from the data under the assumption that $pK''_{eq} = 4.0$. It is then $pK''_{eq} = 5.0$. Further details could only be obtained, if the experiments would be extended to at least pH 6.0. However, earlier experiments on the absorption changes at 695 nm indicated (7) that the system starts to become unstable below pH 5.0.

Fig. 2 reveals that the abscissa intercept for pH 7.0 is very close to the ideal value. One computes $\Delta_{450} = 2.17 \times 10^4$ cm$^{-1}$ (with 25% error). The values of $\Delta_{450}$ for the remaining pH values are intermediate between those at pH 7.0 and 5.0 with only the one at pH 5.5 (like the one at pH 5.0) significantly deviating from the “ideal” value of $2.1 \times 10^4$ cm$^{-1}$. It

![Fig. 4. A. reaction system proposed for the explanation of the results, as far as equilibrium experiments are concerned. This scheme also introduces various equilibrium constants to be used in related equations. B. extended reaction scheme, to include slow structural rearrangements, observed for ferricytochrome c and extrapolated to ferrocytochrome c. The scheme of A was used as basis for the extension.](http://www.jbc.org/Downloaded from http://www.jbc.org/)

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was decided to initiate an investigation of this deviation in a separate study (varying other independent parameters of the system).

With reference to the kinetic experiments, the scheme of Fig. 4A would have to be extended to the symmetric scheme of Fig. 4B, where another type of subscripting is indicated for the various forms of cytochrome c, to the extent of ignoring any explicit indication of protonation. Different subscripting is necessary, as the quantitative connection to the schematic of Fig. 4A can only be partially established. Reasonably firm is only one relation, namely (7).

\[ pK'_{III} = pK'_H + p(1 + k_3/k_4) \]  

(23)

Similarly, one may write

\[ pK''_{III} = pK''_H + p(1 + k'_3/k'_4) \]  

(24)

Furthermore, the two cycles allow the relations:

\[ K'' = K' \left( k_3/k_4 \right) \left( k'_3/k'_4 \right) \]  

(25)

\[ K''' = K' \left( k_3/k_4 \right) \left( k'_3/k'_4 \right) \]  

(26)

These relations are equilibrium relations and should be fulfilled for the condition of the photometrically determined equilibrium constant.

If one assumes constant electron transfer, we may assume \( K'_0 = K''_0 \). The pH dependence of the over-all equilibrium then is given by \( pK''_H \) (with \( pK''_H \) not effective in the experimental range of pH 5 to 7). The data require \( K''_0 \gg K''_0 \). However, as \( pK''_H \) cannot be determined from the experiments, it is not appropriate to compute \( [c^{III}] \) and correct the total ferriytochrome c concentration. It is most likely that \( pK''_H < 6 \) as most likely \( pK'_{III} < 6 \).

Using flow calorimetry, Watt and Sturtevant (14) determined the enthalpy change accompanying the oxidation of ferrocyanochrome c by ferricyanide in the pH range 6 to 11 at 25°C. They obtained about 14 Cal per mole at pH 6 and converged toward 28 Cal per mole at pH 11. Their sigmoidal pH curve is associated with an inflection point at 9.3. They ascribe the structural and protonic dissociations do not contribute any significant increase at pH 5.0, compared to the values at higher pH. This rate constant may be expected to increase further until pH 4, although the values are expected to be different from those in the presence of chloride ion.

As far as the earlier experiments of Brandt et al. (5) are concerned, we obtained essentially the same rate constants for the electron transfer. However, it became necessary to conduct experiments much more closely spaced along the pH scale than done by Brandt et al. This need derives from the fact that the coupling-in of protonic dissociations and monomolecular structural interconversions is more complex for the pH range 5 to 7 than for the pH range 7 to 10. As one would expect, the previously measured slow structural rearrangements (7) do not show up in the electron transfer kinetics, but are presumably contained in the spectrophotometric equilibrium constant \( K_{eq} \), determined a few minutes after mixing of the solutions.

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J. Biol. Chem. 1974, 249:6125-6129.

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Additions and Corrections

Vol. 249 (1974) 6125-6129

In Zabinski, Rose Marie, Kathleen Tatti, and George H. Czerlinski. On the Electron Transfer Reaction between Ferricytochrome c and Ferrohexacyanide in the pH Range 5 to 7.

Reference 15, Greenwood, C. & Wilson, M. T. (1971), should be deleted

Reference 15 should be:

Kaminsky, L. S., Miller, V. J. & Davison, A. J. (1973) Biochemistry 12, 2215

Reference 16 should be:

Yandell, J. K., Fay, D. P. & Sutin, N. (1973) J. Am. Chem. Soc. 95, 1131

Vol. 249 (1974) 7018-7023

In Yang, David C. H., W. Einar Gall, and Gerald M. Edelman. Rotational Correlation Time of Concanavalin A after Interaction with a Fluorescent Probe.

Addendum to page 7022

We obtained a rotational correlation time of 57 ns for the TNS-Con A complex by measurement of the nanosecond decay of the fluorescence polarization anisotropy and stated that this was in agreement with the 58 ns obtained by Inbar, Shinitzky, and Sachs (J. Mol. Biol. 81, 245 (1973)) by static measurements of the fluorescence anisotropy of fluorescein labeled Con A. Re-examination of the paper by Inbar et al. indicates that they obtained a rotational relaxation time of 58 ns, corresponding to a rotational correlation time of 19 ns, much lower than the value we observed for the TNS-Con A complex but comparable to the maximum value of 17 ns we observed for dansyl-Con A. Accordingly, the results of Inbar et al. provide further evidence for our suggestion that these covalent derivatives are not rigidly attached to the protein or that there is a high degree of local flexibility in the Con A molecule in the region of the attached dye.

Vol. 249 (1974) 7282-7289

In Van Der Weyden, Martin B., and William N. Kelly. Human Adenylosuccinate Synthetase. Partial Purification, Kinetic and Regulatory Properties of the Enzyme from Placenta.

Page 7282, co-author's name should be William N. Kelley

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