Reduction of Ferricytochrome c by Dithionite Ion: Electron Transfer by Parallel Adjacent and Remote Pathways*

(horse-heart cytochrome c/stopped flow/kinetics/mechanism)

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ABSTRACT The kinetics of the reduction of horse-heart ferricytochrome c by sodium dithionite (phosphate buffer–sodium chloride; pH 6.5, μ = 1.0, 25°) features two reaction pathways; one with the rate constant k_3 = 1.17 × 10^4 M^{-1} sec^{-1}, the other with the rate constant k_1k_2/k_{-1} = 6.0 × 10^4 M^{-1} sec^{-1}. These pathways are interpreted in terms of remote attack (possibly by way of the exposed edge of the porphyrin system) and adjacent attack (requiring the opening of the heme crevice). The limiting rate for the adjacent pathway (k_1 = 30 sec^{-1}) is in good agreement with the rate of heme-crevice opening of ferricytochrome c determined in other studies. The implication of the adjacent attack pathway to the function of cytochrome c *in vivo* is discussed.

Cytochrome c, an electron carrier in the mitochondrial respiratory chain, is a relatively stable metalloprotein consisting of one heme group and one polypeptide chain. The heme group is covalently bonded to the protein by thioether bridges between the porphyrin ring and two cysteine residues in the peptide chain; in ferricytochrome c (Fe^{III}cyt c) the heme group lies in a crevice of the essentially globular protein with an edge of the porphyrin ring located at the surface of the molecule (1). The iron atom is situated in the plane of the porphyrin ring with its fifth and sixth coordination sites occupied by a ring nitrogen atom of histidine-18 and the sulfur atom of methionine-80.

Recent studies of substitution (2) and electron transfer (3) reactions of ferricytochrome c have revealed two pathways for electron transfer: an *adjacent attack* pathway requiring the rupture of the iron–sulfur bond or the opening of the heme crevice (with a rate constant of 60 sec^{-1} at 25°), and a *remote attack* pathway involving an indirect route, possibly the exposed edge of the porphyrin ring system. This paper is concerned with the reaction of ferricytochrome c with the potent, frequently used reducing agent, dithionite [E° for the reduction of sulfur(IV) to dithionite is −0.46 v at pH 7 (4)]. This work extends the earlier studies (3), and shows that the mechanism of reduction of ferricytochrome c may depend upon both the nature of the reducing agent and its concentration.

MATERIALS AND METHODS

Solutions 5–10 μM in Sigma horse-heart Type III cytochrome c (normally used without further purification) were prepared in 0.04 M phosphate–0.91 M NaCl, having a pH of 6.5. Cytochrome c, purified in the following manner, was used in a few runs. A 60-mg sample of the commercial hemeprotein was subjected to gel filtration on a 2 × 20-cm column of Sephadex G-50 (fine) in 0.04 M phosphate (pH 6.5)–0.91 M NaCl at 4°. The center fraction (about 3 ml) of the filtrate was loaded onto a 4 × 20-cm column of Bio-Rex 70 (100–200 mesh, Na^+ form) and eluted with a linear gradient of NaCl according to Chan and Margoliash (5). The middle fraction (about 50 ml, 40 μM) of this eluate was dialyzed against distilled water at 4° and mixed with sodium chloride and phosphate before the kinetic experiments. Dithionite solutions (0.2–20 mM) were freshly prepared by addition of solid Fisher Purified "sodium hydrosulfite" to deaerated 1.00 M NaCl. The purity of the sodium dithionite was ascertained by spectrophotometrically determining the loss of Fe(III) (420 nm, ϵ 1026) when solid sodium dithionite was added to an excess of deaerated $K_3Fe(CN)_6$ solution. The rate of reduction of ferricytochrome c by dithionite was studied by following the absorbance changes at 550, 420, 400, and 370 nm on a Durrum Stopped Flow Spectrophotometer. Observed rate constants were obtained by least-squares analysis of the time dependence of $\log(A_t - A_\infty)$ where A_t and A_∞ are the absorbance values at time t and at infinite time, respectively. The reaction exhibited an induction period whose length increased with decreasing dithionite concentration, and also when O_2 (at the 10 μM level) was deliberately introduced into the cytochrome c solution; the first A_t point was taken at the end of this induction period. All of the kinetic measurements were performed at 25°.

