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Electron Transport by *C*-Type Cytochromes

I. The Reaction of Horse Heart Cytochrome *c* with Anionic Reductants

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Abstract. The kinetics of reduction of horse heart cytochrome *c* have been investigated using the reductants sodium dithionite and potassium ferrocyanide. Sodium dithionite reduction at pH 7.0 yields rate constants of 2.8×10^8 M^{-1} sec^{-1} for SO_2^- and 6×10^5 M^{-1} sec^{-1} for $S_2O_4^=$ at infinite dilution. Moreover, the data presented demonstrates the participation of positively charged amino acid side chains at the site of electron transfer. The effect of pH on the reduction of ferricytochrome *c* requires a minimum of two pK values for description ($pK_1 = 7.0 \pm 0.4$, $pK_2 = 9.3 \pm 0.3$). Based on the pK values determined, one or more lysines and a residue(s) with a low pK are implicated as the positively charged residues participating in electron transfer. From a comparison of the rates of reduction of various denatured forms of cytochrome *c* we feel that the most viable conclusion is that electron transfer takes place at the exposed heme edge in the vicinity of the amino acid side chains indicated above.

Ferrocyanide reduction of ferri-horse heart cytochrome *c* takes place in a kinetically complex manner. A mechanism is described which includes complexes of ferrocyanide and ferricytochrome *c* and ferricyanide and ferrocytochrome *c*. As was found for dithionite reduction a positively charged region of the cytochrome *c* participates in electron transfer. Combining our results with ferrocyanide and dithionite we conclude that available data is compatible with a single mechanism of electron transfer. It is suggested that the kinetic distinction between different reductants lies in the lifetime of the transient complex formed, with the order ferrocyanide $\gg S_2O_4^= > SO_2^-$.

Key words: Electron Transport — Cytochrome *C* — Kinetics.

Introduction

The reduction of cytochrome *c* has been studied with a variety of reducing agents including potassium ferrocyanide (Greenwood and Palmer, 1965; Sutin and Christman, 1961 and Havsteen, 1965), sodium ascorbate (Greenwood and Palmer, 1965; Wilson and Greenwood, 1971 and Brandt *et al.*, 1966), tetrachlorohydroquinone (Greenwood and Palmer, 1965 and Williams, 1963), hydrated electrons (Land and Swallow, 1971, Pecht and Faraggi, 1971 and Lichtin *et al.*, 1973), Cr(II) (Kowalski, 1969; Dawson *et al.*, 1972 and Yandell *et al.*, 1973) and sodium dithionite (Creutz and Sutin, 1973; Lambeth and Palmer, 1973, and Lambeth *et al.*, 1973). The data reported to date has not lead to a unified picture as to the mechanism of reduction of cytochrome *c*. Conformational changes, electrostatic interactions and hydrophobic interactions have all been implicated at one time or another.

In order to obtain information useful in discerning between the various mechanistic possibilities the reduction of ferricytochrome *c* by sodium dithionite

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and potassium ferrocyanide has been studied. Sodium dithionite was used as it is of sufficiently low reducing potential to be reactive with cytochromes of a wide range of oxidation-reduction potentials. This was important for the work to be reported as we have investigated several denatured forms of cytochrome *c* and several cytochromes *c* from bacterial sources. Ferrocyanide was utilized as it is representative of outersphere reducing agents without many of the complexities of sodium dithionite.

The effect of specific ions, ionic strength, pH and denaturation on the reduction of cytochrome *c* by sodium dithionite are reported. Studies on ferrocyanide reduction were conducted over a more limited range of conditions as the difference in oxidation reduction potential between the ferri-ferrocyanide couple and cytochrome *c* tends to limit the rates which can be observed. The work outlined above was designed to yield specific information on the participation of the protein moiety of cytochrome *c* in the reduction process.

While our work was in progress, four papers were published which bear on our results. Creutz and Sutin (1973) reported on the reduction of cytochrome *c* by sodium dithionite finding a kinetically complex reaction. The reduction process was interpreted as two reactions taking place, one direct reduction at the heme edge, the other displacement of the iron sulfur bond followed by reduction. Lambeth and Palmer (1973) obtained similar results with a quite different conclusion. They presented evidence for competitive reduction of $S_2O_4^=$ and SO_2^- leading to a kinetically complex reaction. Lambeth *et al.* (1973) extended studies of dithionite reduction of cytochrome *c* to alkaline pH. Although not determining second-order rate constants they identified a transient species formed on reduction which converted in a first-order process to the stable alkaline form of cytochrome *c*. Finally, Stellwagen and Shulman (1973) investigated the rate of electron transfer between cytochrome *c* and iron hexacyanides using NMR. They concluded that the reaction is mediated by the formation of stoichiometeric complexes between the various oxidation-reduction species participating. The formation of a complex during electron transfer had not been noted by previous investigators (Sutin and Christman, 1961; Havesteen, 1965; and Greenwood and Palmer, 1965).

In regards to dithionite reduction our results are in general in agreement with Lambeth and Palmer (1973) although somewhat more extensive. Moreover, our data obtained from stopped-flow measurements with ferrocyanide are in excellent agreement with the NMR data of Stellwagen and Shulman (1973) and demonstrate clearly the formation of complexes during electron transfer between cytochrome *c* and iron hexacyanide.

