# Kinetics and Mechanism of Reduction of Horse Heart Cytochrome *c* by Hexaammineruthenium(II) Ion. Reactivities of the Electronic Isomers of Cytochrome *c*

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**Abstract:** The rate constant for the reduction of cytochrome *c* by $Ru(NH_3)_6^{2+}$ shows a complex dependence on pH. At pH $\leq 2$ in a chloride medium, the rate constant at 25 °C, $I = 0.1$ M NaCl, is $(350/[H^+])$ $M^{-1}$ $s^{-1}$, while at pH $\geq 4.0$ the rate constant is $(3.9 \times 10^4 + 1.0 \times 10^8[H^+])$ $M^{-1}$ $s^{-1}$. A maximum redox reactivity exists around pH 3, and in this region, neither of these limiting expressions describes the dependence of the rate constant on pH. In perchlorate medium, the rate constant for the region $[H^+] = 0.02$–$0.1$ M is $(1.9 \times 10^4 + 4.6 \times 10^2[H^+])$ $M^{-1}$ $s^{-1}$. The electron transfer from the reductant to the ferric atom in the protein is supposed to proceed by the heme edge mechanism. The results in the different media suggest that the reactivity order for the reduction of the acid-induced "electronic" isomers of cytochrome *c* by the heme edge mechanism is low spin > mixed spin > high spin. It is proposed that in vivo electron transfer may be preceded by a conformational change such that cytochrome *c* adopts the most reactive form, which contains one more redox-linked ionizable proton than the native conformer.

## Introduction

Cytochrome *c* is a relatively small metalloprotein (mol wt 12 384) that acts as an electron carrier in the respiratory chain of all aerobic organisms.[1] X-ray studies[2-4] have revealed that the heme group is located in a crevice of the essentially globular protein. The iron atom lies in the plane of the porphyrin ring and the fifth and sixth coordination positions are respectively occupied by a nitrogen atom of the imidazole ring of His-18 and the sulfur atom of Met-80. In aqueous solutions, the coordination environment of the heme iron depends on the pH, the ionic strength, and the anionic composition of the medium.[5,6] At physiological pH, the coordination environment of the iron is believed to be the same as in the solid. However, at low pH the Fe–N and Fe–S bonds are both broken, and these coordination positions are probably occupied by water.[7] At pH <2 in a perchlorate medium or at high ionic strength (1.0 M) in a chloride medium, spectral changes, particularly an increase in absorbance at the 695-nm peak, suggest that the methionine sulfur is still coordinated to the heme iron, but that the Fe–N (His-18) bond is broken.[5] These three coordination situations around the ferric ion in the heme are associated with cytochrome *c* "electronic" isomers that are called the low-spin, high-spin, and mixed-spin species with the iron atom possessing one, five, and three unpaired electrons, respectively.[8]

As a means of understanding the in vivo mechanism of electron transfer by cytochrome *c*, the mechanisms of its redox reactions with inorganic reagents have been investigated in aqueous solutions.[9-16] The results of such investigations show that two modes of electron transfer mechanism are possible with cytochrome *c*, viz., the adjacent attack mechanism typified by the reduction of the metalloprotein by chromous and dithionite ions.[13,14] In this mechanism, the electron transfer is governed by a rate-limiting event in the protein such as the opening of the crevice and/or substitution on the iron center. The alternative mechanism is the remote attack in which electron transfer occurs via the exposed edge of the heme to the metal ion. This latter path has been suggested for homogeneous redox reactions of ferricytochrome *c* and simple inorganic reagents.[15]

A characteristic feature of the redox reactions of ferri- and ferrocytochrome *c* by $Cr^{2-}$ or $Co(phen)_3^{3+}$ is the complexity of the hydrogen ion dependence for both oxidation and reduction rates.[13,17,18] For instance, recent studies of the chromium(II) reduction of cytochrome *c* in acidic media led to the conclusion that the reactivity sequence for the adjacent mode of electron transfer for the three identified electronic isomers of this protein is low spin > high spin > mixed spin.[17] It was also speculated that the relative rates for the mixed- and high-spin species may be inverted in electron transfer by the remote attack mechanism.[17] Ewall and Bennett[16] have reported their findings on the reduction of cytochrome *c* by $Ru(NH_3)_6^{2+}$. Their results do show that electron transfer from this ion to cytochrome *c* proceeds by the remote attack mechanism. However, their studies were conducted in the range $3.3 \leq pH \leq 7.0$, and, therefore, do not show the rate constant–pH profile that seems general for the redox reactions of this protein. In this work, we studied the rate of reduction of this metalloprotein from pH 1.0 to 6.2. By changing the anionic composition of the reduction medium from chloride to perchlorate, we could measure the reaction rates of the different electronic isomers of the protein.

