On the Elucidation of the pH Dependence of the Oxidation-Reduction Potential of Cytochrome c at Alkaline pH*

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SUMMARY

Rate constants were measured for the reversible oxidation-reduction reaction between ferricytochrome c and ferrohexacyanide.

\[
\text{Cyt-III} + \left[\text{Fe}^{II}(\text{CN})_6\right]^- \xrightarrow{k(\text{red})} \text{Cyt-III} + \left[\text{Fe}^{III}(\text{CN})_6\right]^- \xrightarrow{k(\text{ox})} \text{Cyt-II} + \left[\text{Fe}^{II}(\text{CN})_6\right]^2
\]

Kinetic measurements in the pH range 7.0 to 9.4 were made by the temperature jump technique, and comparative values of \(K_{eq}\) were determined by a spectrophotometric titration method based on absorption difference at 550 nm between ferrir- and ferrocytochrome c. Also, stopped flow experiments at 550 nm were performed at pH 9.5. In all of these experiments, excess sodium ferrocyanide (8 \(\times\) \(10^{-3}\) M) was mixed with varying concentrations (5 \(\times\) \(10^{-4}\) to 1.6 \(\times\) \(10^{-3}\) M) of ferricytochrome c at a constant ionic strength of 0.2.

The quotient \(k(\text{ox})/k(\text{red})\) is essentially pH-independent in the pH region investigated; \(k(\text{red})\) was found to be 2.4 \(\times\) \(10^4\) M\(^{-1}\) sec\(^{-1}\), and \(k(\text{ox})\), 0.87 \(\times\) \(10^7\) M\(^{-1}\) sec\(^{-1}\).

In the pH region investigated, the over-all equilibrium constant for the reaction, \(K_{eq}\), increased by a factor of 4 with increasing pH.

The stopped flow experiments indicated that a slow process with a half-time of several seconds is responsible for the difference between \(k(\text{ox})/k(\text{red})\) and \(K_{eq}\) values at alkaline pH values.

The experiments indicate that pH has no effect on the electron transfer process itself. The pH dependence of the oxidation-reduction potential of cytochrome c at alkaline pH is evidently due to the effect of pH on a slow isomerization of ferrocytochrome c, with increasing pH decreasing the concentration of cytochrome c which participates in the electron transfer process.

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Previous investigations have shown that the oxidation-reduction potential of cytochrome c is pH-dependent (1-4). The reason for this pH dependence is not known (1), even though the phenomenon has been investigated, under equilibrium conditions, in a great number of laboratories. We have now started to investigate the kinetics of oxidation-reduction reactions mediated by this biologically important protein, since such studies may reveal aspects of the electron transfer mechanism that are not revealed by equilibrium measurements.

In this paper we are reporting investigations in the pH region 7 to 9.5 of the reaction

Ferricytochrome c + ferrohexacyanide \(\rightarrow\) ferrocytochrome c + ferrirhexacyanide

The kinetics of this reaction was investigated by both chemical relaxation and stopped flow techniques. The over-all equilibrium constant for the reaction was obtained from spectrophotometric determinations of the concentration of ferrohexacyanide at 550 nm. A preliminary account of some of these experiments has been published (5).

It has been reported (1, 2) that the oxidation-reduction potential of cytochrome c decreases by about 60 mv per pH unit above pH 8, while the oxidation-reduction potential of iron-hexacyanide is pH-independent in this pH region (6). Previously reported kinetic experiments on the reduction and oxidation of ferrihexacyanide c have indicated the order of magnitude of the rate constants. Chance (7) and Beetlestone (8) reported the rate constant of oxidation of ferrocytochrome c by the hydrogen peroxide complex of yeast cytochrome c to be greater than \(10^6\) M\(^{-1}\) sec\(^{-1}\). Sutin and Christman (9) reported a bimolecular rate constant of 1.6 \(\times\) \(10^7\) M\(^{-1}\) sec\(^{-1}\) for the oxidation of ferrocytochrome c by ferrihexacyanide at pH 6.0, and Havsteen (10), for the same reaction at pH 7.0, reported a bimolecular rate constant of 1.2 \(\times\) \(10^8\) M\(^{-1}\) sec\(^{-1}\). Kinetic experiments of Greenwood and Palmer (11) on the reduction of ferricytochrome c by ascorbate gave a rate constant of 7.5 \(\times\) \(10^5\) M\(^{-1}\) sec\(^{-1}\) at pH 7.0, and indicated the existence of two functionally distinct forms of ferri- cytochrome c at alkaline pH.

