Alkaline Isomerization of Oxidized Cytochrome c

EQUILIBRIUM AND KINETIC MEASUREMENTS*

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SUMMARY

Equilibrium and kinetic studies of the isomerization of horse heart ferricytochrome c at alkaline pH have been carried out using difference spectroscopy and stopped flow techniques. The minimal reaction scheme is:

\[
\begin{align*}
\text{(NC)} & \quad \text{Cyt-c-Fe}^{III} \\
0.05 \text{ s}^{-1} & \quad 0.0 \text{ s}^{-1} \\
\text{(C)} & \quad \text{H}^+ + \text{Cyt-c-Fe}^{III} \\
\text{pK}_H = 11 \\
\text{(CH)} & \quad \text{HCyt-c-Fe}^{III} + [\text{Fe}^{II}(\text{CN})_6]^3^- \\
& \quad \frac{2.6 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}}{8 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}} \\
& \quad \text{HCyt-c-Fe}^{II} + [\text{Fe}^{II}(\text{CN})_6]^3^-
\end{align*}
\]

where CH and C are species of ferricytochrome c which exhibit a 695 nm absorbance band, and NC (no color) is a form without this band; the oxidation-reduction potentials of CH and NC differ significantly. The values of the constants in this scheme were determined at 25° except for the oxidation and reduction rate constants, which were determined at 22°. The pH dependence of the observed rate constant for the conformational changes was measured as the change in the 695 nm band absorbance and as the release and uptake of hydrogen ions. From this it was calculated that the ionizing group in the scheme above has an apparent pK of 11. It is suggested that the difference in oxidation-reduction potential and the absorbance band at 695 nm arises by substituting the sulfur of Met-80 as one of the ligands of the iron porphyrin in CH by the ε-amino group of Lys-79 in NC. This suggestion, consistent with structural information, is accessible to experimental verification.

The oxidation-reduction potential of cytochrome c drops from +0.26 volt at pH 7 to +0.12 volt at pH 10, as though it depends on the ionization of a protonated group with pK 9.3 in ferricytochrome c (1). Two groups reported independently that a pH-dependent isomerization of cytochrome c is responsible for the drop in the oxidation-reduction potential.

Greenwood and Palmer (2), in an investigation of the pH dependence of the rate of reduction of ferricytochrome c by ascorbate, found that at alkaline pH the reaction curve is biphasic, consisting of a fast bimolecular reaction followed by a slow, first order process. They concluded that the slow step represents the conversion of an inactive isomer of ferricytochrome c which cannot be reduced by ascorbate to an isomer which can be reduced.

Brandt et al. (3) investigated the pH dependence of the rates of oxidation and reduction of cytochrome c in the system

\[
\text{Cyt-c}^{III} + \text{Fe}^{II} \xrightarrow{k_{(red)}} \text{Cyt-c}^{II} + \text{Fe}^{III}
\]

using the temperature-jump technique. In this scheme, Cyt-c^{III} and Cyt-c^{II} represent ferri- and ferrocytochrome, and Fe^{II} and Fe^{III} represent ferro- and ferrihexacyanide. It was observed that \(k_{(ox)}\) and \(k_{(red)}\) are independent of pH in the range pH 7.0 to 9.4, thereby demonstrating that the pH dependence of the oxidation-reduction potential is not due to a change in the rate of electron transfer. In a stopped flow experiment at 550 nm in which neutral ferricytochrome c was mixed with alkaline sodium ferrocyanide so that the pH after mixing was 9.4, a fast initial increase in absorbance was observed, which is indicative of the rapid formation of ferrocyanochrome c. This rapid formation of reduced cytochrome c was followed by a slow decrease in absorbance, indicating a decrease in the concentration of ferrocyanochrome c and the reappearance of ferricytochrome c. These experiments led to the suggestion that a slow isomerization of...
ferricytochrome c to a form not reducible by ferrocyanide was responsible for this observed decrease in the concentration of ferricytochrome c. The authors proposed the following scheme to explain their results and the pH dependence of the oxidation reduction potential of cytochrome c at alkaline pH:

\[
\begin{align*}
\text{Cyt-c}^{III} + H^+ & \rightarrow \text{H-Cyt-c}^{III} + Fe^{II} \\
\text{H-Cyt-c}^{III} + Fe^{II} & \rightarrow \text{H-Cyt-c}^{II} + Fe^{III}
\end{align*}
\]

Cyt-c is a ferricytochrome c isomer which is not reducible by ferrohexacyanide. The experiments of Brandt et al. (3) are consistent with the recent results of Pecht and Faraggi (4), who investigated the reduction of ferricytochrome c by pulse radiolysis.

In this paper we report equilibrium and kinetic studies of the pH-dependent isomerization of horse heart ferricytochrome c. The spectral changes at 605 nm and the changes in hydrogen ion equilibria, which accompany this isomerization, were measured.

