Kinetics of Ligand-binding and Oxidation-Reduction Reactions of Cytochrome c from Horse Heart and Candida krusei*

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SUMMARY

Reactions of cytochromes c from horse heart and Candida krusei (a yeast) were studied at 25° under pseudo-first order conditions by a flow technique. The rate law for the reduction of C. krusei by Cr(II) (pH 6.0, μ = 1.0 m) in chloride is \( k_{obs} = k[aCr(II)]/(1 + b[Cr(II)]) \), where \( a \) and \( b \) are \( 1.0 \times 10^6 \) m\(^{-1}\) s\(^{-1}\) and \( 167 \pm 38 \) m\(^{-1}\), respectively. The binding of imidazole (Im) to C. krusei conforms to the rate law \( k_{obs} = k_L + k_{Im} \). At 695 nm, pH 7.1, \( μ = 1.00 \) m which is consistent with the reaction C. krusei + Im \( \rightarrow \) C. krusei-Im. Under the same conditions the equilibrium constant for formation of the C. krusei-imidazole complex determined from absorbance changes is \( 11.0 \pm 0.6 \times 10^6 \) which, within experimental error, is identical with the ratio \( k_L/k_{Im} = 9.9 \pm 0.9 \) m\(^{-1}\). For reduction of ferricytochrome c by pentaamminebenzimidazole-ruthenium(II) (Ru(II)) and oxidation of ferrocyanochrome c by ferricyanide (Fe(III)), the rate laws are of the form \( k_{obs} = k[X] \), which is consistent with the simple process, 

\[ \text{cyt} + X \xrightarrow{k} \text{products} \]

For C. krusei and horse heart, respectively, the rate constants \( (k) \) measured are as follows: \( X = Ru(II), k = (1.0 \pm 0.3) \times 10^4 \) m\(^{-1}\) s\(^{-1}\), \( 4.7 \times 10^5 \) s\(^{-1}\) (pH 6.1, \( μ = 1.00 \) m); \( X = Fe(III), k = (2.1 \pm 0.2) \times 10^7 \) s\(^{-1}\), \( 1.2 \times 10^9 \) s\(^{-1}\) (pH 7.2, \( μ = 1.00 \) m). The rate law found for the reduction of cyanoferricytochrome c by dithionite (pH 6.4, \( μ = 1.00 \) m) is \( k_{obs} = k[S_2O_4^{2-}]^{1/2} \), where \( k' = 9.2 \times 10^3 \) s\(^{-1}\) (C. krusei) and 25.9 s\(^{-1}\) (horse heart). This rate law is consistent with a rate-determining reduction by \( S_2O_4^{2-} \) formed in a rapidly established equilibrium dissociation of \( S_2O_4^{2-} \) into \( S_2O_3^{2-} \) radicals. The immediate products of the dithionite reduction of the cyano-derivatives are the cyanoferricytochromes c. Their conversion to the native ferrocyanochromes c, monitored by conventional techniques, was found to be a first order process (pH 6.4, \( μ = 1.00 \) m) with rate constants \( 6.8 \times 10^4 \) s\(^{-1}\) (C. krusei) and \( 5.0 \times 10^4 \) s\(^{-1}\) (horse heart). As might have been predicted from their structural similarities, the cytochromes from the two species exhibit no major reactivity differences.

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Cytochrome c, a heme protein of molecular weight ~12,000, functions as an electron transport unit in the mitochondria of all aerobic organisms and yeasts, as well as in the chloroplasts of plants. Amino acid sequences (1) and x-ray crystallographic studies of cytochromes (2-4) have shown the high evolutionary conservatism of a crucial region surrounding the heme group. Thus, in all of the species studied, the fifth and sixth coordination positions of the iron are occupied by histidine-imidazole and methionine-sulfur, the first four coordination sites being provided by protoporphyrin IX. Further, the heme unit is attached covalently to the protein which sheaths it almost entirely, with only the porphyrin edge being exposed to solution.