EXPERIMENTAL PROCEDURE

Materials and Apparatus

The crystalline, monomeric horse heart cytochrome c used in these experiments was a generous gift of Dr. Emanuel Margoliash, Abbott Laboratories.
Glycine (ammonia free) was obtained from Eastman. Sodium phosphates, sodium sulfate, and sodium hydroxide were Baker analyzed reagents, and sodium hydrosulfite (purified) was also obtained from Baker. The buffer Tris was obtained from Sigma. All other chemicals were reagent grade and were obtained from Mallinckrodt.

The chemical relaxation experiments were performed on the temperature jump apparatus of Czerlinski (12), a coaxial modification of the original apparatus of Czerlinski and Eigen (13). A special small volume, stopped flow apparatus designed by Chance and Legallais (14), was also used for rate measurements. Measurements of pH were made on a Radiometer Type TTT1c pH meter, relative to Beckman pH 7.0 standard buffer. Spectroscopic measurements were made on a Cary recording spectrophotometer, model 15.

Methods

Preparation of Solutions for Temperature Jump and Spectrophotometric Experiments—Stock solutions of pH buffer, ethylenediaminetetraacetic acid, sodium sulfate, and sodium ferrocyanide were prepared at concentrations either 5 or 10 times the concentration desired in the final solutions (see Table I). The sodium ferrocyanide was prepared fresh daily in oxygen-free water and was protected from the light. A stock solution of ferricytochrome c, about 5 X 10^{-4} M, at pH 8.0 (NH₄OH), was prepared for each series of experiments, and its concentration was determined spectrophotometrically at 526.5 mp with the use of an extinction coefficient of 1.1 X 10^{4} M cm⁻¹ (15). Before each series of experiments, the electron transfer property of the ferricytochrome c stock solution was tested spectrophotometrically. Measurements of the spectra were made before and after complete reduction with sodium dithionite in order to make certain that the stock solution was completely oxidized, and to check the molar difference extinction coefficient at 550 mp.

The test solutions were made up by pipetting the proper amount of all the stock solutions except that of cytochrome c into a 10-ml volumetric flask, adding acid or base to produce the desired final pH, and then, at the last moment, adding ferricytochrome c stock solution and diluting the mixture to 10 ml. As quickly as possible after mixing any given solution, an aliquot was removed for the temperature jump measurement; the remaining portion was used for both spectrophotometric and pH measurements.

Test solutions contained a constant initial concentration of sodium ferrocyanide (8 X 10^{-4} M) and varying initial concentrations (5 X 10^{-4} to 1.6 X 10^{-4} M) of ferricytochrome c. The experiments were performed in the pH range 7.0 to 9.4 at an ionic strength of about 0.18 M. Details of solution composition at the various pH values are given in Table I.

Temperature Jump Measurements—Each experimental solution at 22° was perturbed by a 4° jump in temperature, and the change in transmittance at 560 mp following this perturbation was displayed on an oscilloscope. Photographs of this oscilloscope traces were enlarged by a factor of 2, and values of τ⁻¹ were determined from the half-time of these curves. Also, for several experiments at each pH value, the data were plotted on semilog paper and τ values were obtained from the slopes of the plots. Only a single τ value was observed for any given experiment. For each experiment, measurements were made as soon as possible after mixing (within 2 min). In several cases, measurements were made again after about 15 min; results at the two time intervals were identical within experimental error.

The determination of rate constants from the τ⁻¹ values is discussed in “Results.”