RESULTS

The results of the kinetic measurements are summarized in Fig. 1. The rate data plotted there were obtained at 550 nm; the commercial Fe^{III}cyt c gave k_{obsd} values that were some 10–30% slower (the discrepancy being greatest at high dithionite) at 400 than at 550 nm. This apparent wave-length dependence of the rates was traced to curvature in the log $(A-A_\infty)$ against t plots for the data at 400 nm. This curvature and the wave-length dependence of the rate were absent when the purified cytochrome c was used. Purification did not alter the rate at 550 nm.

It is apparent from Fig. 1 that the reaction features different mechanisms at high and low dithionite concentrations; the reaction is less than first order in dithionite at low dithionite concentrations and becomes first order in dithionite at higher dithionite concentrations. The curve drawn through the experimental points was calculated on the basis of the following scheme.

$$Fe^{III}\text{cyt } c \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Fe^{III}\text{cyt } c^* \qquad [1]$$

Abbreviations: Fe^{III} cyt c, ferricytochrome c; Fe^{II} cyt c, ferrocytochrome c.

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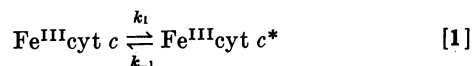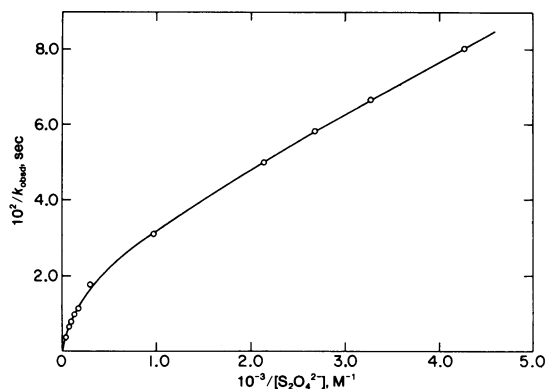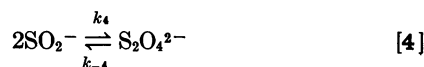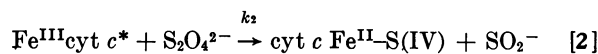
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FIG. 1.   Plot of $1/k_{obsd}$ against $1/[S_2O_4{}^{2-}]$ for the reaction of ferricytochrome c with dithionite at 25°, pH 6.5, 550 nm, and an ionic strength of 1.0 M. Each rate constant is the average of at least five runs.

$$Fe^{III}cyt\ c^* + S_2O_4{}^{2-} \xrightarrow{k_2} cyt\ c\ Fe^{II}\text{-}S(IV) + SO_2{}^- \quad [2]$$

$$Fe^{III}cyt\ c + S_2O_4{}^{2-} \xrightarrow{k_3} Fe^{II}cyt\ c + S(IV) + SO_2{}^- \quad [3]$$

$$2SO_2{}^- \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} S_2O_4{}^{2-} \quad [4]$$

The above scheme yields Eq. **5** for the observed pseudo-first-order rate constants, provided that the steady-state approximation for the concentration of $Fe^{III}cyt\ c^*$ is valid. The following values for the rate constants give the best fit to our

$$k_{obsd} = \left[\frac{k_1k_2}{k_{-1} + k_2[S_2O_4{}^{2-}]} + k_3 \right] [S_2O_4{}^{2-}] \quad [5]$$

data: $k_1k_2/k_{-1} = (6.0 \pm 1.0) \times 10^4\,M^{-1}sec^{-1}$, $k_1 = (30 \pm 5)$ sec^{-1}, $k_3 = (1.17 \pm 0.06) \times 10^4\,M^{-1}sec^{-1}$.