## Experimental Section

**Materials.** Sigma Horse heart cytochrome *c* Type III was used without further purification. $Ru(NH_3)_6Cl_3$ (Johnson Matthey) was purified as follows: 1 g of the commercial sample was dissolved in $10^{-3}$ M hydrochloric acid (40 mL). Any solid impurity was filtered off, and the filtrate was cooled to 0 °C in an ice bath. An equal volume of chilled concentrated hydrochloric acid was added to the cold filtrate, whereby the $Ru(NH_3)_6Cl_3$ was precipitated. The resulting precipitate was filtered and recrystallized twice from $10^{-3}$ M HCl. The final purified product was air-dried. $Ru(NH_3)_6^{2+}$ was prepared from the purified $Ru(NH_3)_6Cl_3$ by zinc amalgam reduction in an anaerobic atmosphere maintained by bubbling argon gas previously scrubbed in chromous towers, to remove the last traces of oxygen. The concentrations of $Ru(NH_3)_6^{2+}$ ions were determined by adding excess acidic solutions of iron(III) to a known volume of the $Ru(NH_3)_6^{2+}$ solution. The iron(II) formed was complexed with 1,10-phenanthroline, and the concentration of the resulting $Fe(phen)_3^{2+}$ was determined spectrophotometrically from its absorbance at 510 nm where its extinction coefficient is $1.1 \times 10^4$ $M^{-1}$ $cm^{-1}$. Trifluorosulfonic acid (BDH) was doubly distilled under reduced pressure before use. $CF_3SO_3Li$ was prepared by neutralization of $Li_2CO_3$ with $CF_3SO_3H$; dissolved carbon dioxide was removed by bubbling argon into the $CF_3SO_3Li$ solution at 70 °C. The concentration of the stock solution of $CF_3SO_3Li$ was determined by passing an aliquot of the solution through a cation exchange column (Amberlite IR 120(H)), and the total acid content of the eluant was determined by titration with standard base. Sodium perchlorate (BDHAR Grade) was purified by recrystallization from water. Sodium chloride, sodium acetate, hydrochloric acid, and perchloric acid were all BDH (AR Grade) and were used without further purification. Triply distilled water was used in preparing all solutions.

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**Table I.** Second-Order Rate Constants for the Reaction of Cytochrome *c* with $Ru(NH_3)_6^{2+}$ at Different pHs[a]

| pH | $10^{-4}K$, $M^{-1}$ $s^{-1}$ | buffer |
|---|---|---|
| 1.0 | 0.30 | NaCl + HCl |
| 1.2 | 0.34 | NaCl + HCl |
| 1.5 | 1.17 | NaCl + HCl |
| 1.7 | 1.75 | NaCl + HCl |
| 1.9 | 1.73 | NaCl + HCl |
| 2.0 | 3.90 | NaCl + HCl |
| 2.2 | 4.65 | NaCl + HCl |
| 2.7 | 5.75 | sodium formate |
| 3.0 | 6.75 | sodium formate |
| 3.2 | 8.29 | sodium formate |
| 3.35 | 7.27 | sodium formate/sodium acetate |
| 3.65 | 7.19 | sodium formate/sodium acetate |
| 3.70 | 6.71 | sodium formate/sodium acetate |
| 3.8 | 6.03 | sodium formate/sodium acetate |
| 4.0 | 5.10 | sodium acetate |
| 4.3 | 4.50 | sodium acetate |
| 5.0 | 4.08 | sodium acetate |
| 5.2 | 3.79 | sodium acetate |
| 5.3 | 3.80 | sodium acetate |
| 5.4 | 3.75 | sodium acetate |
| 6.15 | 3.82 | sodium acetate |

[a] $[Ru(NH_3)_6^{2+}] = (0.5\text{-}4.0) \times 10^{-3}$ M. [Cytochrome *c*] = 3-6 $\mu$M. All rate constants were obtained at $\lambda$ 550 nm; *I* = ionic strength = 0.10 M NaCl; *t* = 25 °C; buffer concentration = 10 mM.