In the past, relatively simple oxidizing and reducing agents have been used to probe the reactivity of horse heart cytochrome c with the hope that the behavior of the protein in such reactions might advance understanding of its in vivo functioning. These studies have revealed, for example, a characteristic heme crevice-opening rate of ~60 s\(^{-1}\) (25°, pH 6 to 7, \( μ = 1.00 \) m) for horse ferricytochrome c (5). The crevice-opened form appears to be kinetically important in ligand-binding reactions (5) and in some reduction reactions (6, 7). From studies of other reduction reactions (6-9), the operation of remote attack reduction pathways, possibly involving attack at the exposed porphyrin edge, has been deduced. The similarity of the physical properties of all of the cytochromes c studied and the structural invariance of certain regions of the molecule seem to indicate that the mode of function of the hemeprotein in vivo has not greatly altered in the course of evolution. We undertook the present study, a comparison of the reactions of cytochromes c from horse heart and Candida krusei (a yeast), in order to acquire more information about the reactivity of cytochrome c and to learn to what extent the structural invariance of the molecule is manifest in its reactivity toward simple inorganic probes. The results of our ligand binding, reduction and oxidation studies are reported here.

EXPERIMENTAL PROCEDURES

Except where noted, chemicals used were reagent grade. Commercial horse heart (Sigma, type III) and C. krusei (Calbiochem, twice recrystallized) cytochrome c were used without further purification. Reported cytochrome c concentrations are based on weights. Sodium dithionite (Fisher sodium hydrosulfite) was ascertained (7) to be 82.4% pure. Pentaamminebenzimidazole-ruthenium(II) chloride was obtained by amalgamated zinc reduction (at pH 3) of its ruthenium(III) chloride salt which had been prepared according to the method of Sundberg et al. (10). Stock Cr(II) chloride solution was prepared by dissolving 99.99%
pure chromium metal (Varlaoid Chemical Co.) in a deoxygenated solution of hydrochloric acid and sodium chloride. Analysis of the chromium solution is described elsewhere (6). In order to avoid "swamping" the buffer in experiments with high Cr(II), the excess acid from the stock solution was neutralized by adding deoxygenated sodium hydroxide solution. Argon was used as the blanket gas in all cytochrome c reduction studies.

All of the buffers described were initially present in only the cytochrome c solutions. For the imidazole runs, solutions of imidazole were adjusted to pH 7.1 by the addition of the required amount of hydrochloric acid. Solutions of cyanoferriicytochrome e were prepared as follows: the cytochrome c, dissolved in 0.15 M dihydrogen phosphate, 0.05 M monohydrogen phosphate, 0.60 M sodium chloride, was bubbled with argon for 1 hour. After removing the gas exit needle, solid sodium cyanide was added via a side arm in the flask (final pH 8.4, 60 mM hydrogen cyanide).

The mixture was stirred under positive argon pressure for 1/2 hour before use in the kinetic runs. (The half-life for formation of the cyano-derivative is -30 min under these conditions.) Ferrocyanochrome c solutions were prepared by sodium dithionite reduction of the ferric protein. The reduced solution was loaded onto a Bio-Rex 70 cation exchange resin, washed with water, eluted with a few milliliters of 1.00 M sodium chloride, and diluted to the desired volume with buffer-sodium chloride solution.

The rate of cyanide loss from ferrocyanochrome c was studied on a Cary model 14 using 10 cm cells containing a magnetic stirring bar and sealed with a serum cap. A Beckman Research pH meter was used for the pH determinations. Most of the kinetic measurements were made on a Durrum D-110 stopped flow spectrophotometer; all of the measurements were performed at 25.0° ± 0.2°.

RESULTS

Values of the observed rate constants were found from a weighted least squares analysis of the time dependence of log 

\[
(A_t - A_w)
\]

(the solution absorbance values at times \( t \) and \( \infty \), respectively). The quality of the above fits established that the reactions were first order in cytochrome c. Reported \( k_{obs} \) values are averages from 6 to 10 runs in which the deviation of individual values from the mean was usually less than 10%.

The data analysis also yielded values of \( (A_s - A_w) \), the difference between the absorbance of the reaction mixture at zero and infinite times. Equilibrium constants for imidazole binding were evaluated from the ratio of the intercept to the slope of a plot of the reciprocal of this absorbance difference versus the reciprocal of the ligand concentration. The plots were found to be linear as is expected for the formation of a 1:1 complex.