Methods

Horse heart cytochrome *c* (type VI) was purchased from Sigma Chemical Corporation and purified on Sephadex G-100. Cytochrome *c* denatured by ethanol was prepared as described by Matsubara and Smith (1963). Cytochrome *c* was photo-oxidized according to the method of Jori *et al.* (1971). Amino acid analysis indicated that one histidine residue (his-18 according to Jori *et al.*, 1971) was oxidized in agreement with the findings of Jori *et al.* (1971). The 1 to 65 residue heme peptide (HP1-65) was prepared by cyanogen bromide cleavage of cytochrome *c* (Corradin and Harbury, 1970). Sodium hydroxide treated cytochrome *c* was

prepared by suspending ferri-cytochrome *c* in 1.0 M NaOH for 18 hrs followed by dialysis against 0.02 M potassium phosphate until the pH was 7.0.

Rhodopseudomonas capsulata cytochrome c_2 (Meyer, 1970), *Rhodopseudomonas spheroides* cytochrome c_2 (Meyer, 1970) and cytochrome c_3 (Meyer *et al.*, 1971), and *Rhodospirillum rubrum* cytochrome c_2 (Bartsch *et al.*, 1971) were isolated as described in the indicated references. Sodium dithionite (Hardman and Holden Limited), ascorbic acid (Mallinckrodt) and potassium ferrocyanide (Mallinckrodt) were reagent grade and used without further purification.

All kinetic studies were conducted in a Durrum-Gibson stopped-flow spectrophotometer with a mixing time of 2.1 msec. The driving syringe holders were modified to permit purging the backside of the plungers with nitrogen to reduce oxygen diffusion into the syringe. The temperature was maintained at 20° C and all kinetic data was obtained at least in duplicate.

All solutions were exhaustively deoxygenated by bubbling (0.75 to 1 hr) with water saturated nitrogen gas purified by passage over a column of BASF R3-11 catalyst (Wyandotte Corporation) prior to use. Solid sodium dithionite or potassium ferrocyanide was added to deoxygenated buffers via a side arm. Sodium dithionite solutions were stable as long as maintained anaerobically.

Electrodialysis of cytochrome *c* was performed at 200 V for 3 hrs in a vessel of 300 ml capacity against 4 l of constantly flowing 0.02 M tris-cacodylate buffer, pH 7.0. Electrodialyzed cytochrome *c* was used in all experiments performed in Tris-cacodylate buffer.

Amino acid analysis of photo-oxidized cytochrome *c* was performed with a Beckman Model 120C Amino Acid Analyser. Electron paramagnetic resonance (EPR) spectra were measured at room temperature with a Varian E-3 Spectrometer. EPR spectra of aqueous solutions of sodium dithionite were measured using a flat quartz cell with a path length of approximately 0.1 mm. Sodium dithionite solutions (usually 10 mM) were prepared anaerobically and the sample tube (approximately 1 ml) filled under a stream of N_2 and then flushed with 10 ml of anaerobic solution. The tube was sealed with a ground quartz stopper on the bottom and a layer of mineral oil on the top to limit oxygen diffusion during the measurement.

Studies in the pH range 5.5 to 11 were performed in 0.025 M tris-sodium acetate-glycine-potassium phosphate (TAGP) buffer. Experiments at pH 12 and pH 13 were performed in 0.01 M and 0.1 M NaOH, respectively.

Results

Reduction by Sodium Dithionite

From the proposal of Lambeth and Palmer (1973), the reduction of ferricytochrome *c* by sodium dithionite can be represented as described by Eqs. (1) to (3):

$$S_2O_4^{=} \overset{K_{eq}}{\rightleftharpoons} 2\,SO_2^{-} \tag{1}$$

$$\text{ferricyto. } c + S_2O_4^{=} \overset{k_1}{\rightarrow} \text{ferrocyto. } c + P \tag{2}$$

$$\text{ferricyto. } c + SO_2^{-} \overset{k_2}{\rightarrow} \text{ferrocytoc. } c + P' \tag{3}$$

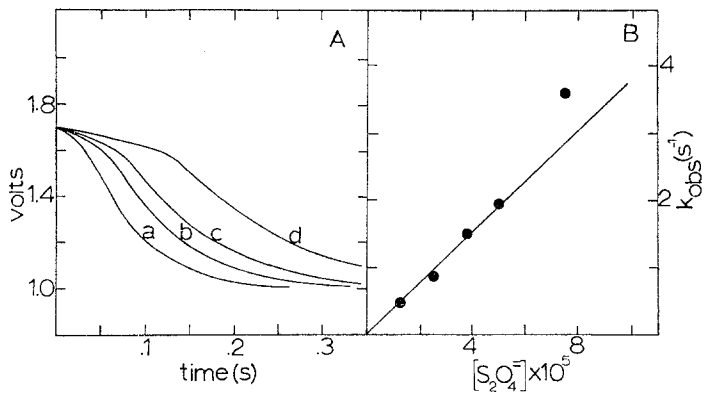

Fig. 1. A. Time course for the reduction of ferricytochrome *c* by sodium dithionite. Heme concentration 3 μM, 0.1 potassium phosphate, pH 7.0, 20°, oxygen concentration 1 μM, dithionite concentrations: a = 75 μM, b = 50 μM, c = 37 μM, d = 25 μM. The reaction was monitored at 417 nm. B. Plot of k_{obs} determined from semi-log plots of the absorbance change taking place during the induction period (data taken from Fig. 1 A), vs. dithionite concentration

Where P and P' represent oxidation products of $S_2O_4^=$ and SO_2^-, respectively. As relative to the rate of reduction of ferricytochrome *c*, the formation of SO_2^- is not rate limiting (Lynn *et al.*, 1964; Lambeth and Palmer, 1973), the rate law for Eqs. (1) to (3) is given by Eq. (4).

$$k_{obs} = k_1[S_2O_4^=] + k_2 K_{eq}^{1/2}[S_2O_4^=]^{1/2}. \quad (4)$$

From Eq. (4) it can be seen that a plot of $k_{obs}/[S_2O_4^=]$ vs. $1/[S_2O_4^=]^{1/2}$ will yield k_1 and $k_2 K_{eq}^{1/2}$.