**Kinetics.** Rates of reaction were measured at 25 °C by monitoring the absorbance changes due to ferrocytochrome *c* at 550 nm using a Durrum 110 stopped-flow spectrophotometer. The rate data were obtained by analyzing Polaroid photographs of absorbance-time curves recorded on the oscilloscope. For each run, at least three of such traces were analyzed. The temperature of the reaction medium was maintained constant at 25.0 ± 0.1 °C by passing water from a thermostated bath through the cell compartment of the spectrophotometer. In order to avoid kinetic complications arising from sudden changes in ionic strength or pH of the protein solution as reported by previous workers, the ionic strength and pH of both reactants were adjusted to the same values (0.1 M) before rate measurements were made. All kinetic measurements were made under pseudo-first-order conditions with the concentration of $Ru(NH_3)_6^{2+}$ at least 50-500 times that of the protein. The concentration of cytochrome *c* was determined by dissolving known weights of the protein in a known volume of the solution.

## Results

**Spectra.** The spectral data obtained are in excellent agreement with those previously reported.[8,17] Thus, at pH 5.2 (*I* = 0.1 M NaCl) the protein exists in its native low-spin form with a peak at 530 nm; at pH ≥4.0 both the mixed-spin and the low-spin isomers are present. At pH 1.0 (*I* = 0.1 M NaCl), the high-spin isomer is dominant with a peak at 620 nm, and the amount of mixed-spin species has decreased significantly. In perchlorate acid medium, the spectra show that at pH 1.7 both the low-spin and mixed-spin forms are present, but as the pH decreases to 1.0, the amount of the high-spin species increases and the low-spin species decreases.

**Reduction of Ferricytochrome in 0.1 M Chloride.** The kinetics of the reduction of ferricytochrome *c* were studied at an ionic strength of 0.10 M NaCl and from pH 1.0 to 6.2. Above pH 2, the first-order plots obtained from the absorbance-time data were linear for more than 90% reaction. The pseudo-first-order rate constants obtained from such plots varied linearly with ruthenium(II) concentration ($0.5 \times 10^{-3}$ to $4.0 \times 10^{-3}$ M). The calculated second-order rate constants varied with pH. The second-order rate constants are presented in Table I. The rates were also found to be independent of buffer concentrations in the range 5.0-10.0 mM for acetate (pH 3.5-6.15) and formate (pH 2.7-3.8) buffer. Between pH 3.5 and 3.8, the rate constants were insensitive to the buffer medium.

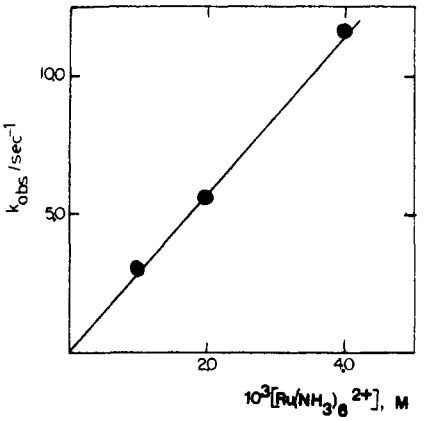


**Figure 1.** Variation of pseudo-first-order rate constant with $Ru(NH_3)_6^{2+}$ concentration in acid medium. $[H^+]$ = 0.1 M = 0.10 M NaCl; *t* = 25 °C.

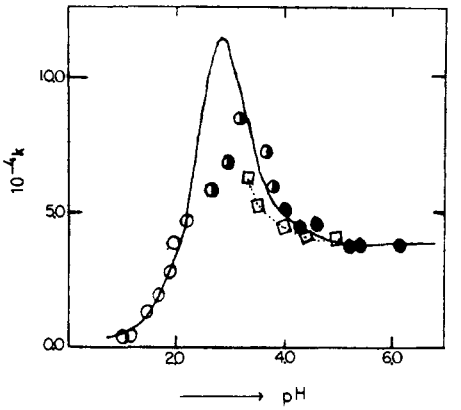


**Figure 2.** Variation of *k* with pH for the reaction of $Ru(NH_3)_6^{2+}$ with cytochrome *c*. Experimental points are in circles. The curve was based on rate constants calculated with eq 1: (□) points obtained by Bennett and Ewall,[16] *I* = 0.10 M NaCl, *t* = 25 °C; (○) HCl + NaCl medium; (◑) sodium formate buffer; (●) acetate buffer.

At pH <2, the rate curves showed biphasic kinetics with an initial fast step followed by a much slower reaction. At $[H^+]$ = 0.1 M, the rates were investigated as a function of the concentration of the reductant. The observed first-order rate constants of the fast step of the biphasic kinetics varied linearly with the concentration of the reducing ion (Figure 1), while the constant for the slower part gave values of 0.096, 0.092, and 0.093 $s^{-1}$ for a fourfold variation in $Ru(NH_3)_6^{2+}$ concentration. Thus, for the region 1.0 ≤ pH ≤ 1.9, the reported rate constants were obtained from the initial parts of the curve.