**Imidazole Binding**—Slope to intercept ratios derived from \( (A_0 - A_w) \) versus [total imidazole]-1 plots at 690 nm gave \( K' = 11.0 ± 0.6 \) M\(^{-1}\) for the binding of imidazole (1 mM) to C. krusei ferriicytochrome c at pH 7.1, \( \mu = 1.00 \) M, 25.0° (Equation 1).

\[
P_{III, cyt c} + Im \xrightleftharpoons[k_f\text{ Ferricytochrome } c \text{ at pH } 7.1, \mu = 1.00 \text{ M, 25.0°} (Equation 1).
\]

When this effective \( K \) value is corrected for the fraction of the total imidazole which is protonated at this pH (using 7.03 as the imidazole pK (5)), the "true" equilibrium constant for Reaction 1 is found to be \( K' = 19 ± 1 \) M\(^{-1}\). Observed rate constants for Reaction 1 are given in Table 1. When these are plotted as a function of total imidazole concentration, the resulting plot is linear, with slope 16.4 ± 0.3 M\(^{-1}\) s\(^{-1}\) and intercept 1.65 ± 0.15 s\(^{-1}\). Equation 1, describing a simple approach to equilibrium, is the simplest kinetic scheme which can account for this behavior. In this interpretation, the slope and intercept of the \( k_{obs} \) versus [total imidazole] plot are \( k_f \) and \( k_w \), respectively. The ratio \( k_f/k_w \) gives \( K = 9.9 ± 0.9 \) M\(^{-1}\) at pH 7.1 and the value for \( K' \) is 16.7 ± 1.0 M\(^{-1}\), which is the same, within experimental error, as that found from the absorbance changes.

The reaction was also studied in the Soret region and yielded data of quality comparable with that of the data recorded in Table 1. To conserve space, the results are only summarized here: at 402 nm, pH 7.1, \( k_f = 34.7 \) M\(^{-1}\) s\(^{-1}\), \( k_w = 1.0 \) s\(^{-1}\) (from absorbance changes); at 416 nm, \( k_f = 22.2 \) M\(^{-1}\) s\(^{-1}\), \( k_w = 1.2 \) s\(^{-1}\) (from absorbance changes).

**Reduction of C. krusei Ferricytochrome c by Cr(II)**—Rate constants found for the Cr(II) reduction of C. krusei at 25° in solutions buffered at pH 7.1 with 20 mM phosphate. The ionic strength was maintained at 1.00 M with sodium chloride. The cytochrome c concentration was -40 μM.

<table>
<thead>
<tr>
<th>( 10^3 \text{[total imidazole]} ) M</th>
<th>( k_{obs} ) s(^{-1})</th>
<th>( 10^3 (A_w - A_s) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.00</td>
<td>1.91</td>
<td>5.52</td>
</tr>
<tr>
<td>2.50</td>
<td>2.83</td>
<td>6.33</td>
</tr>
<tr>
<td>5.00</td>
<td>2.29</td>
<td>11.0</td>
</tr>
<tr>
<td>10.0</td>
<td>3.45</td>
<td>16.4</td>
</tr>
<tr>
<td>20.0</td>
<td>5.00</td>
<td>21.3</td>
</tr>
<tr>
<td>40.0</td>
<td>7.80</td>
<td>26.1</td>
</tr>
<tr>
<td>50.0</td>
<td>9.81</td>
<td>26.3</td>
</tr>
<tr>
<td>59.0</td>
<td>11.1</td>
<td>26.7</td>
</tr>
<tr>
<td>80.4</td>
<td>14.7</td>
<td>27.0</td>
</tr>
<tr>
<td>90.0</td>
<td>15.7</td>
<td>28.6</td>
</tr>
<tr>
<td>100.0</td>
<td>17.8</td>
<td>28.0</td>
</tr>
</tbody>
</table>

The measurements were made at 695 nm, 25.0° in solutions buffered at pH 7.1 with 20 mM phosphate. The ionic strength was maintained at 1.00 M with sodium chloride. The cytochrome c concentration was -40 μM.
TABLE II
Rate constants ($k_{obs}$) for reduction of C. krusei ferricytochrome c by chromium(II) at 26°

The solutions were buffered at pH 6.0 with 50 mM cacodylate and maintained at $\mu = 1.00$ M by the addition of sodium chloride. The cytochrome c concentration was ~2.5 $\mu$M.