K_{eq} in Eq. (4) can be evaluated by measuring the concentration of SO_2^- radical in equilibrium with a known amount of sodium dithionite by EPR. Using a value of 5.68×10^{-10} (Burlamacchi *et al.*, 1967) for K_{eq}, the EPR peak to peak signal can be standardized in 0.1 M NaOH. The concentration of SO_2^- can then be determined for other solvents by comparison of the magnitude of the peak to peak signal to that observed in 0.1 M NaOH. In all cases a single symmetrical signal was observed at $g = 2.006$ with the peak width at half-height 2.3 Gauss.

By determining K_{eg} for a particular buffer the values of k_1 and k_2 can be obtained from Eq. (4) after measurement of the pseudo first-order rate constants (k_{obs}) as a function of $[S_2O_4^=]$. When experiments are conducted in the absence of O_2 we obtain data consistent with Eq. (4) as reported by Lambeth and Palmer (1973). However, if the kinetic experiments are conducted in the presence of a known amount of O_2 added to the cytochrome solution an initial induction period is observed followed by a rapid pseudo first-order process (Fig. 1 A). The length of the induction period is directly proportional to the O_2 concentration (0.1 to 20 μM). During the induction period some reduction takes place, in fact, semilog plots of the absorbance change during the induction period yields pseudo first-order rate constants. The rate constants obtained from the induction period when plotted as a function of $[S_2O_4^=]$ are directly proportional to the dithionite concentration

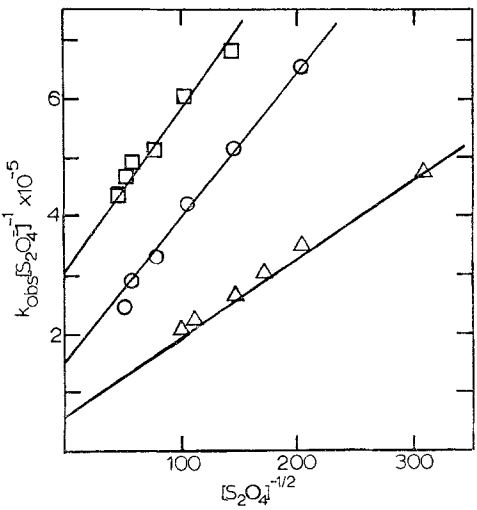

Fig. 2. Typical data plotted according to Eq. (4). The pseudo first-order rate constant (k_{obs}) was determined from semi-log plots of data obtained from the rapid reaction following the induction period. Heme concentration, 3 μM, 20°. The reaction was monitored at 417 nm. ○-buffer, 20 mM potassium phosphate, pH 7.0: □-buffer, 20 mM Tris-cacodylate, pH 7.0: △-buffer, 0.1 M potassium phosphate, pH 7.0

(Fig. 1 B) and yield second-order rate constants. We find that the second-order rate constant obtained from the induction period is the same as k_1 ($\pm$ 30%) obtained from Eq. (4) when the data derived from the rapid reaction (Fig. 1 A) (or in the absence of O_2) is plotted. Thus we conclude that Eqs. (1) to (3) described the mechanism of reduction of cytochrome *c* by dithionite and have analyzed all data to be presented in terms of this mechanism.

For the results to be presented each k_{obs} was obtained at least in duplicate and for the calculation of k_1 and k_2, k_{obs} was measured for at least 6 different dithionite concentrations. We found that k_{obs} could be measured to $\pm$ 10% for separate experiments and that Eq. (4) was obeyed over the concentration range 10^{-5} to 5×10^{-3} M dithionite. Fig. 2 is presented to give an idea of the quality of the data. It is our experience that k_1 was good to approximately $\pm$ 30% and k_2 to $\pm$ 20%.

Effect of Specific Ions and Ionic Strengths

Table 1 summarizes a portion of the results we have obtained in regards to the reduction of cytochrome *c* by sodium dithionite. All of the data presented in Table 1 were obtained at pH 7.0, 20°. Two points can be made in regard to the data presented; 1) When comparing the rate of reduction by $S_2O_4^{=}$ (k_1) in Tris-cacodylate to that in the presence of potassium phosphate or sodium chloride at a particular ionic strength it is found that Tris-cacodylate always yields a larger rate constant. This result is obtained independent of ionic strength up to $\mu = 0.5$ (data not shown). No similar effect was noted for SO_2^- reduction. 2) A strong general ionic strength effect is observed for both SO_2^- (k_2) and $S_2O_4^{=}$ (k_1) reduction of cytochrome *c*. Fig. 3 presents a plot of the logarithm of k_1 and k_2 versus the square root

Table 1. Effect of Ionic Strength and Specific Ions on the Reduction of Ferri-Horse Heart Cytochrome *c*

Buffer[a]	μ	k_1[b] (M^{-1} sec^{-1}) × 10^{-4}	k_2[b] (M^{-1} sec^{-1}) × 10^{-7}	K_{eq}[c] (M) × 10^{10}
Tris-cacodylate	0.018	30.00	11.0	12.1
potassium phosphate	0.018	19.50	11.5	7.5
Tris-cacodylate	0.063	21.00	8.3	3.8
Tris-cacodylate + potassium chloride	0.068	9.00	8.3	5.7
potassium phosphate	0.088	9.00	5.8	5.9
potassium phosphate	0.176	5.50	6.0	5.4

[a] All experiments were conducted at pH 7.0, 20°, heme concentration 2 to 4 μM.