**EXPERIMENTAL PROCEDURE**

**Materials and Apparatus**

The crystalline, monomeric horse heart ferricytochrome c used in these experiments was a generous gift of Dr. Emanuel Margoliash, Department of Biology, Northwestern University.

Sephadex G-25 was obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N. J. The indicators, phenolphthalein, thymol blue, and chlorphenol red, were obtained from the Fisher Scientific Co., Rochester, N. Y., and sodium hydrosulfite was purchased from J. T. Baker Chemical Co., Phillipsburg, N. J. All other reagents were of analytical grade and were supplied by Mallinckrodt, St. Louis, Mo.

A Durrum-Gibson stopped flow spectrophotometer was used. Photomultiplier output compensation was provided by an external potentiometer. Transmittance changes were recorded on a Tektronic storage oscilloscope, either a type 564 with a type 2A63 differential amplified plug-in unit, or a type 549 with a type 1A7 high gain differential amplifier plug-in unit. The temperature was regulated by means of a Lauda thermostat, type K2-R.

A Radiometer pH meter, model TTT1c, with glass combination electrodes (A. H. Thomas, No. 4858-L15), attached to a Tektronic storage oscilloscope, either type 564 or type 549, provided with an external potentiometer, was used to measure the hydrogen ion release which accompanied the conformational changes in ferricytochrome c. The pH changes were recorded in terms of millivolts.

A Corning model 12 research pH meter equipped with glass-calomel combination electrodes (A. H. Thomas, No. 4838-L15) was also used. All pH measurements were made relative to Beckman standard buffers of values no more than 1 pH unit from the pH of the solutions to be measured. Spectrophotometric measurements were made on a Cary recording spectrophotometer, model 14. The sample compartment was thermostated and the temperature was regulated with a Haake constant temperature circulating water bath.

**Methods**

**Preparation of Ferricytochrome c**

The ferrocyanochrome c was prepared by dissolving 12 to 36 mg of crystalline ferricytochrome c in 1 ml of distilled water at room temperature and reducing it with sodium hydrosulfite. The resulting solution was desalted by placing it on a column of Sephadex G-25, 1 cm × 20 cm, and eluting with distilled water at room temperature.

**Difference Spectrum**

The difference spectrum between alkaline and neutral horse heart ferricytochrome c from 650 nm to 230 nm was measured on a Cary model 14 recording spectrophotometer. A 2-ml aliquot of ferricytochrome c, 40 μM in 0.066 M Na₂SO₄, pH 6.8, was used as the reference solution. The remainder of the sample was adjusted to pH 9.9 with 5 N NaOH; a 2-ml aliquot was withdrawn and used as the sample solution. The dilution of the cytochrome c solution by the added NaOH was less than 1%, and no correction was made. The spectrum was measured at 20°.

**Kinetic Studies**

The conversion of ferricytochrome c, from a form exhibiting the 605 nm band to one with no 605 band, was studied using the stopped flow spectrophotometric method. The rate of the interconversion was investigated using several experimental approaches. In all cases, conditions were chosen so that the transmittance change was less than 5%. The kinetic constants could, therefore, be evaluated directly from the change in transmittance as a function of time.

**pH-Jump: Disappearance of 605 nm Band—Ferricytochrome c** (40 μM in 0.066 M Na₂SO₄, pH 7, ionic strength = 0.2) was mixed in the stopped flow apparatus with either 0.2 M glycine adjusted to varying pH values from pH 0.0 to pH 10.4 with 5 N NaOH, or with glycine-NaOH buffers of varying pH values within the same range and ionic strength = 0.2. The rate of disappearance of the 605 nm band was measured at 16°, 21°, and 25°.

The final pH of the solution was determined by measuring the pH either of a mixture of equal volumes of the protein and buffer solutions, or of the effluent from the stopped flow machine. The results obtained by the two methods did not differ significantly.

**pH-Jump: Reappearance of 605 nm Band—Ferricytochrome c** (40 μM in 0.066 M Na₂SO₄) was adjusted to pH 10.0 with 1 N NaOH and mixed in the stopped flow apparatus with 0.2 M glycine buffer adjusted to varying pH values less than pH 10 with 5 N NaOH or with 0.2 M Tris-Cl, pH 7.0. The rate of reformation of the 605 nm band was measured at 16°, 21°, and 25°. The final pH was determined as above.

**pH-Jump: Hydrogen Ion Release—Horse heart ferricytochrome c** (40 μM in 0.066 M Na₂SO₄, pH 7) was mixed in the stopped flow apparatus with a solution of 0.5 mM glycine, 0.066 M Na₂SO₄, and 40 μM phenolphthalein in 1% methanol which had been adjusted to varying pH values in the range 9 to 10 with 1 N NaOH and had a final methanol content of 0.5% v/v. These solutions were prepared in distilled water which had been boiled and then cooled under nitrogen to remove carbon dioxide. The pH adjustment and all transfers were carried out under nitrogen. Measurements were made at 21°. The rate of the hydrogen ion release was observed at 546 nm, which is an isosbestic point for the low and high pH forms of horse heart ferricytochrome c (Fig. 1).