Spectrophotometric Measurements—Spectra of aliquots of the test solutions prepared as described above were routinely recorded in the range 450 to 600 mp at 22°. Total cytochrome c concentration was obtained from values at 526.5 mp. Values of ΔĐΔΔξ, which corresponds to a difference measurement at 550 mp between a sample containing an equilibrium mixture of ferri- and ferroferricytochrome c with respect to a corresponding solution of ferricytochrome c, were obtained as follows. For any given sample, the difference in extinction between 541 mp, an isosbestic point, and 550 mp was measured. This observed ΔĐΔξ value for the sample solution was then converted to ΔĐΔξ. The conversion was made by adding a correction for the difference in absorbance of ferricytochrome c between 550 mp and 541 mp; this correction was obtained from plots according to Beer’s law of the extinction of ferricytochrome c at the two wave lengths. This complex method for obtained ΔĐΔξ value was used in order to conserve cytochrome c, for with this procedure it is unnecessary to prepare, for each test solution, a corresponding solution of ferricytochrome c as reference.

Stopped Flow Measurements—Stock solutions for the stopped flow experiments were made up in the same way as for the temperature jump and spectrophotometric experiments. The reactant solutions were prepared so as to produce, in the mixing chamber of the apparatus, final solutions comparable in composition to the pH 9.4 solutions used in the temperature jump experiments. Reactant solutions containing varying amounts of ferricytochrome c (1.6 X 10^{-4} to 9.2 X 10^{-4} M) were prepared in 2 X 10^{-3} M ethylenediaminetetraacetic acid and 6.6 X 10^{-2} M Na₂SO₄ so that ionic strength was 0.2, and were adjusted to pH 7.0 with dilute NaOH. Sodium ferrocyanide reactant solution,

### Table I

<table>
<thead>
<tr>
<th>pH</th>
<th>Material</th>
<th>Concentration</th>
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<tr>
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<td>Sodium phosphate</td>
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<td>0.18</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>0.001</td>
<td></td>
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<tr>
<td></td>
<td>Sodium sulfate</td>
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<td></td>
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<tr>
<td></td>
<td>Sodium ferrocyanide</td>
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<td>Glycylglycine</td>
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<td>0.19</td>
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<tr>
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<td>EDTA</td>
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</tr>
<tr>
<td></td>
<td>Sodium sulfate</td>
<td>0.035</td>
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</tr>
<tr>
<td></td>
<td>Sodium ferrocyanide</td>
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<td></td>
</tr>
<tr>
<td>9.0</td>
<td>Glycine</td>
<td>0.02</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
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<td>Sodium sulfate</td>
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<td></td>
<td>Sodium ferrocyanide</td>
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<tr>
<td>9.4</td>
<td>Glycine</td>
<td>0.02</td>
<td>0.18</td>
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<td>Sodium sulfate</td>
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<tr>
<td></td>
<td>Sodium ferrocyanide</td>
<td>0.008</td>
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</table>
P. H. Dependence of Oxidation-Reduction Potential of Cytochrome c

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Cyt-CIII + FeII $\xrightarrow{k(\text{red})/k(\text{ox})}$ Cyt-CIII + FeIII

(1)

where Cyt-CIII is ferricytochrome c, Cyt-CII is ferrocytochrome c, FeII is ferrohexacyanide, and FeIII is ferrihexacyanide. The relationship between the relaxation time, $\tau$, and the equilibrium concentrations of the components of the system is given by (16)

$$\tau^{-1} = k(\text{red})[\text{Cyt-CII}] + k(\text{ox})[\text{Ferrocyanide}]$$

(2)

A bar over the symbols denotes equilibrium concentration. Under the conditions of the experiments, $[\text{Ferrocyanide}] \gg [\text{Ferricytochrome c}]$, where the subscript zero denotes initial concentration; and since $[\text{Cyt-CII}] = [\text{FeII}] = 0$, it follows that $[\text{Cyt-CII}] = [\text{Ferrocyanide}]$. Thus Equation 2 becomes, under the conditions of the experiment,

$$\tau^{-1} = k(\text{red})[\text{Ferrocyanide}] + 2k(\text{ox})[\text{Ferricytochrome c}]$$

(3)

Since the initial concentration of ferrocyanide is known, and the equilibrium concentration of ferrocytochrome c can be determined spectrophotometrically from aliquots of the solutions used in the temperature jump experiments, $k(\text{ox})$ and $k(\text{red})$ can be determined from the intercept and slope of plots of the data according to

$$\tau^{-1} = k(\text{red})[\text{Ferrocyanide}] + 2k(\text{ox})[\text{Ferricytochrome c}]$$

(4)

Typical data obtained at pH 7.0 and 9.4 and plotted according to Equation 4 are shown in Fig. 1. The solid lines through the points were computed from the data by linear regression analysis.