[b] k_1 is given by Eq. (2) and k_2 is given by Eq. (3).

[c] K_{eq} is as given by Eq. (1) and was determined from EPR measurements as described in the text.

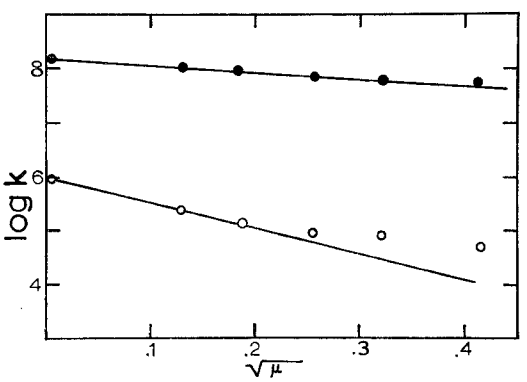

Fig. 3. Relation of the logarithm of k_1 (○) and k_2 (●) to $\sqrt{\mu}$. The buffer was different concentrations of potassium phosphate, pH 7.0, 20°, heme concentrations 3 μM

of the ionic strength (μ). The slope of the plots at low ionic strength should yield the product of the charges on the reactants and the intercept the rate constant at infinite dilution (Frost and Pearson, 1961). The data presented in Fig. 2 yields a slope of -1.3 for SO_2^- reduction with a rate constant of 2.8×10^8 M^{-1} sec^{-1} at infinite dilution. Similary, a slope of -4.8 is obtained for $S_2O_4^=$ reduction and a rate constant of 6×10^5 M^{-1} sec^{-1} in the presence of phosphate or chloride (a somewhat higher value is obtained in Tris-cacodylate) at infinite dilution. The data deviates at high ionic strength as expected (Frost and Pearson, 1961). As the charge on SO_2^- is -1 and that of $S_2O_4^=$ is -2, active site charges of $+1.3$ and $+2.4$ can be obtained for SO_2^- and $S_2O_4^=$ reduction. Although the experimental conditions are not rigorous for application of Debye-Huckel theory the results yield minimum estimates of the charge on cytochrome *c* at the site of electron transfer and indicate that the effective active site charge is much less than that of the overall charge of cytochrome *c* ($+7$ to $+8$) at this pH.

Effect on pH: Fig. 4 presents the rate constants for reduction of ferricytochrome *c* by $S_2O_4^=$ and SO_2^- as a function of pH. As observed by Lambeth *et al.*

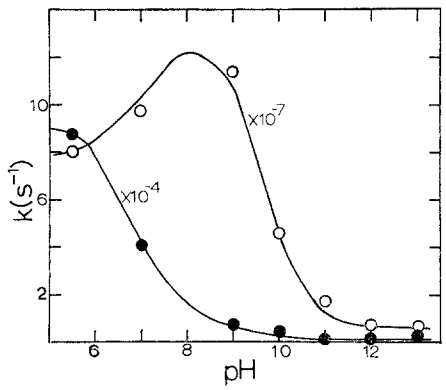

Fig. 4. Effect of pH on the second-order rate constants for the reduction of ferricytochrome *c* by SO_2^- (○) and $S_2O_4^=$ (●). Solid lines were calculated from Eq. (5) using the constants given in the text. Buffer 0.025 M TAGP, heme concentrations 3 μM, 20° and the reaction was monitored at 417 nm

(1973) we find that above pH 8 the reduction is biphasic with the second (slower) phase independent of reductant concentration. The slow phase has been attributed to a change in the sixth ligand of cytochrome *c* (Lambeth *et al.*, 1973). However, after substracting out the second phase we find that Eq. (4) applies at all pH values with no indication of a third phase or other complexities.

The solid lines given in Fig. 4 were calculated from Eq. (5) which was derived from the mechanism given by Eqs. (6) to (10).

$$k_{\text{obs}} \frac{k_a + k_b K_1/[\text{H}^+] + k_c K_1 K_2/[\text{H}^+]^2}{1 + K_1/[\text{H}^+] + K_1 K_2/[\text{H}^+]^2} \tag{5}$$

$$\text{H}_2(\text{Fe}^{\text{III}})\ \text{cyto.}\ c \overset{K_1}{\rightleftharpoons} \text{H}(\text{Fe}^{\text{III}})\ \text{cyto.}\ c + \text{H}^+ \tag{6}$$

$$\text{H}(\text{Fe}^{\text{III}})\ \text{cyto.}\ c \overset{K_2}{\rightleftharpoons} (\text{Fe}^{\text{III}})\ \text{cyto.}\ c + \text{H}^+ \tag{7}$$

$$\text{H}_2(\text{Fe}^{\text{III}})\ \text{cyto.}\ c + \text{reductant} \overset{k_a}{\rightarrow} \text{products} \tag{8}$$

$$\text{H}(\text{Fe}^{\text{III}})\ \text{cyto.}\ c + \text{reductant} \overset{k_b}{\rightarrow} \text{products} \tag{9}$$

$$(\text{Fe}^{\text{III}})\ \text{cyto.}\ c + \text{reductant} \overset{k_c}{\rightarrow} \text{products}\ , \tag{10}$$

where reductant represents either SO_2^- or $S_2O_4^=$ and products represent the appropriate form of ferrocytochrome *c* and the oxidation products of either SO_2^- or $S_2O_4^=$.