The rate of the hydrogen ion release was also observed at 557 nm. In this experiment horse heart ferricytochrome c (80 μM in 0.066 M Na₂SO₄, pH 7.0) was mixed with a solution of 2 mM glycine, 0.066 M Na₂SO₄, and 125 μM phenolphthalein in 1% methanol, which had been adjusted to varying pH values from pH 9 to 10 with 1 N NaOH. Although 557 nm is not an isos-
bestie point for the pH conformers of horse heart ferricytochrome c, it can be seen from Fig. 1 that at this wave length, the contribution of the protein to the observed absorbance change is negligible.

In all cases the total absorbance change due to the release of hydrogen ions was less than 0.03 (absorbance unit), corresponding to a pH change of less than 0.05 pH unit. Calibration of the indicator solutions showed that within this range the relationship between absorbance change and the number of protons released is linear. The calibration was performed by preparing solutions identical with those used in the stopped flow experiments and adding varying amounts of HCl in the concentration range 0 to 60 μM. The change in absorbance was recorded.

pH-Jump: Hydrogen Ion Uptake—Horse heart ferricytochrome c (40 μM in 0.066 M Na2SO4) was adjusted to pH 9.6 with 1 N NaOH and mixed in the stopped flow apparatus with 62.5 μM chlorphenol red, 1 mM Tris-HCl, and 0.066 M Na2SO4, pH 7.0. The rate of hydrogen ion uptake was followed at 546 nm. Measurements were made at 21°C.

Rapid Oxidation: Disappearance of 695 nm Band—Horse heart ferrocytochrome c (40 μM in 0.066 M Na2SO4) was adjusted to pH 9.6 with 1 N NaOH and mixed in the stopped flow apparatus with 62.5 μM chlorphenol red, 1 mM Tris-HCl, and 0.066 M Na2SO4, pH 7.0. The rate of disappearance of the 695 nm band was measured at 21°C. The rate of disappearance of the 695 nm band was measured at 21°C. The ferricyanide concentration was chosen so that the ferrocytochrome c was completely oxidized within the mixing time of the instrument.

Rapid Oxidation: Hydrogen Ion Release—Horse heart ferricytochrome c (40 μM in 0.066 M Na2SO4) was adjusted to pH 9.0 with 1 N NaOH and mixed in the stopped flow apparatus with a solution of 0.4 mM Na3Fe(CN)6, 5 mM glycine, and 0.066 M Na2SO4 adjusted to the same pH as the ferrocytochrome c solution with 1 M NaOH. The rate of disappearance of the 695 nm band was measured at 21°C. The ferrocytochrome c was completely oxidized within the mixing time of the instrument.

Absorbance Changes at 243 nm—The kinetics of the change in absorbance at 243 nm (the peak of the difference spectrum between ionized and protonated tyrosine (5)) was observed using a Cary model 14 recording spectrophotometer. Horse heart ferricytochrome c (90 μM in 0.066 M Na2SO4) was added from a Hamilton syringe to 2 ml of a solution of glycine-NaOH, pH 0.8, ionic strength 0.2. Rapid mixing (within 10 s) was accomplished using a Teflon plunger, 1 × 1 cm in area, with holes drilled in the base. The reference solution was horse heart ferricytochrome c, 17 μM in Tris-HCl, pH 7.0, ionic strength 0.2. The sample compartment was thermostated at 21°C.

Equilibrium Experiments

Hydrogen Ion Release—A solution of ferricytochrome c (40 μM in 0.066 M Na2SO4, 2.0 μl) was adjusted to the desired pH with 1 M NaOH in a stoppered, nitrogen-flushed reaction vessel. The vessel was thermostated at 21°C. The solution was rapidly oxidized by the addition of 10 μl of 0.1 M Na3Fe(CN)6. The change in millivolts was recorded on a storage oscilloscope attached to the Radiometer pH meter. Millivolts were converted to Δ[H+]obs using calibration curves obtained by adding various amounts of HCl to the oxidized solution (Fig. 2). The total pH change did not exceed 0.1 pH unit. The control experiment consisted of adding 10 μl of 0.1 M Na3Fe(CN)6 to a solution of ferricytochrome c at the same pH and concentration as the experimental solution. No change in pH was observed.

Spectrophotometric Titration Curves—Measurements for titration curves were made at 16°C, 21°C, and 25°C on the Cary recording spectrophotometer set at a constant wavelength of 695 nm. The reference solution was distilled water. Sample solutions were prepared by mixing equal volumes (either 0.5 or 1 ml) of horse heart ferrocytochrome c (0.4 mM in 0.066 M Na2SO4) and buffer (from pH 7 to pH 8.8, Tris-HCl, ionic strength = 0.2; from pH 9.0 to pH 10.8, glycine-NaOH, ionic strength = 0.2).