<table>
<thead>
<tr>
<th>[Cr(II)] (mM)</th>
<th>$k_{obs}$ (550 nm s$^{-1}$)</th>
<th>$k_{obs}$ (450 nm s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.211</td>
<td>1.86</td>
<td>-</td>
</tr>
<tr>
<td>0.423</td>
<td>4.04</td>
<td>3.46</td>
</tr>
<tr>
<td>0.634</td>
<td>5.71</td>
<td>5.85</td>
</tr>
<tr>
<td>0.845</td>
<td>7.45</td>
<td>7.42</td>
</tr>
<tr>
<td>1.69</td>
<td>13.1</td>
<td>12.4</td>
</tr>
<tr>
<td>2.56</td>
<td>16.8</td>
<td>18.4</td>
</tr>
<tr>
<td>3.38</td>
<td>21.2</td>
<td>22.2</td>
</tr>
<tr>
<td>5.06</td>
<td>25.7</td>
<td>28.4</td>
</tr>
<tr>
<td>6.75</td>
<td>31.1</td>
<td>34.3</td>
</tr>
</tbody>
</table>

The saturation behavior parallels that found for the horse heart ferricytochrome c-Cr(II) reaction (6). Scheme a (Equations 2 and 3), which leads to Equation 4 as rate expression

\[
\text{Fe}^{III}\text{cytc} \frac{k_2}{k_2} \text{Fe}^{III}\text{cytc}^* \quad \text{(2)}
\]

\[
\text{Fe}^{III}\text{cytc}^* + \text{Cr(II)} \frac{k_3}{k_3} \text{products} \quad \text{(3)}
\]

\[
k_{obs} = \frac{k_2 k_3 [\text{Cr(II)}]}{k_3 [\text{Fe}^{III}\text{cytc}^*] + k_2} \quad \text{(4)}
\]

was the preferred description of the latter reaction. It was pointed out, however, that Scheme b (Equations 5 and 6) which leads to Equation 7 as rate expression, was also in accord with the observed behavior.

\[
\text{Fe}^{III}\text{cytc} \frac{k_6}{k_6} \text{Fe}^{II}\text{cytc}^* \quad \text{(5)}
\]

\[
\text{Fe}^{II}\text{cytc}^* - \text{Cr(II)} \frac{k_6}{k_6} \text{products} \quad \text{(6)}
\]

\[
k_{obs} = \frac{k_6 k_5 [\text{Cr(II)}]}{k_6 [\text{Fe}^{III}\text{cytc}^*] + k_5} \quad \text{(7)}
\]

Rearrangement of Equation 4 yields Equation 8, while similar rearrangement of Equation 7 gives Equation 9.

\[
\frac{1}{k_{obs}} = \frac{1}{k_2} + \frac{k_2^{-1}}{k_3 [\text{Cr(II)}]} \quad \text{(8)}
\]

\[
\frac{1}{k_{obs}} = \frac{1}{k_6} + \frac{k_6^{-1}}{k_5 k_6 [\text{Cr(II)}]} \quad \text{(9)}
\]

Equations 8 and 9 suggest the construction of a plot of ($k_{obs}$)$^{-1}$ versus [Cr(II)]$^{-1}$. The resulting plot is linear, as is expected for either Scheme a or b, and has (intercept)$^{-1} - 65 \pm 15$ s$^{-1}$ and (slope)$^{-1} = 1.0 \times 10^4$ s$^{-1}$. In terms of Scheme a, these are identified with $k_3$ and $k_2 k_3 / k_5$, respectively; for Scheme b, they are $k_4$ and $k_5 k_6$, respectively. Both schemes are consistent with the kinetic data for the C. kruusei ferricytochrome c-Cr(II) reaction. The relative merits of the two descriptions will be discussed later.

Reduction of Ferricytochrome c by Ru(II)—The kinetic data of the reaction of ferricytochrome c from both horse heart and C. kruusei with pentaamminebenzimidazoleruthenium(II) were studied at 25.0° in solutions 20 mM in cacodylate and 0.99 M in sodium chloride at pH 6.1. Cacodylate was used as buffer instead of phosphate because the latter precipitates the Ru(III) product. The results are presented in Table III. For both species, plots of $k_{obs}$ versus [Ru(II)] are linear with zero intercepts indicating that Equations 10 and 11 obtain.

\[
\text{Fe}^{III}\text{cytc} + \text{Ru(II)} \frac{k_7}{k_7} \text{Fe}^{II}\text{cytc} + \text{Ru(III)} \quad \text{(10)}
\]

\[
k_{obs} = k_7 [\text{Ru(II)}] \quad \text{(11)}
\]

From such plots the second order rate constants $4.7 \times 10^4$ s$^{-1}$ M$^{-1}$ (horse) and $(1.0 \pm 0.3) \times 10^6$ s$^{-1}$ M$^{-1}$ (C. kruusei) are found. The C. kruusei data are subject to a large amount of scatter caused by the low (down to 20 $\mu$M) concentrations of the air-sensitive reducing agent needed and to the problems associated with measuring the rapid rates observed. The reported rate constant is the arithmetic average over all the measurements made; the error given is the standard deviation.