The solid curves (Fig. 4) were obtained using $K_1 = 10^{-7}$ M and $K_2 = 2 \times 10^{-10}$M with $k_a = 8 \times 10^7\ \text{M}^{-1}\ \text{sec}^{-1}$, $k_b = 1.3 \times 10^8\ \text{M}^{-1}\ \text{sec}^{-1}$ and $k_c = 6 \times 10^6\ \text{M}^{-1}\ \text{sec}^{-1}$ for SO_2^- reduction and $k_a = 9 \times 10^4\ \text{M}^{-1}\ \text{sec}^{-1}$, $k_b = 8 \times 10^3\ \text{M}^{-1}\ \text{sec}^{-1}$ and $k_c = 10^3\ \text{M}^{-1}\ \text{sec}^{-1}$ for $S_2O_4^=$ reduction. These results indicate a *minimum* of two ionizations. Further, the pK values and rate constants can be varied somewhat (pK values ± 0.4, rate constants ± a factor of two) yielding similar quality fits. The point is that two similar pK values are required for both SO_2^- and $S_2O_4^=$

Table 2. Reduction of Denatured Cytochrome *c* by Sodium Dithionite[a]

Condition	Relative $S_2O_4^=$ Rate Constant	Relative SO_2^- Rate Constant
Native	1.0	1.0
NaOH treated[b]	1.0	1.3
Heat treated[c]	0.76	1.1
6 M guanidine[d]	0.50	0.65
Photo-oxidized[e]	0.45	1.1
Ethanol treated[f]	0.22	1.0
1-65 peptide[g]	0.021	2.9

[a] Reaction in 0.1 M or 0.20 M potassium phosphate, pH 7.0, and monitored in Soret region; cytochrome *c* approximately 3 μM, temperature 20°.

[b] Ferricytochrome *c* was suspended in 1.0 M NaOH for 18 hrs at 4° and then dialyzed against 0.02 M potassium phosphate until the pH was 7.0.

[c] Ferricytochrome *c* was suspended in 0.02 M potassium phosphate, pH 7.0 and heated to 70° for 2 hrs.

[d] Ferricytochrome *c* was suspended in 0.10 M potassium phosphate — 6 M guanidine pH 7.0 and reduced with sodium dithionite dissolved in the same buffer.

[e] Ferricytochrome *c* was photo-oxidized by the method of Jori *et al.* (1971) by irradiation with white light. The cytochrome was suspended in 0.1 M potassium phosphate, pH 8.2.

[f] Ferricytochrome *c* was denatured with ethanol as described by Matsubara and Smith (1963) by incubation in 80 % ethanol for 24 hrs.

[g] The heme peptide of ferri-HHC containing amino acids 1 to 65 was prepared by cyanogen bromide cleavage (Corradin and Harbury, 1970).

reduction. Due to the rapid rated and the complex kinetics it is unlikely that data can be obtained with sufficient percision to generate a more quantitative description.

Reduction of Denatured HHC

Ferricytochrome *c* can be denatured by several methods and by measuring the rate of reduction of the various denatured forms an estimate of the degree of molecular integrity required for efficient electron transfer can be obtained. Table 2 summarizes the relative rates obtained for reduction of denatured ferricytochrome *c* by $S_2O_4^=$ and SO_2^-. All of the denatured forms of the cytochrome bind carbon monoxide in the ferrous state. Presented in Table 2 are relative rate constants which were determined by dividing the rate constant measured under the indicated conditions by the rate constant obtained with native cytochrome *c* in the buffers indicated in the legend to the table.

Reduction of Other Cytochromes c by Sodium Dithionite

Reduction by dithionite of several *c*-type cytochromes was investigated to determine if the results obtained with cytochrome *c* were of a general nature. Table 3 presents the results of a survey of cytochromes *c* which all contain low-spin heme-iron and represent a variety of midpoint potentials and functions. In all cases the reaction involves competitive reduction by $S_2O_4^=$ and SO_2^-. The rate constants vary and do not correlate quantitatively with the midpoint potentials or isoelectric points of the cytochromes *c* investigated.

Table 3. Reduction of Various Cytochromes *c* by Dithionite[a]

Cytochrome	pI	E_m, 7 (mV)	k_1 (M^{-1} sec^{-1}) × 10^{-4}	k_2 (M^{-1} sec^{-1}) × 10^{-7}
Horse heart cytochrome *c*	1 0.0	+ 260	5.5	6.0
R. rubrum cyt. c_2	5.9	+ 330	35.5	3.7
R. capsulata cyt. c_2	7.1	+ 320	29.5	1.2
R. spheroides cyt. c_2	6.3	+ 330	9.4	1.6
R. spheroides cyt. c_3	4.3	− 260	1.0	15.3

[a] Reaction in 0.1 M potassium phosphate, pH 7.0 (except *R. rubrum* cytochrome c_2 which was in 0.01 M Tris, pH 7.3), monitored in the Soret region; heme approximately 3 μM temperature 20°.