RESULTS

Kinetic Studies

The kinetics of the reaction

\[ \text{H Cyt-c}^{III} \rightleftharpoons \text{Cyt-c}^{II} + \text{H}^+, \]

where H Cyt-cIII is the form with the 695 nm band, and Cyt-cII is the form with no 695 nm band, were studied by observing the approach to equilibria from both the acid and the alkaline side of the pH range; from the acid side by observing the decrease in 695 nm absorbance and the release of hydrogen ions, and from the alkaline side by observing the increase in 695 nm absorbance and the uptake of hydrogen ions.

In all experiments the ionic strength was kept in the range of

![Fig. 1](image-url)  
**Fig. 1.** Difference spectrum, horse heart ferricytochrome c (40 μM, 0.066 M Na2SO4), pH 9.9 versus pH 6.8, at 20°C. The dilution resulting from the addition of 5 N NaOH to adjust the sample solution to pH 9.9 was less than 1% and no correction was made for it.

![Fig. 2](image-url)  
**Fig. 2.** Calibration of the buffer capacity of horse heart ferrocytochrome c solutions, 41.6 μM in ferricytochrome c, 0.066 M in Na2SO4, 21°C. The pH change, measured using a glass electrode, is expressed in millivolts. a, pH 10.3; b, pH 9.5.
Variation within this range had no effect on the $k_{\text{obs}}$ values (Table I).

It was also found that at a given pH, $k_{\text{obs}}$ is independent of the cytochrome c concentration in the range used in the investigations (Table II). The composition of the solutions used in the experiments is given in Table III.

Two first order processes were observed in the pH-jump experiments in which the rate of proton release from ferricytochrome c was measured with phenolphthalein as an indicator (Fig. 3). The faster of the two processes is associated with the indicator.

**Table I**

<table>
<thead>
<tr>
<th>pH</th>
<th>Ionic strength</th>
<th>$k_{\text{obs}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.50</td>
<td>0.2$^a$</td>
<td>0.16</td>
</tr>
<tr>
<td>9.65</td>
<td>0.2$^a$</td>
<td>0.28</td>
</tr>
<tr>
<td>9.65</td>
<td>0.1$^b$</td>
<td>0.30</td>
</tr>
</tbody>
</table>

$^a$ Ferricytochrome c, 20 μM; Na$_2$SO$_4$, 0.066 M; sodium glycinate, 1 mM.

$^b$ Ferricytochrome c, 20 μM; Na$_2$SO$_4$, 0.033 M; sodium glycinate, 1 mM.

When phenolphthalein alone was mixed with 0.066 M Na$_2$SO$_4$ (40 μM in 1 mM glycine, 0.066 M Na$_2$SO$_4$, pH 9.2), a first order change in absorbance was observed. This change was of the same rate and direction and approximately the same magnitude as the faster of the two first order processes observed in the presence of ferricytochrome c. It did not occur when the indicator was diluted with a solution of the same pH and ionic strength.

**Table II**

<table>
<thead>
<tr>
<th>pH</th>
<th>$[\text{Cyt-c}]$</th>
<th>$k_{\text{obs}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0</td>
<td>2.0</td>
<td>0.034 ± 0.002$^a$</td>
</tr>
<tr>
<td>9.05</td>
<td>2.0</td>
<td>0.20 ± 0.01$^c$</td>
</tr>
<tr>
<td>9.90</td>
<td>2.0</td>
<td>0.34 ± 0.02$^d$</td>
</tr>
</tbody>
</table>

$^a$ See Table III for details of solution composition.

$^b$ pH-jump up, 695 nm.

$^c$ pH-jump down, hydrogen ion release, 546 nm.

$^d$ pH-jump up, 546 nm.

$^e$ Hydrogen ion release, pH-jump up, 537 nm.

**Table III**

**Composition of solutions for stopped flow experiments**

For each measurement, Solution A was mixed with Solution B. The two solutions were adjusted to the pH indicated or to various pH values within the pH range indicated with 1 N or 5 N NaOH.