The rate constant-pH profile for this reaction in the pH region investigated is shown in Figure 2. This type of curve has been previously reported for the reduction[13] of the protein by $Cr^{2+}$ and the oxidation[18] of ferrocytochrome *c* by $Co(phen)_3^{3+}$. In the present work, the rate constant was found to fit the form $k = a/[H^+]$ at low pH (pH 2.0), and $k = c + d[H^+]$ at high pH (4.0 < pH < 6.2). The same functional form is found for the pH dependence of the rate constant for the reduction of the ferricytochrome *c* by Cr(II).[13] Ewall and Bennet,[16] working in the range pH 3.3-7.0, obtained a pH dependence of the same form as that found here at high pH. The curve drawn in Figure 2 was calculated from the equation:

$$k = \frac{a/[H^+] + ac/d[H^+]^2}{1 + a/d[H^+]^2} \quad (1)$$

using $a = 350\ s^{-1}$, $c = 3.9 \times 10^4\ M^{-1}\ s^{-1}$, and $d = 1.0 \times 10^8\ M^{-2}\ s^{-1}$. The value of *c* is in excellent agreement with $3.78 \times 10^4\ M^{-1}\ s^{-1}$ previously reported by Ewall and Bennett,[16] while

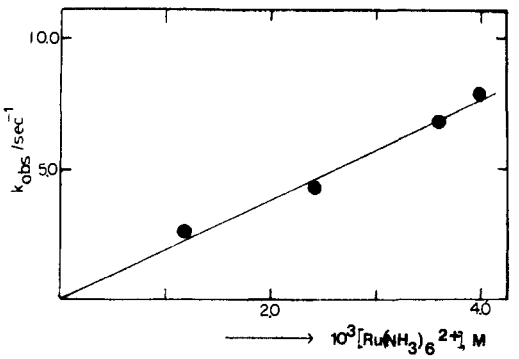


Figure 3. Pseudo-first-order rate constant ($k_0$) as a function of $Ru(NH_3)_6^{2+}$, in perchlorate medium. $[H^+] = 0.10$ M, $[ClO_4^-] = 0.1$ M, $t = 25$ °C.

our $d$ value is slightly higher than theirs ($0.7 \times 10^8$ $M^{-2}$ $s^{-1}$). As in the $Cr^{2+}$ reduction of the protein or the oxidation of the reduced form by $Co(phen)_3^{3+}$, eq 1 correctly gives the limiting forms at high and low pH values, but it gives a poor fit in the intermediate region (pH 2.5-3.5).

**Rate Studies in Perchlorate Medium.** Spectral investigations by Aviram[8] have shown that in perchlorate medium and at low pH, the predominant form of the protein is the mixed-spin form. In order to determine the rate of reduction of this isomer of cytochrome $c$ by $Ru(NH_3)_6^{2+}$, rate measurements were made in the acid range 0.02-0.1 M. The cytochrome $c$ medium was 0.1 M $NaClO_4$. Since $Ru(NH_3)_6^{2+}$ reacts with $ClO_4^-$ in acid solution,[19] the ionic strength and acid concentrations of $Ru(NH_3)_6^{2+}$ were maintained by adding desired amounts of $CF_3SO_3Li$ and $CF_3SO_3H$. Except at 0.02 M where pseudo-first-order plots are linear to more than 80%, the reaction is complex for all acid concentrations, being biphasic for $[H^+]$ = 0.025-0.08 M, and probably triphasic at 0.1 M. However, as in the chloride medium, the electron transfer rates between the predominant protein species in this acid range and the reducing ion are much faster than the side reactions, which are probably a mixture of isomerization rates and reduction of other less reactive cytochrome $c$ species which are present in these high acid media. That the fast step corresponds to a redox reaction is confirmed by (a) the dependence of the rate constant for the fast step on the concentrations of reducing ion (Figure 3) at a constant acid; and (b) the independence of the rate constant for the slower steps on acid concentration. As a result of the large difference in the rates of the component reactions, it was possible to obtain pseudo-first-order rate constants for the component paths by selecting appropriate sweep times on the oscilloscope. For the fast part, rate constants were obtained from the initial rates, while for the slower reactions, the initial points were ignored. The results obtained are presented in Table II. The results show that second-order rate constants are independent of the concentration of either the oxidant or cytochrome $c$, but the rate decreases as the hydrogen ion concentration increases. A plot of the second-order rate constant for the redox reaction against $1/[H^+]$ is linear with the data described by:

$$k_0 = \left(e + \frac{f}{[H^+]}\right) \qquad (2)$$

where $e = 1.3 \times 10^4$ $M^{-1}$ $s^{-1}$ and $f = 4.6 \times 10^2$ $s^{-1}$, for $[H^+]$ at 0.01-0.1 M.