Oxidation of Ferrocytochrome c by Ferricyanide—The observed rate constants for oxidation of ferrocytochrome c from both horse heart and C. kruusei by ferricyanide are reported in Table IV. The reaction was carried out in 2 mM phosphate, 91 mM sodium chloride ($\mu = 0.10$ M) at pH 7.2 and 25.0°. For both cytochromes c, plots of $k_{obs}$ versus [Fe(CN)$_6$]$^{3-}$ are linear with zero intercepts. The kinetic data are thus consistent with the
The solutions were buffered at pH 7.2 with 2 mM phosphate and maintained at \( \mu = 0.10 \) mM with sodium chloride and were \( \sim 0.6 \mu M \) in cytochrome c. Measurements were made at 250° and 550 nm.

<table>
<thead>
<tr>
<th>([\text{Fe(CN)}_6]^{3-})</th>
<th>(k_{\text{obs}})</th>
<th>(k_{\text{obs}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\mu M)</td>
<td>s(^{-1})</td>
<td>(s(^{-1})</td>
</tr>
<tr>
<td>3.95</td>
<td>47.7</td>
<td>87.7</td>
</tr>
<tr>
<td>5.92</td>
<td>68.9</td>
<td>127</td>
</tr>
<tr>
<td>7.90</td>
<td>85.6</td>
<td>143</td>
</tr>
<tr>
<td>9.87</td>
<td>113</td>
<td>188</td>
</tr>
</tbody>
</table>

\(a\) Rates measured at 418 nm were \( \sim 10\% \) slower.

\(b\) The same rates were observed at 415 nm.

**Table V**

**Rate constants (k\(\text{obs}\)) for reaction of dithionite with the cyano-derivative of ferrocyanochrome c**

Solutions were 25 mM in HCN, buffered at pH 6.4 with 0.2 mM phosphate and maintained at \( \mu = 1.00 \) mM with sodium chloride. The measurements were made at 250° and 550 nm. Solutions were \( \sim 8 \mu M \) in cytochrome c.

<table>
<thead>
<tr>
<th>([S_2O_4]^{2-})</th>
<th>(k_{\text{obs}})</th>
<th>(k_{\text{obs}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\mu M)</td>
<td>s(^{-1})</td>
<td>(s(^{-1})</td>
</tr>
<tr>
<td>0.633</td>
<td>0.661</td>
<td>-</td>
</tr>
<tr>
<td>0.703</td>
<td>0.689</td>
<td>0.261</td>
</tr>
<tr>
<td>1.20</td>
<td>1.65</td>
<td>0.313</td>
</tr>
<tr>
<td>3.86</td>
<td>4.01</td>
<td>0.634</td>
</tr>
<tr>
<td>11.4</td>
<td>2.78</td>
<td>1.02</td>
</tr>
<tr>
<td>39.6</td>
<td>5.12</td>
<td>1.69</td>
</tr>
</tbody>
</table>

**Discussion**

Comparison of Reactivity Patterns of Cytochromes c from Horse Heart and C. krusei—Rate constants for the horse heart and C. krusei cytochrome c reactions are summarized in Table VII. Comparison of the Cr(II) and imidazole reactions will be made later. The redox reactions with Ru(II) and ferricyanide manifest simple second order rate laws. For these, in contrast to the Cr(II) reaction, there is no reason to invoke a crevice opening mechanism and the reactions may be said to proceed by remote attack. For the redox reactions featuring remote attack on the native cytochrome, the \( C. \) krusei rate constants are \( \sim 2 \) times larger than those for horse heart. As Margalit and Schejter (13) have found the reduction potentials for the two cytochromes to be the same within experimental error (260 \( \pm \) 2 mV), none of these differences can be attributed to differences in driving force. From NMR studies Gupta (14) has concluded that the electron self-exchange rate for \( C. \) krusei cytochrome c is a factor of 10 smaller than that for horse heart cytochrome c. Since this difference is expected to be manifested in slower rates of outer sphere oxidation and reduction for \( C. \) krusei, it is difficult to reconcile his results with ours. Moreover, since more rapid oxidation rates are observed for both anionic (Fe(CN)\(_{6}\)\(^{3-}\)) and cationic (tris(1,10-phenanthrolinecobalt(III)) reagents with \( C. \) krusei, differences in charges of the cytochromes in some region critical to the redox process cannot account for the enhanced rates. To further complicate matters, the rate differences are in the opposite direction for reduction of the cyano-derivatives with \( S_2O_4^{2-}\)

Horse heart and \( C. \) krusei cytochromes c differ in about half of their amino acid residues (1). Despite these differences, the reactivities of the two cytochromes toward the reagents used here are remarkably similar. These studies thus confirm the view (1) that cytochrome c is a highly evolutionarily conservative molecule and extend the realm of this conservatism to its reactivity with simple inorganic reagents.