<table>
<thead>
<tr>
<th>Method</th>
<th>Solution A</th>
<th>Solution B</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH-jump up, 695 nm</td>
<td>7.0</td>
<td>&gt;8</td>
</tr>
<tr>
<td>PH-jump down, 695 nm</td>
<td>10.0</td>
<td>9-9.9</td>
</tr>
<tr>
<td>Hydrogen ion release, PH-</td>
<td>7.0</td>
<td>&gt;9</td>
</tr>
<tr>
<td>jump up, 546 nm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrogen ion release, PH-</td>
<td>7.0</td>
<td>&gt;9</td>
</tr>
<tr>
<td>jump up, 537 nm</td>
<td>9.6</td>
<td>&gt;9</td>
</tr>
<tr>
<td>Hydrogen ion release, PH-</td>
<td>9.0</td>
<td>&gt;9</td>
</tr>
<tr>
<td>jump down, 546 nm</td>
<td>9-10</td>
<td>9-10</td>
</tr>
<tr>
<td>Rapid oxidation, 695 nm</td>
<td>9.0</td>
<td>9.0</td>
</tr>
<tr>
<td>Rapid oxidation, hydrogen</td>
<td>9.0</td>
<td>9.0</td>
</tr>
<tr>
<td>ion release, 537 nm</td>
<td>9.0</td>
<td>9.0</td>
</tr>
</tbody>
</table>

$^a$ pH-jump down, 695 nm.

$^b$ pH-jump up, 695 nm.

$^c$ pH-jump down, hydrogen ion release, 546 nm.

$^d$ pH-jump up, 546 nm.

$^e$ Hydrogen ion release, pH-jump up, 537 nm.
FIG. 3. Indicator relaxations, stopped flow experiments, pH 9.3, 21°C, 546 nm. a, a solution 40 μM horse heart ferricytochrome c, 0.066 M in Na₂SO₄, pH 7.0, was mixed with a solution of 0.5 mM glycine, 40 μM phenolphthalein, 1% v/v methanol, 0.066 M Na₂SO₄, and containing sufficient NaOH to produce a pH of 9.3 after mixing. The first rapid change of the oscilloscope trace is seen with the indicator alone (in the absence of cytochrome c). b, a solution of 0.066 M Na₂SO₄ at pH 7.0 was mixed with a solution of 0.25 μM phenolphthalein, 1% v/v methanol, 1 mM glycine, 0.066 M Na₂SO₄, and containing sufficient NaOH to produce a pH of 9.3 after mixing.

The rate constant for the process observed with phenolphthalein was about 1.5 s⁻¹ between pH 9.0 and pH 10.0, 10 times faster than that for the ferricytochrome c conformational change measured at 695 nm. The indicator relaxation, therefore, did not interfere with observation of the proton release associated with the isomerization of ferricytochrome c.

The rate constants associated with the interconversion of the two forms of ferricytochrome c, in the pH range studied, are less than 1 s⁻¹ and involve either a slow uptake or a slow release of protons (Fig. 4), depending upon the direction from which the equilibria are attained. The changes in both 695 nm absorbance and hydrogen ion activity appear as single, first order reactions. At a given pH, kobs is independent, within experimental error, both of the method of measurement and of the hydrogen ion concentration before mixing (Fig. 4). Two possible reaction schemes can be written to explain these observations.

Only those forms designated by C or CH have the 695 nm band. In Scheme 1, the loss of the 695 nm band occurs during a conformational change of the deprotonated form C, whereas, in Scheme 2, the 695 nm band is lost in an isomerization of the protonated form.

The kinetic equations pertinent to both schemes are derived below, with the following assumptions. Following pH changes, the hydrogen ion equilibria of the protein are adjusted quickly compared to the observed isomerization and are considered to be in pre-equilibrium. The use of buffered solutions allows one to consider the pH to be constant during the course of isomerization. In the equations below, C₇ represents the total concentration of cytochrome c.

**Scheme 1**

\[
\begin{align*}
\text{CH} & \xrightleftharpoons{K_H} \text{C} + \text{H}^+ \\
k_f & \quad k_b
\end{align*}
\]

**Scheme 2**

\[
\begin{align*}
\text{CH} & \xrightleftharpoons{K_H} \text{C} + \text{H}^+ \\
k_f & \quad k_b
\end{align*}
\]

**Scheme 1**

\[
\begin{align*}
\frac{-d([695])}{dt} &= \frac{d([\text{CH}] + [\text{C}])}{dt} = \frac{d([\text{NC}])}{dt} = k_b[\text{C}] - k_b[\text{NC}] \\
[\text{NC}] & = k_b(k_r([\text{C}][\text{H}^+] + k_f) + k_f(k_r + [\text{H}^+] / (1 + K_r(1 + [\text{H}^+] / K_r))) \\
k_{obs} &= k_b + k_f(k_r + [\text{H}^+]) / (K_r + [\text{H}^+])
\end{align*}
\]

**Scheme 2**

\[
\begin{align*}
\frac{-d([695])}{dt} &= \frac{d([\text{NCH}] + [\text{NC}])}{dt} = \frac{d([\text{CH}])}{dt} = k_f([\text{CH}] - k_b[\text{NCH}]) \\
[\text{CH}] & = \frac{C_T}{1 + C_T(1 + K_r(1 + [\text{H}^+] / K_r))} \\
& + \left[ C_T \left( 1 + K_r \frac{C_T}{1 + K_r(1 + [\text{H}^+] / K_r)} \right) e^{-k_{obs}t} \right] \\
k_{obs} &= k_f + k_b(k_r + [\text{H}^+]) / (K_r + [\text{H}^+])
\end{align*}
\]
The rate of the conformational change as indicated by the disappearance of the 695 nm band was measured at 16°, 21°, and 25°, in order to obtain a rough estimate of the thermodynamic parameters which characterize the conformational change. The pH dependence of $k_{obs}$ for the conformational change of ferricytochrome c was also determined at both 16° and 25°, and the data at 25° are shown in Fig. 6. The individual kinetic parameters were calculated as above (see Fig. 5). The results are shown in Table IV.