It is to be noticed in Fig. 1 that for these experiments, in which the ionic strengths of the solutions were the same, the same slopes and intercepts are obtained, within experimental error, at both pH 7.0 and pH 9.4. However, the $\tau^{-1}$ values, compared at essentially equal initial concentrations of ferricytochrome c and ferrocyanide, are considerably smaller at pH 9.4 than they are at pH 7.0. This can be seen in Fig. 2, which presents a plot of $\tau^{-1}$ with respect to pH for solutions of different pH but the same initial composition.

A summary of the rate constants obtained from the temperature jump experiments is given in Table II. All values were obtained by linear regression analysis of the data. It is to be noticed that both the oxidation and reduction rate constants for the system are independent of hydrogen ion concentration in the pH region from 7.0 to 9.4.

Spectrophotometric Titration

Aliquots of the solutions used in the temperature jump experiments were used for spectrophotometric determinations of the equilibrium concentration of ferrocytochrome c. These concentrations were used for plotting the data obtained in the temperature jump experiments and for the determination of the over-all equilibrium constant, $K_{eq}$, of the reaction. In all cases, absorbance measurements were taken within 5 min after the components were mixed, or roughly at the same time the relaxation experiments were performed. The measurements were independent of time for the period investigated, 15 min.

For the system investigated, the over-all equilibrium constant may be defined by

$$K_{eq} = \frac{[\text{Ferricytochrome c}]_{\text{Ferrocyanide}}}{[\text{Cyt-CIII}]_{\text{FeII}}$$

(5)

Under the conditions of the experiments, in which excess ferro-
cyanide is initially mixed with ferricytochrome c, and with substitution of ADsso (AeM 5& -1 for the equilibrium concentration of ferrocytochrome c, Equation 5 becomes

\[ K_\text{eq} = \left[ \text{Cyt-C}^{\text{III}} - \left( \frac{\Delta D_{950}}{\Delta E_{470}} \right) \right] \frac{\text{Fe}^{3+}}{\text{Fe}^{2+}} \left[ \frac{\Delta D_{950}}{\Delta E_{470}} \right] \]  

(6)

When Equation 6 is written in the form

\[ \frac{\Delta D_{950}}{\Delta E_{470}} = \frac{\text{Cyt-C}^{\text{III}}}{K_\text{eq}} \]  

(7)

\[ K_\text{eq} \text{ and } \Delta E_{470} \text{ can be obtained from the slopes and intercepts of plots of the experimental data. Typical plots of the data at pH 7.0 and 9.4 according to Equation 7 are shown in Fig. 3. The solid lines through the points in Fig. 3 were computed from the data by linear regression analysis. A value for AeM ~0 of 2.1 \times 10^4 was used in the computer program. This value represents an average obtained from difference spectra between oxidized and completely reduced (by dithionite) cytochrome c at the pH values used.}

It can be seen that the slopes of the two lines are different and thus correspond to different $K_\text{eq}$ values at the two pH values: 410 at pH 7.0 and 2050 at pH 9.4.

Table III lists the $K_\text{eq}$ values obtained from a number of experiments in the pH region from 7.0 to 9.4. All values were obtained from linear regression analysis of the data.

**Stopped Flow Experiments**

The difference between $K_\text{eq}$ determined from spectrophotometric experiments, and the quotient of the rate constants, $k'(\text{ox})/k'(\text{red})$, was thought to be due to an isomerization of ferricytochrome c which is slow compared to the observed relaxation processes. To obtain evidence for this hypothesis, a series of stopped flow experiments was conducted as described in "Experimental Procedure." A typical trace of transmittance at