## Discussion

In 0.1 M chloride, the kinetic data obtained for the reduction of cytochrome $c$ show the same type of complex dependence on pH as in the previously reported reduction[13,17] of the protein by $Cr^{2+}$ or oxidation[18] of ferrocytochrome $c$ by $Co(phen)_3^{3+}$.

Table II. Rate Constants for the Reaction of Ferricytochrome $c$ with $Ru(NH_3)_6^{2+}$ in Perchlorate Medium[a]

| $[H^+]$, M | $10^3[Ru(NH_3)_6^{2+}]$, M | $k_1^1$, $s^{-1}$ | $10^{-4}k_0$, $M^{-1}$ $s^{-1}$ |
|---|---|---|---|
| 0.02 | 1.2 | 3.5 | 3.8 |
| 0.025 | 1.2 | 3.5 | 2.8 |
| 0.035 | 1.2 | 2.6 | 2.7 |
| 0.06 | 1.2 | 2.0 | 2.3 |
| 0.08 | 1.2 | 3.1 | 2.2 |
| 0.10 | 1.2-4.0 | 4.0 | 1.9 |

[a] $t = 25$ °C; $I = 0.10$ M ($NaCO_4 + CF_3SO_3Li$). $k_1^1$ is the first-order rate constant for the slow step associated with isomerization rates of the protein, and $k_0$ is the second-order rate constant for the electron transfer reaction between cytochrome $c$ and $Ru(NH_3)_6^{2+}$.

In all three cases, a maximum rate exists around pH 3. The previously reported two cases were interpreted in terms of acid-dependent equilibrium by three forms of cytochrome $c$, with each having a different reaction rate. The data obtained here for the reduction of the protein by $Ru(NH_3)_6^{2+}$ can also be interpreted in this manner (Scheme I).

In Scheme I, cyt $c^0$ is the native form of the protein (stable at pH 6), while cyt $c^1$ and cyt $c^2$ have added one and two protons, respectively. Provided the two equilibria are fast relative to the electron transfer rate, the series of steps outlined in Scheme I leads to the following expression for the second-order rate constant:

$$K = \frac{k_a + k_b K_1^1/[H^+] + k_c K_0^1 K_1^1/[H^+]^2}{1 + K_0^1/[H^+] + K_0^1 K_1^1/[H^+]^2} \qquad (3)$$

This expression is similar to an earlier one reported by Brunschwig and Sutin[18] for the oxidation of ferrocytochrome by $Co(phen)_3^{3+}$. Provided $k_a$ and $K_0^1/[H^+]$ are negligible, eq 3 is identical with eq 1; then, $a = k_b K_1^1$, $ac/d = k_c K_0^1 K_1^1$, and $a/d = K_0^1 K_1^1$. On this basis, $k_c = 3.9 \times 10^4$ $M^{-1}$ $s^{-1}$, $K_0^1 > 10^{-3}$ M, $K_1^1 < 10^{-3}$ M, and $k_b > 3\text{-}5 \times 10^5$ $M^{-1}$ $s^{-1}$. It therefore follows from the above that cyt $c^1$, which is a singly protonated and a more readily protonated species than the native protein, is the most reactive form of cytochrome $c$ in solution, and it is responsible for the observed maximum at about pH 3.

The rate data obtained for the range 0.02-0.1 M acid in perchlorate medium can be interpreted in terms of Scheme II, where cyt $c^2$ and cyt $c^3$ are different conformations of the protonated cytochrome protein. If we make a steady-state approximation for cyt $c^3$, we obtain for the rate expression, provided the first equilibrium is fast compared to the redox reaction:

$$-\frac{d}{dt}[\text{cyt } c^{III}] = \frac{k_1 K_1/[H^+] + k_2 + k_3 k_{23}/(k_{32} + k_3[Ru(II)])}{K_1/[H^+] + 1 + k_{23}/(k_{32} + k_3[Ru(II)])} \times [\text{cyt } c^{III}][Ru(II)] \qquad (4)$$

where $[\text{cyt } c^{III}] = [\text{cyt } c^1] + [\text{cyt } c^2] + [\text{cyt } c^3]$ and $K_1 = [\text{cyt } c^1][H^+]/[\text{cyt } c^2]$. If $k_2$ is small and $k_{32} \gg k_3[Ru(II)]$, then this simplifies the expression for the second-order rate constant to:

$$k = \frac{K_1 k_1/[H^+] + k_3 K_{23}}{K_1/[H^+] + 1 + K_{23}} \qquad (5)$$

where $K_{23} = k_{23}/k_{32}$. If $K_1/[H^+] \ll 1$, then eq 5 is of the same form as eq 2 with $e = k_3 K_{23}/(1 + K_{23})$ and $f = K_1 k_1/(1 + K_{23})$. If we assume that $K_1 \approx K_1^1 \approx 10^{-3}$, $K_{23} \simeq 10$ in 0.1 M $ClO_4^-$ (Aviram[8] shows that $K_{23} \simeq 1$ and 4 at 0.01 and 0.04 M $ClO_4^-$, respectively, given a value of $K_{23}/[ClO_4^-] \simeq 100$ $M^{-1}$), then $k_3 \simeq 10^4$ $M^{-1}$; $k_1 \simeq 4 \times 10^6$ $M^{-1}$ $S^{-1}$.

**Scheme I**

$$\text{cyt } c^1 \rightleftharpoons \text{cyt } c^0 + H^+,\ K_0^1$$

$$\text{cyt } c^2 \rightleftharpoons \text{cyt } c^1 + H^+,\ K_1^1$$

$$\text{cyt } c^2 + Ru(NH_3)_6^{2+} \xrightarrow{k_a} \text{products}$$

$$\text{cyt } c^1 + Ru(NH_3)_6^{2+} \xrightarrow{k_b} \text{products}$$

$$\text{cyt } c^0 + Ru(NH_3)_6^{2+} \xrightarrow{k_c} \text{products}$$

**Scheme II**

$$\text{cyt } c^1 + H^+ \rightleftharpoons \text{cyt } c^2,\ K_1$$

$$\text{cyt } c^2 \overset{k_{23}}{\rightleftharpoons} \text{cyt } c^3$$

$$\text{cyt } c^1 + Ru(II) \xrightarrow{k_1} \text{products}$$

$$\text{cyt } c^2 + Ru(II) \xrightarrow{k_2} \text{products}$$

$$\text{cyt } c^3 + Ru(II) \xrightarrow{k_3} \text{products}$$

The results in 0.1 M $Cl^-$ can be compared with those in 0.1 M $ClO_4^-$ if we assume that the species cyt $c^1$ and cyt $c^2$ are the same in both media. This requires that $K_1^1 \approx K_1$, $k_a \approx k_2$, $k_1 \approx K_b$. We have assumed the first condition, and noting that both schemes require that $k_a$ and $k_2$ be small, we calculate $k_b > 3.5 \times 10^5$ and $k_1 \approx 4 \times 10^6$ $M^{-1}$ $s^{-1}$. Thus, the two schemes are consistent: in both perchlorate and chloride media the most reactive species is one that contains one proton more than the native form; however, in percholate medium there is a protein species which has gained two protons that is reactive and that is not present in the chloride medium.

One would like to discuss the possible structures of cyt $c^1$, cyt $c^2$, cyt $c^3$, and cyt $c^4$ with respect to the work of others. Unfortunately, this is difficult to do. At least three forms of the protein are known:[8,17,18] a low-spin native form, a mixed-spin form that is stable in acid at high anion concentrations (1.0 M $Cl^-$, 0.1 M $ClO_4^-$), and a high-spin form stable at low ionic strength and high acid.

We can associate cyt $c^2$, cyt $c^3$, and cyt $c^0$ with the high-spin, mixed-spin, and native low-spin forms, respectively. Then the relative rate of the reduction of the protein by $Ru(NH_3)_6^{2+}$ is cyt $c^1$ > cyt $c^0$ (low spin) > cyt $c^3$ (mixed spin) > cyt $c^2$ (high spin). However, Przystas and Sutin[17] have identified another species, cyt $c^4$, with three more protons than cyt $c^0$ as the high-spin species in 1.0 M $Cl^-$. If another species exists in our solution, its reactivity is small and the same order of reactivity holds. That $k_3$ refers to the reaction of the mixed-spin species is further confirmed by the results of a few kinetic measurements we made at $I$ = 1.0 M NaCl and $[H^+]$ = 0.1 M. Under these conditions, we obtained $1.9 \times 10^4$ $M^{-1}$ $s^{-1}$ as the bimolecular rate constant for the reduction of the protein by $Ru(NH_3)_6^{2+}$. This value is the same as $1.9 \times 10^4$ $M^{-1}$ $s^{-1}$ we obtained at 0.1 M perchlorate and 0.1 M acid, and it is consistent with the fact that the predominant cytochrome $c$ species in both media are the mixed-spin form. This result further shows that the effect of changing from chloride to perchlorate medium is not a mere anion effect on rate, but a change in the nature of the cytochrome $c$ species present in solution.