The enthalpy, $\Delta H_{eq}$, and entropy, $\Delta S_{eq}$, of the conformational change were calculated from the temperature dependence of $K_e$ using the following thermodynamic relationships:

$$\Delta G_e = -RT \ln K_e$$

$$\Delta G_e = \Delta H_e - T\Delta S_e$$

$$\ln K_e = -\frac{\Delta H_e}{R} + \frac{\Delta S_e}{R}$$

From the data, plotted as log $K_e$ versus $1/T$ (Fig. 7), it was calculated that $\Delta H_e$ is approximately 13 Cal per mole, and $\Delta S_e$ was calculated to be 37 e.u. at 21°. The scatter in the values of $K_e$ made it impossible to determine the enthalpy of ionization from the kinetic data.

The activation energies of the forward and reverse reactions, $E_a^f$ and $E_a^r$, respectively, were calculated from the temperature dependence of their respective rate constants using the logarithmic form of the Arrhenius equation:

$$\ln k = -\frac{E_a}{RT} + \ln A$$
The data are shown in Fig. 7, a and b. From the slopes of the lines, a value for $E_a^i$ of 28 Cal per mole and a value for $E_a^b$ of 16 Cal per mole were calculated.

In one experiment the rate of the isomerization was followed at 243 nm. A slow, first order increase in absorbance was seen with $k_{obs} = 0.060 \pm 0.006 \text{ s}^{-1}$ at pH 8.8, 16°. The magnitude of the absorbance increase was 0.016 absorbance unit. Under the same experimental conditions, the rate constant for the disappearance of the 695 nm band is about the same, $k_{obs}$ having a value of $0.067 \pm 0.006 \text{ s}^{-1}$.

**Equilibrium Studies: Horse Heart Cytochrome c**

Studies of the conformational change at equilibrium were undertaken both to provide an independent check of the kinetic data and to provide data to use in the calculation of the enthalpy of ionization. In agreement with the literature (6), it was found that the pH dependence of the loss of the 695 nm band can be described by assuming an equilibrium between two forms of ferricytochrome c. The colored form of ferricytochrome which has a 695 nm absorption band is represented by $CH^+$; the form which does not have this absorption band is designated by NC. The assumption is that in the $CH^+$ form, a particular group of the protein is protonated and in the NC form it is not. The pH dependence of the 695 nm band was measured at 16°, 21°, and 25° and the data at 21° are shown in Fig. 8a. In the equations below, $C_T$ represents the total concentration of cytochrome c, $A_{695}$ the absorbance of the solution at 695 nm at a particular pH, $A_{695}^H$ the absorbance of the solution at 695 nm at pH 6.8, and $A_{695}^H$ the absorbance of the solution at 695 nm at pH 9.9.

The apparent equilibrium constants pertaining to the isomerization of ferricytochrome c were calculated from the pH dependence of the absorbance as follows:

$$\begin{align*}
CH + H^+ & \rightleftharpoons K_{H}(app) NC \\
C_T & = CH + NC \\
K_{H}(app) & = \frac{[NC][H^+]}{[CH]} \quad (12) \\
[NC] & = [C_T] - K_{H}(app) + H^+ \quad (13) \\
A_{695} & = [CH]_{695}^C + [NC]_{695}^C = [CH]_{695} - [NC] + [C_T NC]_{695}^C \quad (14) \\
[CH] & = \frac{A_{695} - [C_T NC]_{695}^C}{[NC]_{695}^C} \quad (15) \\
[NC] & = C_T - CH \quad (16) \\
& = \frac{[C_T NC]_{695} - A_{695}}{[NC]_{695}^C} \quad (17)
\end{align*}$$

At pH 6.8 $[H^+] \gg K_{H}(app)$, $[CH] = [C_T]$ and the absorbance of the solution at pH 6.8 and at 695 nm, $A_{695}^H$, is given by:

$$A_{695}^H = [C_T NC]_{695}^C \quad (18)$$

At pH 9.9 $[H^+] \ll K_{H}(app)$, $[NC] = [C_T]$ and the absorbance of the solution at pH 9.9 and at 695 nm, $A_{695}^H$, is given by:

$$A_{695}^H = [C_T NC]_{695}^C \quad (19)$$
Taking the quotient of Equations 16 and 17 and substituting the relationships of Equations 18 and 19 gives:

\[
\frac{[CH]}{[NC]} = \frac{A_{065} - A_{065}^H}{A_{065}^H - A_{065}}.
\]