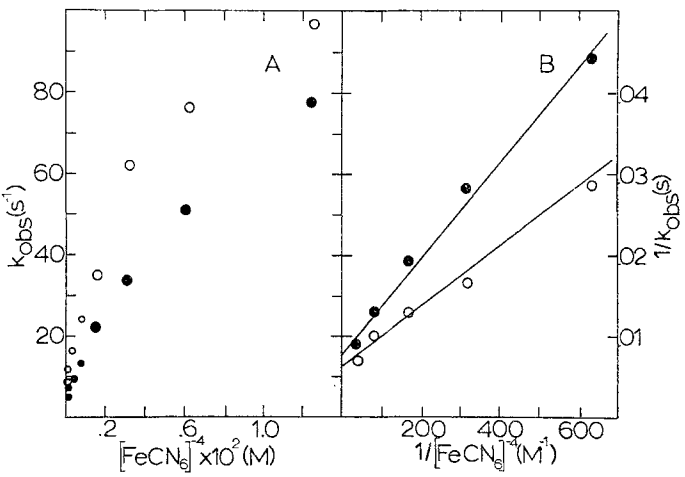

Fig. 5. A. Second-order plot for the reduction of ferricytochrome *c* by potassium ferrocyanide. ○- μ = 0.242, ●- μ = 0.400. Buffer 0.10 M potassium phosphate, pH 7.0 supplemented with NaCl to give a constant ionic strength. Heme concentration 3 μM, 20° and the reaction was monitored at 417 nm. B. Plot of $1/k_{obs}$ vs. 1/[Ferrocyanide] for the data given in part A

Reduction by Potassium Ferrocyanide

Reduction of ferricytochrome *c* by ferrocyanide yielded a monophasic pseudo first-order reaction with a plot of ln absorbance change versus time linear for at least 4 half-lifes. Fig. 5 presents the variation of k_{obs} with ferrocyanide concentration at two different ionic strengths. For the data presented here, constant ionic strength was obtained by adding sodium chloride to compensate for the decrease in [ferrocyanide] as it was diluted. The rate of reduction is not linear with [ferrocyanide] and appears to approach a limiting value at high reductant concentrations. In the absence of dissociation of ferrocyanide to form another species as was observed for dithionite reduction of cytochrome *c*, this non-linearity is suggestive of a change in rate-limiting step at high reductant concentrations and implies at least a two step mechanism as shown in Eq. (11).

$$\text{ferricyto. } c + \text{F} \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} \text{FH} \underset{k'_{-2}}{\overset{k'_2}{\rightleftharpoons}} \text{ferrocyto. } c + 0. \qquad (11)$$

Table 4. Reduction of Ferricytochrome *c* by Potassium Ferrocyanide

μ[a]	k'_1 (M^{-1} sec^{-1} $\times 10^{-4}$	k'_2 (sec^{-1})	K'_{eq}	k'_{-1} (sec^{-1})
0.035	> 18	133	6.8×10^3	> 26
0.242	3.2	154	183	20
0.321	1.9	167	108	8
0.400	1.7	133	128	5

[a] The buffer was 0.02 potassium phosphate supplemented with NaCl to give the indicated ionic strength, pH 7.0, 20°, heme concentration 2 to 4 μM.

In this mechanism F represents ferrocyanide, FH represents a ferrocyanide-ferricytochrome complex, and 0 is ferricyanide. With sufficiently large excess of ferrocyanide (100- to 1000-fold) over cytochrome *c*, k'_{-2} [0] [ferrocyto. *c*] becomes negligible as compared to k'_1[F][ferricyto. *c*] and solution of the rate equations yields:

$$k_{obs} = \frac{k'_2 K'_{eq} [F]}{1 - K'_{eq} [F]}, \tag{12}$$

where $K'_{eq} = k'_1/(k'_{-1} + k'_2)$ and F is the concentration of ferrocyanide. According to Eq. (12) a plot of $1/k_{obs}$ vs. 1/[F] will be linear with a slope of $(1/k'_2) \times K'_{eq}$ and an intercept of $1/k'_2$. Fig. 5 B presents plots of $1/k_{obs}$ vs. 1/[F] for reduction of ferricytochrome *c* as given in Fig. 5 A. Table 4 summarizes the rate constants obtained from Fig. 5 and also presents other data.

Given in Table 4 is k'_1 derived from the initial slope of a plot of k_{obs} vs. [ferrocyanide], k'_2 obtained from Eq. (12) and k'_{-1} calculated from k'_1 and K'_{eq}. Applying Debye-Huckel theory (Frost and Pearson, 1961) we obtain a value of 10^6 M^{-1} sec^{-1} for k'_1 at infinite dilution with an active site charge of + 1.3. Measuring k'_{-1} and k'_2 at nine different ionic strengths in the range $\mu = 0.035$ to 0.4 we find these rate constants to be independent of ionic strength with an average value of 132 $\pm$ 22 sec^{-1} for k'_2 and 7 $\pm$ 10 sec^{-1} for k'_{-1}.

Discussion

In agreement with Lambeth and Palmer (1973) we conclude that sodium dithionite reduces cytochrome *c* via both SO_2^- and $S_2O_4^=$. Creutz and Sutin (1973) considered the possibility of competitive reduction of cytochrome *c* by SO_2^- and $S_2O_4^=$ but rejected it on the grounds that plots of $k_{obs}/\sqrt{S_2O_4^=}$ vs. $\sqrt{S_2O_4^=}$ curve downward at low dithionite concentrations. We have not observed such a phenomenon. In fact, we find that the mechanism outlined in Eqs. (1) to (3) accurately fits available data for a wide range of ionic strengths and pH values for dithionite concentrations ranging from 0.01 to 5 mM.