<table>
<thead>
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<th>TABLE II</th>
<th>Effect of pH on rate constants for oxidation-reduction reaction of cytochrome c with iron-hexacyanide</th>
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</thead>
<tbody>
<tr>
<td>pH</td>
<td>Method</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>6.0</td>
<td>Flow</td>
</tr>
<tr>
<td>7.0</td>
<td>Temperature jump</td>
</tr>
<tr>
<td>8.4</td>
<td>Temperature jump</td>
</tr>
<tr>
<td>9.0</td>
<td>Temperature jump</td>
</tr>
<tr>
<td>9.4</td>
<td>Temperature jump</td>
</tr>
</tbody>
</table>

a Concentrations of the experimental solution at each pH value are the same as those given in Table III. For buffer and ionic strength, see Table I. Temperature was approximately 22°.

b Sutin and Christman (9): phosphate buffer; ionic strength, 0.1; temperature, 25°.

c Havsteen (10): Tris buffer; ionic strength, 0.17; temperature, 12°.

It may be noticed in the figure that the transmittance increases with time, reaching a final equilibrium value within 30 sec. This slow process is preceded by a rapid decrease in transmittance which is too fast to be seen on the time scale used and appears in Fig. 4 as a stepwise change at zero time. The transmittance reading at 550 mp made at the end of the experiment ($t = \infty$) was taken to be a measurement of the equilibrium concentration of ferrocytochrome c. The zero transmittance reading ($t = 0$) was obtained by extrapolation of the slow transmittance change to zero time.

Values of $\Delta D_{950}$ corresponding to $t = 0$ and $t = \infty$ were calculated for each experimental solution from the transmittance readings at 550 mp in the stopped flow apparatus and from an absorption spectrum measurement taken at the end of the experiment (see "Experimental Procedure").

The data obtained in the stopped flow experiments at pH 9.5 are shown, plotted according to Equation 7, in Fig. 5. It may be noticed that $K_\text{eq}$ obtained from the data at $t = 0$ is about
NaOH to produce a pH of 9.5 after mixing. In glycine at an ionic strength of 0.2 and containing sufficient ethylenediaminetetraacetic acid at an ionic strength of 0.2 with NaOH and adjusted to pH 7.0 with dilute NaOH was mixed.

22°. A solution 9.2 X 10⁻⁵ M in ferricytochrome c and 2 X 10⁻³ M in sodium ferrocyanide and 4 X 10⁻³ M in glycine at an ionic strength of 0.2 and containing sufficient NaOH to produce a pH of 9.5 after mixing.

740; this value (obtained at pH 9.5) is in reasonable agreement with the value obtained at pH 7.0 by the spectrophotometric method (see Table III). The Kₚ value obtained from the stopped flow measurements at t = ∞ is about 2000, in close agreement with the value obtained from spectrophotometric experiments at pH 9.4.

DISCUSSION

The results obtained in the temperature jump experiments with the system

\[
\text{Cytc}^\text{III} + \text{Fe}^\text{II} \rightleftharpoons \frac{k(\text{red})}{k(\text{ox})} \text{Cytc}^\text{II} + \text{Fe}^\text{III}
\]

and presented in Fig. 1 and Table II show that the oxidation and reduction rate constants are pH-independent in the pH region from 7.0 to 9.4 when Equation 3 and the spectrophotometrically determined ferrocyanide c concentrations are used for the evaluation of the rate constants. The rate constants obtained in these studies are compared, in Table II, with those obtained by Havsteen (10) at pH 7.0 from temperature jump experiments and those of Sutin and Christman (9) obtained at pH 6.0 from flow experiments. When different cytochrome preparations and different buffer solutions used are taken into account, the data agree well.

The temperature jump data also show (Fig. 2) that τ is pH-dependent at constant initial concentrations of ferricytochrome c and ferrocyanide. These results are also in agreement with previous observations of Havsteen (10).

The data presented in Fig. 3 and Table III show that Kₛₑₒₚ, determined in spectrophotometric experiments, increases with pH in the pH region 7.0 to 9.4, in agreement with the known pH dependence of the oxidation-reduction potential of cytochrome c. It should be noticed that at pH 7.0, Kₛₑₒₚ is about the same as the quotient of the rate constants, k(ox)/k(red) (see Table II), but that while k(ox)/k(red) is pH-independent, Kₛₑₒₚ increases to around 2000 at pH 9.4. The stopped flow experiments (Fig. 4) clearly show that the difference between k(ox)/k(red) and Kₛₑₒₚ at the higher pH values is due to a slow process. Immediately after a fast bimolecular process of reduction of ferricytochrome c by ferrocyanide has gone to completion at pH 9.5 (Figs. 4 and 5), Kₛₑₒₚ has about the same value as the quotient of the rate constants.