Equation 12 can be written as:

\[
pH = pK_H(app) - \log \left( \frac{[CH]}{[NC]} \right)
\]

Substituting the relationship in Equation 20 in Equation 21 gives:

\[
pH = pK_H(app) - \log \left( \frac{A_{065} - A_{065}^H}{A_{065}^H - A_{065}} \right)
\]

The data plotted according to Equation 22 are shown in Fig. 8b. The line was calculated using a least squares computer analysis. Within experimental error, the slope of \(-1.2 \pm 0.1\) at 21°, \(-1.0\) at 25°, and \(-1.1 \pm 0.1\) at 25° is consistent with the slope of \(-1\) predicted by Equation 22.

The equilibrium equations for the three-state system, which were found to be consistent with the kinetic data, are derived below.

\[
CH \xrightleftharpoons{K_H} C + H^+ \xrightleftharpoons{K_e} NC
\]

\[
C_T = [CH] + [C] + [NC]
\]

\[
K_H = \frac{[C][H^+]}{[CH]}
\]

\[
K_e = \frac{[NC]}{[C]}
\]

\[
[NC] = \frac{[C_T]}{K_H K_e} + H^+
\]

It can be seen that if \(K_e \gg 1\), Equation 27 reduces to the equation for the two-state system with \(K_H(app) = K_H K_e\). A comparison of \(pK_H(app)\) with the kinetically determined \(pK_H K_e\) is shown in Table IV. Because of the large error in the kinetic determination over-all \(pK\), all that can be said is that there is no apparent disagreement between the kinetic and equilibrium measurements. The enthalpy change for the over-all reaction, as evaluated from the data plotted according to Equation 10, is 23 Cal per mole. The \(\Delta H\) so obtained is the sum of \(\Delta H_e\), the enthalpy associated with the isomerization of ferricytochrome \(c\), and \(\Delta H_H\), the enthalpy associated with the ionization process. From the kinetic results it has been found that \(\Delta H_e\) is approximately 13 Cal per mole; \(\Delta H_H\) is therefore approximately 10 Cal per mole.

**Hydrogen Ion Release at Equilibrium, Horse Heart Cytochrome c**

The number of protons released concomitant with the conformation change was determined directly by measuring the \(pH\) change resulting from the rapid oxidation of ferricytochrome \(c\) at alkaline \(pH\). An assumption implicit in the method used for this measurement is that the only reaction involving a hydrogen ion in the \(pH\) region investigated is the conformation change of ferricytochrome \(c\). The results of the experiment are shown in Table V. The values of 1.0 and 0.7 moles of \(H^+\) released per mole of cytochrome \(c\) at \(pH\) 10.5 and 9.5, respectively (Table V), are consistent with the apparent \(pK\) observed for the isomerization process (Table IV) and indicate that the isomerization of ferricytochrome \(c\) involves the release of 1 hydrogen ion per mole of cytochrome \(c\).

**Table V**

<table>
<thead>
<tr>
<th>pH</th>
<th>([H^+]/[cytochrome c]^-)</th>
<th>([H^+]/[cytochrome c]^±)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.5</td>
<td>1.0 ± 0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>9.5</td>
<td>0.63 ± 0.02</td>
<td>0.7</td>
</tr>
</tbody>
</table>

* Experimental value. Cytochrome \(c\), 40 \(\mu\)M; sodium ferricyanide, 0.4 mM; \(Na_2SO_4\), 0.066 M.

† Calculated from the spectrophotometrically determined \(pK_H(app)\); \(pK_H(app) = 9.1\) at 21°.

**DISCUSSION**

The results of the stopped flow experiments indicate that the alkaline isomerization of horse heart ferricytochrome \(c\) can be described by the following minimal reaction scheme:

\[
CH \xrightleftharpoons{K_H} C + H^+ \xrightleftharpoons{K_e} NC
\]

where \(CH\) and \(C\) are species of ferricytochrome \(c\) with the 665 nm band, and \(NC\) is the form without the 665 nm band. Experimental and theoretical considerations suggest that the presence of the 665 nm band in a heme protein depends on the coordinative binding of a methionine sulfur to the low spin state of ferric iron (7, 8). The oxidation-reduction potentials of \(CH\) and \(NC\) differ significantly (3). Since at \(pH\) 10, there will exist a finite concentration of \(C\) in the above scheme, fast changes in hydrogen ion uptake or release are expected to be observed only not when solutions of ferricytochrome \(c\) are adjusted both from \(pH\) 7.0 to \(pH\) 10 but also when the \(pH\) is changed from \(pH\) 10 to \(pH\) 7 where a slow isomerization precedes hydrogen ion release. The observations of fast proton release and uptake in the isomerization have led recently to the suggestion (9) of a much more complex cyclic scheme for the isomerization reaction than the minimal scheme suggested in this paper.