Experimentally we cannot rigorously exclude the proposal of Creutz and Sutin (1973) as their rate law takes on a form similar to that for competitive reduction. However, based on the experimental observations available and on the known physical-chemical properties of cytochrome *c* were feel competitive reduction by SO_2^- and $S_2O_4^=$ is most likely and is consistent with ferrocyanide reduction (see

below). The crux of the matter lies in an explanation for the induction period observed in the presence of O_2. Lambeth and Palmer (1973) concluded the induction period resulted from the reactivity of SO_2^- with O_2 requiring the scavenging of O_2 before reduction of cytochrome *c* by SO_2^-. This interpretation is consistent with our observations and further, explains why we obtain a rate constant for cytochrome reduction during the induction period identical to that obtained for $S_2O_4^=$ reduction ($\pm$ 30%). It can be argued that O_2^- formed from the reaction of O_2 and SO_2^- (Lambeth and Palmer, 1973) could be responsible for reduction during the induction period as O_2^- is reactive with cytochrome *c* (Land and Swallow, 1971). However, at O_2 concentrations below 2 μM and $S_2O_4^=$ concentrations above 10 μM (the conditions used in this work) the rate of reduction of cytochrome *c* by O_2^- would not be competitive with $S_2O_4^=$ reduction.

It can be suggested that the induction period results from the presence at early times of an oxidizing species or an auto-oxidizable form of cytochrome *c* causing a net decrease in the rate of reduction. Two possibilities exist: 1) the reaction of 2 O_2^- and 2 H^+ yields $H_2O_2 + O_2$ and H_2O_2 can then react with ferrocytochrome *c*. However, for this reaction to be competitive at oxygen concentrations of less than 10 μM the rate of H_2O_2 oxidation of ferrocytochrome *c* would have to be $\geq 10^5$ M^{-1} sec^{-1}. This limiting value is at least 3 orders of magnitude greater than that measured (Cusanovich, unpublished observations). 2) Creutz and Sutin (1973) propose a sulfite-ferrocytochrome *c* intermediate resulting from $S_2O_4^=$ attack which would be expected to be autooxidizable and thus account for the induction period. However, in the absence of firm physical-chemical data identifying this species we feel its existence is speculative. In sum the available data is most consistent with simultaneous reduction by SO_2^- and $S_2O_4^=$ and we will use it for subsequent discussion.

Turning to the mechanistic implications of the reduction of cytochrome *c* by SO_2^- and $S_2O_4^=$ it seems fair to conclude that electron transfer takes place by a rather direct route in both cases with only a mild requirement for structural integrity. The effect of ionic strength is consistent in both SO_2^- and $S_2O_4^=$ reduction with interaction at a localized positively charged region of the cytochrome surface. The concept of a localized site of electron transfer is supported by the fact that a variety of *c*-type cytochromes yield rates of reduction by both SO_2^- and $S_2O_4^=$ independent of cytochrome isoelectric points. Thus it can be concluded that the net charge on the particular cytochrome is not rate limiting.

The lack of substantial effect of denaturation on the rate of reduction argues strongly against involving a highly organized arrangement of amino acid side chains (Dickerson *et al.*, 1971; Margoliash *et al.*, 1973). The most obvious interpretation is that both SO_2^- and $S_2O_4^=$ act at or near the exposed heme edge (Dickerson *et al.*, 1971) in close proximity to positive charges (possibly lys-13 and/or lys-79), thus explaining the effect of ionic strength and denaturation. The differences between SO_2^- and $S_2O_4^=$ in regard to the influence of ionic strength and denaturation on the rates can be attributed to charge and steric effects with the bulky, more charged $S_2O_4^=$ anion more susceptible to perturbations. Moreover, the sulfur dioxide anion radical (SO_2^-) is a more vigorous reducing agent (witness the much greater rates of reduction) and as such would be expected to be less sensitive to experimental conditions.

The specific ion effect observed with $S_2O_4^=$ appears to be related to the affinity of cytochrome *c* for anions. Margoliash *et al.* (1970) have shown that anions such as chloride and phosphate bind to cytochrome *c* in a manner which effects the electrophoretic mobility of the cytochrome. On the other hand, Tris-cycodalate does not influence the electrophoretic mobility of cytochrome *c*. Presuming at least one anion is bound at or near the site of electron transfer it can be proposed that displacement of the bound anion must precede reduction. Hence, the more bulky $S_2O_4^=$ anion is less efficient than SO_2^- in displacement of the bound anion(s) and thus is influenced by its presence.

In order to fit the pH profiles for SO_2^- and $S_2O_4^=$ reduction of cytochrome *c* we were required to invoke at *least* two ionizations. The data presented can be fit using pK values of 7.0 ± 0.4 and 9.3 ± 0.4 for the different forms of cytochrome *c*. The rate constants used to fit the pH profile show a substantial difference at low pH when comparing SO_2^- and $S_2O_4^=$ reduction. For $S_2O_4^=$ reduction, the first ionization (pK = 7) results in a decrease in the rate of reduction. This same ionization leads to an increase in the rate of reduction by SO_2^-. Although we have no data to explain this apparent discrepancy we can speculate that the $S_2O_4^=$ anion would be more susceptible to the loss of a positive charge. Further, if the ionization was coupled to a small structural change in the vicinity of the site of electron transfer the loss in positive charge could be compensated for (at least in the case of SO_2^- reduction).