Rate Correlations for Remote Attack Reactions—Remote attack pathways, observed here for reduction by Ru(II) and oxidation by ferricyanide, have also been found for reduction of ferrocyanochrome c by Ru(NH\(_3\))\(_4\)\(^{2+}\) (8), Fe(EDTA)\(^{3+}\) (9), S\(_2\)O\(_4\)\(^{2-}\) (7), and Fe(CN)\(_{6}\)\(^{3-}\) (16) (see Table VII) and for oxidation of ferrocyanochrome c by Co(phen)\(_3\)\(^{3+}\) (15). It has previously been proposed that remote attack occurs at the solution exposed edge of cytochrome c and that such reactions may be amenable to treatment by the Marcus theory (7-9, 15, 16, 18). In terms of the

\[ k_{\text{obs}} = k' [S_2O_4^{2-}]^{1/2} \]  

applies. The coefficients \( k' \) are 25.9 \( M^{-1/2} s^{-1} \) (horse heart) and 9.2 \( M^{-1/2} s^{-1} \) (\( C. \) krusei). The above rate law is consistent with a mechanism involving \( S_2O_4^{2-}\) as the active reducing agent.
Marcus theory (19), the observed rate constant \( k_{12} \) is given by

\[
k_{12} = \left( k_{11} k_{22} K_{12} \right)^{1/2}
\]  

(17)

where \( k_{11} \) and \( k_{22} \) are the appropriate self-exchange rate constants and \( K_{12} \), the equilibrium constant or driving force for the cross reaction, is not too large. For ferricyanide oxidation of horse heart ferrocytochrome c, \( k_{12} \) is calculated from Equation 17, where \( k_{11} \), the horse cytochrome c electron exchange rate, is \((0.2 \text{ to } 1.0) \times 10^4 \text{ M}^{-1} \text{s}^{-1} \) at pH 7.0, \( p = 0.1 \text{ M} \); \( k_{22} \), the electron exchange rate for the ferriyanide-ferrocyanide partners, is \(9.6 \times 10^3 \text{ M}^{-1} \text{s}^{-1} \) (22); and \( K_{12} \), the equilibrium constant for the reaction, is \(450 \) (16). This calculation yields \((2.9 \text{ to } 6.6) \times 10^4 \text{ M}^{-1} \text{s}^{-1} \) as the predicted rate constant. The rate constant measured here for the ferricyanide-ferrocytochrome c reaction \((1.2 \times 10^7 \text{ M}^{-1} \text{s}^{-1}) \), although in reasonable agreement with previously reported values \((0.18 \text{ to } 1.6) \times 10^7 \text{ M}^{-1} \text{s}^{-1} \) (14, 23, 24), is thus much higher than that predicted from Marcus theory. This is not, however, unexpected since the above equation does not take into account specific ion effects on the exchange rates or the rate enhancement arising from complex formation between the anionic oxidant and the positively charged residues on the protein surface in the heme region (3). It is noteworthy that the same apparent anomaly arises for reduction of horse heart ferricytochrome c by ferrocyanide \((k_{12} \) calculated \(= 0.6 \text{ to } 1.4) \times 10^5 \text{ M}^{-1} \text{s}^{-1} \); \( k_{12} \) found \(= 2.6 \times 10^5 \text{ M}^{-1} \text{s}^{-1} \) (16)), as is expected from the principle of microscopic reversibility.

For oxidation of horse heart ferrocytochrome c by tris(1,10-phenanthroline)Co(III) the calculations give \( k_{12} = (1.2 \text{ to } 2.7) \times 10^5 \text{ M}^{-1} \text{s}^{-1} \) as the predicted rate constant, which is in excellent agreement with the experimental rate constant, \(1.6 \times 10^5 \text{ M}^{-1} \text{s}^{-1} \) (pH 7.2, 2 mm phosphate, \( \mu = 0.1 \text{ M} \) (sodium chloride) 25°) (15).