The observed thermodynamic parameters of the isomerization contain the parameters of two reactions: an ionization with an apparent \(pK\) of 11.0 ± 0.1 at 25° and a heat of ionization of approximately 10 Cal per mole, and a conformational change for which the enthalpy change is approximately 13 Cal per mole and the entropy change is 37 e.u. at 25°.

The thermodynamic parameters for the conformational change are strikingly similar to those observed for the temperature-induced conformational change at neutral \(pH\), \(\Delta H = 14.6\) Cal per mole, and \(\Delta S = 43\) e.u. (10). This, together with the fact that the temperature-induced conformational change is also accompanied by the loss, upon heating, of the 695 nm band, suggests that both changes, \(pH\)-induced and temperature-induced, represent identical rearrangements of the protein chain.

It would be of interest to identify the ionizing group with an apparent \(pK\) of 11.0 involved in the alkaline isomerization of ferric cytochrome \(c\). Three different kinds of residues ionize in this \(pH\) region: the phenolic hydroxyl group of tyrosine (pK 11, \(\Delta H_{\text{H}}\) 11 Cal per mole); the guanido group of arginine (pK 12, \(\Delta H_{\text{H}}\) 12 Cal per mole); and the e-amino group of lysine (pK 11, \(\Delta H_{\text{H}}\) 11 Cal per mole (11)).

Tyrosyl-67 has been implicated in the isomerization process as a consequence of studies on sulfated cytochrome \(c\). Treatment of horse heart ferricytochrome \(c\) with tetranitromethane followed by chromatography led to the isolation of 2 molecules in which
one of the 4 tyrosyl residues, Tyr-67, was specifically nitrated (12). Spectroscopic and chemical reactivity evidence indicates that these nitrated cytochrome c molecules exist as two conformational isomers, differing in the position of the nitro group on the phenolic ring (13). One of the isomers is similar to native cytochrome c in its reducibility with ascorbate, but in its spectral properties and chemical reactivity it shows an apparent pK of 5.9 instead of the value for pK(app) of 9 observed in the studies reported here. It has been suggested that the ionization with pK(app) of 5.9 in nitrocyanochrome c involves the Tyr-67 residue and is the counterpart of the ionization of pK 9.0 observed in the isomerization of native ferrocyanochrome c. If a tyrosine were the ionizing group controlling the conformational change of ferrocyanochrome c, the pK(app) value of 9 which is seen in the studies reported here would also be seen in the ultraviolet titration experiments of tyrosines in ferrocyanochrome c (14–16). The lowest pK value reported in these studies for tyrosyl residues in ferrocyanochrome c is 10.8, almost 2 pH units higher than the value found in our studies. However, at least 1 tyrosyl residue appears to be affected by the alkaline isomerization of ferrocyanochrome c. Our kinetic experiments at pH 9.8 indicate a small increase in absorbance at 243 nm, corresponding to the ionization of 0.1 tyrosyl residue per mole of cytochrome c with an apparent pK of about 10.7. Although this change in tyrosyl ionization occurs as a result of the conformational change of ferrocyanochrome c, this ionization must affect the equilibria between the two forms of the protein. The argument which related the pK of tyrosyl-67 with the conformal equilibria still holds, although for different reasons than those given (13).

Structural considerations suggest the possible involvement of a lysyl residue in the conformational equilibria of ferrocyanochrome c. The three-dimensional structure of oxidized horse heart cytochrome c determined by Dickerson and co-workers (17) indicates that the ε-amino nitrogen of lysyl-79 can coordinate to the iron atom by rotating the residue around its α-carbon atom and displacing the methionyl ligand. This reaction may occur where this ε-amino group becomes unprotonated and it may be the pK(app) of this group which is observed in our experiments.

The effects of pH on conformational equilibria of a protein and thereby on its catalytic efficiency have been observed previously and have been investigated in some detail (5, 18, 19). The conformational equilibria of ferrocyanochrome c have an important effect on the oxidation-reduction potential of this protein which may have physiological relevance. In the respiratory chain, the environment of cytochrome c is in all probability less polar than in aqueous solution. This should be reflected in lowering the apparent pK of the lysyl-79 residue and result in a significant amount of cytochrome c molecules without the 605 nm band at physiological pH. Thus, we might expect that the oxidation-reduction potential of cytochrome c in mitochondria is sensitive to pH changes and has a chemical behavior similar to that shown by the cytochrome in alkaline aqueous solution. At least one observation is in keeping with this interpretation; neither aqueous ferricytochrome c above pH 9 nor mitochondrial cytochrome c is able to bind cyanide ions (20, 21).

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Alkaline Isomerization of Oxidized Cytochrome c: EQUILIBRIUM AND KINETIC MEASUREMENTS
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