It is significant to note that in the few cases (all are homogeneous reactions in aqueous solution) where the effect of pH on the redox reaction rate between cytochrome $c$ and simple inorganic ions or radicals has been extensively investigated, an optimum pH for the protein reactivity has always been obtained between pH 3 and 4. Examples of such systems are: the reduction of the protein by $Cr^{2+}$,[13] the carboxylate radical,[20] the ferricytochrome $c$ catalyzed oxidation of pyrogallol to purpurogallin by hydrogen peroxide,[21] and the oxidation[18] of ferrocytochrome $c$ by $Co(phen)_3^{3+}$, as well as our findings in this work. As contained in this report, these results suggest that the most reactive form of cytochrome $c$ toward these redox reagents is the protein species with one more ionizable proton than the native form. Since this conformational change is pH induced, and linked with the redox reactivity of the protein, it is quite feasible that in vivo electron transfer by cytochrome $c$ is preceded by such oxidase- or reductase-induced protonation and conformational changes because this process seems to lead to the minimum barrier to electron transfer to and from the protein. This suggestion implies that electron transfer by cytochrome $c$ in its native environment occurs via the exposed edge of the heme. While we do not suggest any site on the protein for the protonation, this speculation is similar to an earlier suggestion that the physiological reduction of cytochrome $c$ could be facilitated by protonation of Tyr-78 by an acidic function on the reductase.[22]

In conclusion, our findings in this work show that the reduction of cytochrome $c$ by $Ru(NH_3)_6^{2+}$ shows the same complex dependence on pH as in the previously reported redox reactions of $Cr^{2+}$ and $Co(phen)_3^{3+}$ with this protein. The results also show that the three "electronic" isomers, viz., the low-spin, mixed-spin, and high-spin forms of the protein, have the reactivity order by the peripheral mechanism: low spin > mixed spin > high spin. This order is different from that (low spin > high spin > mixed spin) observed in the reduction by $Cr^{2+}$, a reaction that proceeds by the adjacent attack mechanism. It is also noted that for all reactions in aqueous solutions, where extensive studies of the effect of pH on the rate of reaction of cytochrome $c$ have been carried out, an optimum pH of reactivity exists between pH 3 and 4. This is interpreted as implicating a single protonated cytochrome $c$ as the most reactive form of the protein, and that the physiological reactions of this protein might be preceded by reductase/oxidase-sponsored pH-induced conformational change in order that electron transport to and from cytochrome $c$ might proceed via this path of minimum activation barrier.

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(23) One of the referees did point out that although the mixed-spin species is believed to exist in 0.1 M chloride at pH 2, our cyt $c^3$, which we suggest to be the mixed-spin species, is not identifiable from our kinetic data under these conditions. We do acknowledge this point, but also add that this does not affect our interpretation of the data, and, more important, it does not alter the reactivity order we have suggested. Our identification of cyt $c^3$ with the mixed-spin species is based on the fact that our independently measured rate constants in 1.0 M NaCl (pH 1.0) and in 0.1 M $ClO_4^-$ (pH 1) are the same. Under these conditions, the mixed-spin forms are strongly believed[8,17] to be the dominant cytochrome *c* species in solution, and we suggest these to be cyt $c^3$. Our data in 0.1 M chloride (pH 2.0) suggest that, even if the mixed-spin species is present, its reactivity is considerably masked by that of the less protonated but far more reactive cyt $c^1$ (whose existence has always been inferred from rate measurement only!!). This issue confirms the fundamental difficulty of interpretation that can be associated with the complexity of the acid dependence data at low acid in this type of rate study.