Pentaamminebenzimidazoleruthenium(II), a 6-coordinate complex containing 5 ammonia molecules and a benzimidazole group coordinated through its "pyridine" nitrogen (10), is a mild reducing agent \((E^o = 0.15 \text{ volts}) \). For reduction of horse heart cytochrome c, the rate constants for hexaammineruthenium(II) and the benzimidazole complex are very similar (Table VIII). Evidently any rate enhancement derived by replacing one "insulating" ammonia group by the better conducting benzimidazole ligand is compensated for by the lower driving force for the benzimidazole reaction.

**Table VI**

<table>
<thead>
<tr>
<th>Species</th>
<th>Conditions</th>
<th>( k ) (550 nm) ( \text{s}^{-1} )</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse heart</td>
<td>pH 7.0, 0.05 M cyanide, 25°, 0.10 M phosphate</td>
<td>4.3 \times 10^{-3}</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>pH 7.4, 9 mM cyanide, 0.10 M phosphate</td>
<td>3.8 \times 10^{-3}</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>pH 7.4, 9 mM cyanide, 0.03 M trismaleate, 20-25°</td>
<td>4.9 \times 10^{-3}</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>pH 6.4, 9 mM cyanide, 0.18 M phosphate, ( \mu = 1.00 \text{ M (NaCl)} ), 25.0°</td>
<td>5.0 \times 10^{-3}</td>
<td>c</td>
</tr>
<tr>
<td>Candida krusei</td>
<td>pH 6.4, 9 mM cyanide, 0.18 M phosphate, ( \mu = 1.00 \text{ M (NaCl)} ), 25.0°</td>
<td>6.8 \times 10^{-3}</td>
<td>c</td>
</tr>
</tbody>
</table>

* Ref. 11.
* Ref. 12.
* This work.
Dithionite reduction of cyanoferricytochrome c yields the cyanide complex of the ferrous protein which is rather rapidly converted to the native ferrocytochrome. It is noteworthy that cyanide loss from the reduced cytochrome proceeds about 400 times faster than from the oxidized species.

**Cr(II) and Imidazole Reactions**—As was the case for horse heart cytochrome c, rate constants measured for the reaction of *C. krusei* ferricytochrome c with Cr(II) in chloride tend to approach a constant value at high Cr(II) concentrations. Scheme a, a limiting $S_N1$ mechanism which involves first the formation of the highly reactive crevice opened Fe(III)cyt c* (Equation 2) followed by the binding and oxidation of chromium(II) by this intermediate (Equation 3), was proposed to account for the horse heart kinetic data. Analysis of the *C. krusei* results in terms of Scheme a yields $k_3$, the crevice-opening rate constant, $= 60 \pm 15$ s$^{-1}$ and $k_{3}/k_{-3} = 1.0 \times 10^4$ M$^{-1}$ s$^{-1}$; the comparable values for horse were $60 \pm 20$ s$^{-1}$ and $4.0 \times 10^4$ M$^{-1}$ s$^{-1}$, respectively. For horse heart cytochrome c additional experimental support for Scheme a came from the results of ligand binding studies. These rates also showed saturation at high ligand concentrations, and data analysis similar to that suggested by Equation 8 gave a crevice-opening rate constant of 30 to 60 s$^{-1}$ depending on the ligand. By contrast, such an analysis of the rate data for imidazole binding to *C. krusei* ferricytochrome c (Table I) gives $k_2 > 10^3$ s$^{-1}$ for the wavelength used. Because of the discrepancy between the $k_2$ values found from the chromous ion reduction and imidazole binding studies it is necessary to reconsider Scheme b, the alternative Cr(II) reduction mechanism of the over-all reaction. More probably the wavelength dependence arises because the kinetic data of the cytochrome c reaction are more complex than indicated (Equation 1), e.g.

$$\text{cyt c} + \text{Im} \rightarrow \frac{(\text{cyt c}) \cdot (\text{Im})}{(\text{cyt c}) \cdot (\text{Im})} \rightarrow \text{cyt c} \cdot \text{Im}$$