The pK proposed at 9.3 is consistent with the loss of 695 nm absorbance (Wilson and Greenwood, 1971) and the formation of the alkaline form of cytochrome *c* (Lambeth *et al.*, 1973). However, the proposed pK at 7.0 is more difficult to cope with at this time. No well established heme linked ionizations have been reported in the neutral pH region, although, Czerlinski and Bracokova (1973) have reported a spectral transition with a pK of 6.7 for ferricytochrome *c*. The bulk of the data in the literature is from equilibrium studies (oxidation-reduction, EPR and absorbance spectroscopy) and does not preclude an ionization in the transition state during reduction.

It is useful to note that reduction of cytochrome *c* by the hydrated electron appears to require two pK values in the pH region 5 to 10 (Wilting *et al.*, 1972). Salemme *et al.* (1973) have suggested that cytochrome *c* reduction is facilitated by perturbation of the thr-78 tyr-67 hydrogen bond which they postulate to stabilize the cytochrome *c* transition state. Hence, by analogy we can speculate that ionization of tyr 67 in the transition state could yield a kinetic pK around 7 as observed here. Whatever the case more work is required to identify the transition reported.

Combining our results on ferrocyanide reduction with those of Stellwagen and Shulman, (1973) there is no doubt that ferrocyanide can form a complex with cytochrome *c* and that this complex plays an active role in the electron transfer mechanism. Stellwagen and Shulman (1973) have proposed a three step mechanism [Eq. (13)] which also includes a ferricyanide-ferrocytochrome *c* complex. Although our kinetic data cannot discern this second complex, it seems to us to be a logical extension of the mechanism we have applied [Eq. (11)]. Thus, we will adopt Eq. (13) for further discussions:

$$\text{cyto. } c\ (\text{Fe}^{\text{III}}) + \text{Fe(II)} \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} \text{cyto. } c\ (\text{Fe}^{\text{III}}) - \text{Fe(II)} \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} \text{cyto. } c\ (\text{Fe}^{\text{II}})$$
$$- \text{Fe(III)} \underset{k_{43}}{\overset{k_{34}}{\rightleftharpoons}} \text{cyto. } c\ (\text{Fe}^{\text{II}}) + \text{Fe}^{\text{III}}, \qquad (13)$$

where Fe(II) represents ferrocyanide and Fe(III) represents ferricyanide. From Eq. (13) we make the following definitions:

$$K_1 = k_{12}/k_{22}, \qquad K_2 = k_{23}/k_{32} \quad \text{and} \quad K_3 = k_{34}/k_{43}.$$

As we would expect electron transfer between the complexes to be rapid as no collisions are involved we assign in terms of Eq. (13) $k_1' = k_{12}$, $k_2' = k_{34}$ and $k_{-1}' = k_{21}$. In regards to the individual rate constants, at $\mu = 0.40$ we find $k_{12} = 1.7 \times 10^4\ \text{M}^{-1}\ \text{sec}^{-1}$, $k_{34} = 132\ \text{sec}^{-1}$ and $k_{21} = 7\ \text{sec}^{-1}$. Moreover, Stellwagen and Shulman estimate that $k_{32} = 2.1 \times 10^4\ \text{sec}^{-1}$ and k_{43} is known to be approximately $8.7 \times 10^6\ \text{M}^{-1}\ \text{sec}^{-1}$ (Brandt *et al.*, 1966). From the difference in oxidation-reduction potential between cytochrome *c* and the ferri-ferrocyanide couple the overall equilibrium constant for the reaction written [Eq. (13)] is 2.6×10^{-3}. Hence, k_{23} can be calculated and is found to be $1.5 \times 10^3\ \text{sec}^{-1}$. Taking the six rate constants described above we obtain for the individual equilibra: $K_1 = 2.4 \times 10^3\ \text{M}^{-1}$, $K_2 = 7.2 \times 10^{-2}$ and $K_3 = 1.5 \times 10^{-5}\ \text{M}$. Keeping in mind, the differences in temperature, ionic strength and experimental approach our results are in reasonable agreement with those of Stellwagen and Shulman ($K_1 = 400\ \text{M}^{-1}$, $K_2 = 7.1 \times 10^{-2}$, $K_3 = 2.5 \times 10^{-3}\ \text{M}$). The major discrepancy is in K_3 which Stellwagen and Shulman assumed to be the inverse of K_1.

Combining the notion of a ferrocyanide-cytochrome *c* complex with the ionic strength effects we have noted on k_{12}, we feel it is reasonable to extend the interpretations of the SO_2^- and $S_2O_4^=$ reduction to include ferrocyanide. The results will all three reductants are compatible with a mechanism of electron transfer whereby the anionic reductant acts at or near the exposed heme edge in the vicinity of positively charged amino acid side chains. The kinetic distinctions between the various reductants lies in the lifetime of transient complex formed (on displacement of bound anion?). It follows then that the lifetime of the cytochrome *c*-reductant complex has the order ferrocyanide $\gg S_2O_4^= > SO_2^-$. Clearly, the results presented here do not rigorously exclude the various reductants acting at a site other than that proposed, however, our interpretation is consistent with available data and provides us with a working model for further studies. Finally, it is interesting to note that Salemme *et al.* (1973) have arrived at a quite similar conclusion based upon a detailed comparison of the three dimensional structure of cytochrome *c* and cytochrome c_2.

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