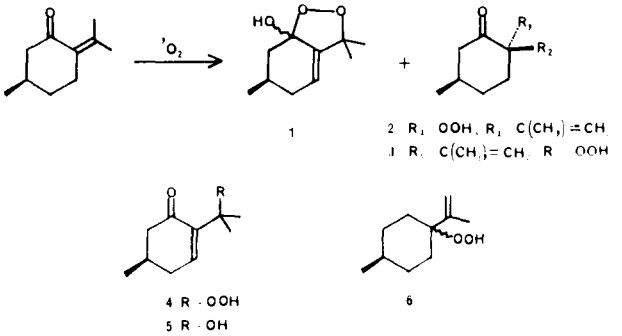
# *Communications to the Editor*

## Reaction of Singlet Oxygen with α,β-Unsaturated Ketones and Lactones

*Sir:*

The reaction of singlet oxygen ($^1O_2$) with alkenes has been extensively studied because of its synthetic utility,[1] its implications in environmental and biological processes,[2] and the mechanistic interest associated with this reaction.[3-6] Despite this intense investigation, there are relatively few examples of the successful oxidation of alkenes that are substituted with electron-withdrawing groups.[7] Numerous examples of the attempted photooxygenation of 3-keto-$\Delta^4$ steroids have shown that these types of enones are unreactive toward singlet oxygen,[1a] a fact which is not surprising since singlet oxygen has been shown to be weakly electrophilic.[8] However, in our previous studies on the conversion of (*R*)-(+)-pulegone into its enantiomer, singlet oxygen proved to be the reagent of choice for the initial allylic oxidation of this enone.[9] We now report that the reactivity of α,β-unsaturated ketones and lactones toward singlet oxygen is strongly dependent on the conformation of the unsaturated system. Those α,β-unsaturated carbonyl systems which prefer (or are constrained to) the s-cis conformation are rapidly oxidized by singlet oxygen, whereas those systems which prefer the s-trans conformation react slowly or not at all.

The photooxygenation of (*R*)-(+)-pulegone using a variety of sensitizers (rose bengal, zinc tetraphenylporphin, methylene blue, and Photox[10]) and in a variety of solvents (benzene, methylene chloride, and methanol) affords, after evaporation of solvent and flash chromatography,[11] **1**,[12] **2**, and **3**[13] in 75, 6, and 6% yields, respectively.[14] Compound **1**, which is the hemiperketal of the hydroperoxyenone **4**, is quantitatively reduced to **5**[15] on exposure to excess triphenylphosphine, triethyl phosphite, or aqueous stannous chloride.




1

2 $R_1$ OOH, $R_2$ $C(CH_3)=CH_2$
3 $R_1$ $C(CH_3)=CH_2$, $R_2$ OOH




4 R = OOH
5 R = OH

6

The regioselectivity of the reaction of **1** with singlet oxygen is surprising since the photooxygenation of the corresponding alkene, isopropylidene-4-methylcyclohexane, occurs exclusively with migration of the double bond away from the ring, to give **6**,[16] and also since singlet oxygen reactions do not normally show a strong Markownikoff effect.[4c,17] To explore the reasons behind this change in regioselectivity, the reaction of singlet oxygen with a series of α,β-unsaturated carbonyl systems was studied. The results are summarized in Table I.

That the products shown in Table I arise from reaction with singlet oxygen and not from radical processes is indicated by the complete inhibition of photooxygenation in the presence of 10 mol % 1,4-diazabicyclo[2.2.2]octane[18] and the lack of inhibition of photooxygenation in the presence of 10 mol % 2,6-di-*tert*-butylcresol, a free-radical inhibitor. Also, in the cases of entries 1 and 6 of Table I the same products are obtained in 58 and 69% yields, respectively, when the enone (5 mmol) is added to a solution of triphenyl phosphite ozonide (25 mmol) in methylene chloride at −78 °C and the solution is allowed to warm to room temperature.[19]

Any mechanistic explanation of the data presented in Table I must account for both the large differences in the β values for s-cis and s-trans α,β-unsaturated carbonyl systems and the preference for the formation of the α,β-unsaturated oxidation product. Both the "approach control" mechanism of Fukui[4b] and the zwitterionic peroxide mechanism of Jefford[3c] lead to incorrect predictions of the major product in the above reactions. The biradical mechanism proposed by Goddard[5] correctly predicts the major product, but does not explain the observed differences in reactivity.

Paquette and Liotta[20] have proposed that the site selectivity observed in the reaction of singlet oxygen with certain polyenes can be predicted from the ionization potentials of the various olefinic systems. Their proposal suggests that differences in reactivity between s-cis and s-trans conformers may be a reflection of conformational effects[21] on the ionization potentials of the α,β-unsaturated carbonyl system. Therefore, we have measured the n and π ionization potential for several of the compounds in Table I. These data are shown in Table II.

Even though the conformation of the π system does have a small effect on the ionization potential as shown by the first two entries of Table II, there is no correlation between ionization potential and reactivity toward singlet oxygen, showing that electronic effects are not the controlling factor in these systems.

The mechanism which we propose to explain the reactivity differences between s-cis and s-trans α,β-unsaturated carbonyl

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