Then, as can be seen from Fig. 4, the transmittance at 550 nm changes slowly, indicating a slow decrease in ferrocytochrome c concentration. The experimental evidence available suggests that this slow process is due to a pH-dependent equilibrium between at least two forms of ferricytochrome c, only one of which participates in the oxidation-reduction reaction. The considerations which lead to this hypothesis, presented graphically in Fig. 6, are summarized below.

1. There are several experimental observations which make a slow process involving isomerization of ferrocyanide c unlikely. (a) The optical rotatory dispersion spectrum of ferrocyanide c is pH-independent in the pH region investigated. (b) The molar absorption difference coefficient between com-

pletely oxidized and completely reduced cytochrome c is pH-independent at 550 μM in the pH region investigated. This indicates that the increase in transmittance at 550 μM observed in the stopped flow experiments is caused by a slow conversion of ferrocyanochrome c to ferricytochrome c rather than to a ferrocytochrome c species having altered spectral properties at 550 μM. (c) The oxidation rate constant, k(red), is pH-independent. Since the experimental conditions used in the temperature jump experiments and the equation used for evaluating k(red) make the determination of k(red) particularly sensitive to the measured equilibrium concentration of ferrocytochrome c, a pH-dependent isomerization of ferrocyanochrome c which involved the electron transfer process would be expected to show up as pH-dependent changes in the value of k(red).

2. There are several experimental observations which make a slow, pH-dependent equilibrium among various forms of ferrocytochrome c, with only one form reducible by ferrohexacyanide, a likely possibility. (a) Although k(ox) and k(red) are pH-independent, τ increases with increasing pH at constant initial concentrations of ferricytochrome c and ferrohexacyanide. The simplest explanation for these observations is that, with increasing pH, a decreasing amount of cytochrome c participates in the oxidation-reduction process, and that the fraction of cytochrome c which does participate in the oxidation-reduction process does so at the same rate throughout the pH region investigated. (b) Under the conditions of the experiments, the concentration of ferricytochrome c does not affect the relaxation time (see Equation 3) and therefore an isomerization of ferricytochrome c which is slow compared to the oxidation-reduction process would not be expected to affect the value of k(red). An isomerization of ferricytochrome c would, however, be expected to affect the spectrophotometrically determined equilibrium constant, for this consists of a combination of equilibrium constants which would include one pertaining to the isomerization of ferrocytochrome c. (c) A pH-dependent protein isomerization of ferrocyanochrome c in the pH region investigated is also suggested by the following observations. Experiments on the temperature dependence of an absorption band at 696 μM, characteristic of native monomeric ferricytochrome c at pH 7, indicated that this band is characteristic of a conformational isomer of ferricytochrome c (17), and this band was found to disappear with increasing pH (18). Moreover, measurements of the optical rotational dispersion parameters of ferricytochrome c showed a pH dependence of these parameters (19). Finally, the kinetics of the reduction of ferricytochrome c by ascorbate indicated the existence of two functionally distinct forms (11).

The hypothesis presented in Fig. 6 adequately accounts for the data presented here and for results reported previously by others. The important conclusion to be drawn from the experiments is that the pH dependence of Keq of the reaction studied, and therefore the pH dependence of the oxidation reduction potential of cytochrome c, is not due to the pH dependence of the electron transfer process per se. The pH dependence of the oxidation-reduction potential is adequately accounted for by a slow, pH-dependent process which changes the over-all equilibrium constant of the electron transfer process without changing the rate with which electrons are transferred between oxidized and reduced reactants.

It is interesting to note that only recently there has been found another example of a pH-mediated reaction in which the pH dependence of the reaction appears to be due to the effect of pH on the conformation of the protein. Evidence has been presented (20–22) which indicates that the decrease in catalytic activity and altered spectral properties at alkaline pH of a proteolytic enzyme, chymotrypsin, is due to a pH-dependent equilibrium between catalytically active and inactive conformations of the enzyme and not to the effect of pH on the hydrolysis step per se.

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