where the formation of an imidazole-cytochrome c adduct precedes the rupture of the Fe-S bond and subsequent Fe-imidazole bond-making step (this is analogous to Scheme b). The slower rate found at 695 nm where the Fe(III)—S chromophore absorbs could reflect only the rate of the second step. This sequence does not, however, account for the wavelength dependence of the equilibrium constant for the over-all reaction. More probably the wavelength dependence results from impurities which contribute differently to the absorbances at the three wavelengths monitored.
anismon proposed for horse heart cytochrome c (0). From the Scheme b analysis of the C. krusei data, $k_kK_2$ is $1.0 \times 10^4 \text{M}^{-1} \text{s}^{-1}$ (horse $4.0 \times 10^4 \text{M}^{-1} \text{s}^{-1}$) and $k_8$ is $65 \pm 15 \text{ s}^{-1}$ (horse $60 \pm 20 \text{ s}^{-1}$), where $K_2$ is the equilibrium constant for formation of the Cr(II)-cytochrome precursor complexes and $k_8$ is the rate of intra-complex rearrangement to yield products. The analog of Scheme b could also be operative for imidazole binding to C. krusei, but, for this reaction, the rearrangement of the precursor complex (the $K_2$ value of which would be $\leq 11.0 \text{ M}^{-1}$) must proceed more rapidly ($k_8 > 110 \text{ s}^{-1}$) than for the chromium-cytochrome or the imidazole-horse heart ($k_8 = 60 \pm 20 \text{ s}^{-1}$) precursor complexes.

With the presently available information it is not possible to distinguish between Schemes a and b for these cytochrome c reactions. The 2-fold variation of $k_k$ for the different reactions is not necessarily strong evidence against Scheme a. By analogy with substitution processes in both organic and inorganic systems, rupture of the iron-sulfur bond is not expected to be limiting $S_81$ in character (that is to say, entirely free of incoming group assistance). Further, differences in the degree of assistance possible for cytochromes of the two species are not unexpected. Although the immediate (first coordination sphere) iron environments are identical in the two cytochromes, the "lining" of the heme crevice differs at several points (1), i.e. positions 60, 64, 81, 83, 92, 95, and 96. With the exception of residues 64 and 96, the effect of the substitutions is to fill the crevice of horse heart with bulkier amino acids than are found in that region of C. krusei (e.g. alanine 81 in C. krusei becomes isoleucine 81 in horse heart). Conceivably imidazole has greater access to the roomier crevice of C. krusei, and consequently is able to provide greater assistance in the rupture of the iron-sulfur bond.

Finally, in support of Scheme a, the importance of the crevice-opening process may be inferred from the work of Grimes et al. (27), who determined the location of the chromium in the Cr(III)-ferrocytochrome c (horse heart) product formed at pH 4. The chromium was found to cross-link residues 40 to 53 and residues 61 to 73, probably binding at asparagine 52 and tyrosine 67, both of which form part of the crevice lining. Grimes et al. (27) have proposed that electron transfer succeeds the binding of Cr(II) to tyrosine-67. An alternative interpretation is that chromium(II) enters the crevice-opened cytochrome c (perhaps, but not necessarily, binding to tyrosine-67) and then transfers its electron to the porphyrin ring system either directly or via an Fe(III)-Cr-Cr(II) bridge (6). After reduction, the Fe(II) would exclude the CrCrC complex from its coordination sphere. As a consequence Cr(III) would be trapped in the protein's interior if not initially coordinated to tyrosine-67 and asparagine-52 and tyrosine-67, both of which form part of the crevice lining. Convesely imidazole has greater access to the roomier crevice of C. krusei, and consequently is able to provide greater assistance in the rupture of the iron-sulfur bond.

CONCLUSIONS

The results of this study corroborate the results of earlier work (0,7) which suggested that ferrocytochrome c offers at least two ways by which its heme group may participate in electron transfer reactions. For the adjacent attack mode (exemplified by the Cr(II) reaction), attack on the heme from the cytochrome c crevice has been suggested. For the remote attack mode (exemplified by the reactions listed in Table VIII), the solution-exposed heme edge is likely to be involved. Both of these pathways may lead to rapid reduction of the iron. Which is employed by a given reagent depends on its particular properties. To date direct evidence (as distinct from microscopic reversibility arguments) for only the latter reaction mechanism has been found in oxidations of ferrocytochrome c (15). With the possible exception of the imidazole binding reaction, reactions of cytochromes c from horse heart and C. krusei follow common mechanisms for a given reaction and proceed at similar rates. Thus it seems sound to conclude that the structural conservatism of cytochrome c is accompanied by conservatism with respect to both reaction mechanism and rate.

4. Such mobility for Cr(III) is not unreasonable, as this metal ion could be quite substitution labile at this relatively high pH.
REFERENCES

1. Dickerson, R. E. (1972) Sci. Amer. April, 58–72
Kinetics of Ligand-binding and Oxidation-Reduction Reactions of Cytochrome c from Horse Heart and *Candida krusei*

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