The above scheme neglects the reaction of the reducing agents sulfite and $SO_2{}^-$. At millimolar sulfite in 1 M NaCl (pH 7), sulfite reduction of ferricytochrome c has a half-life of hours. The extent to which the radical path is important depends upon the rate of radical production and the relative rates of reactions **2**, **3**, and **6**. The rate of radical production is

$$SO_2{}^- + Fe^{III}cyt\ c \xrightarrow{k_6} products \quad [6]$$
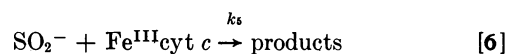
equal to $2k_{-4}[S_2O_4{}^{2-}]$ with $k_{-4} = 40\ sec^{-1}$ (6).‡ This rate is several orders of magnitude faster than the rate of reduction of ferricytochrome c, so that the radical formation rate is not a limiting factor under the conditions of our experiments.‡ This rate comparison (as well as the fact that a rate-limiting dissociation of dithionite would introduce a term in the rate law zero-order in ferricytochrome c and thus require a departure from pseudo-first-order kinetics) shows that $2k_4[SO_2{}^-] \gg k_6[Fe^{III}cyt\ c]$, as has, in fact, been assumed in the derivation of Eq **5**. The radical path thus introduces into the rate law a term proportional to $\sqrt{[S_2O_4{}^{2-}]}$, as is, for example, found for the reaction of dthionite with oxygen (7) and with the cyano-

‡ From $K_4 = 1.54 \times 10^9\,M^{-1}$ and $k_4 = 40\ sec^{-1}$ reported by Lynn *et al.* (6), k_{-4} is calculated to be $6 \times 10^{10}\,M^{-1}\ sec^{-1}$, which is about 10-times higher than a reasonable estimate of the diffusion controlled limit for $SO_2{}^-$ recombination. A more reasonable value for k_4 would be about $5\ sec^{-1}$. Even with this k_4, the rate of radical production is an order of magnitude greater than the $Fe^{III}\ cyt\ c$ reduction.

derivative of ferricytochrome c [Creutz and Sutin, unpublished results (1973)]. Our kinetic data can indeed be fit to a rate law consisting of two terms,§ the first proportional to $\sqrt{[S_2O_4{}^{2-}]}$ and the second proportional to $[S_2O_4{}^{2-}]$ with coefficients of $7 \times 10^2\,M^{-1/2}\,sec^{-1}$ and $8 \times 10^3\,M^{-1}\,sec^{-1}$, respectively. However, we favor the interpretation advanced above (Eqs. 1 to 4) since the plot of $k_{obsd}/\sqrt{[S_2O_4{}^{2-}]}$ against $\sqrt{[S_2O_4{}^{2-}]}$ curves downward in the low dithionite region as though to approach the zero intercept predicted by Eq. **5**, and because of the behavior of the reaction at high cytochrome c concentrations. Thus, at high concentrations of purified cytochrome c (80–200 μM), with dithionite in at least 10-fold excess, the rate of the absorbance change at 550 nm is 3- to 5-fold larger than the rate found at low ferricytochrome c concentrations, and the absorbance passes through a maximum. This effect is discussed further below.

DISCUSSION

The rate law shows that the reduction of ferricytochrome c by dithionite proceeds by two parallel paths. The remote attack pathway (reaction **3**) is the simpler and so will be discussed first.

Remote attack pathway

Reaction **3** proceeds faster than the rate of crevice opening [60 sec^{-1} at 25°, (2, 3)], and therefore necessarily involves remote attack by dithionite, probably on the exposed porphyrin edge. By this criterion, remote pathways also obtain in the reaction of ferricytochrome c with the following reducing agents: hydrated electrons, $1.3 \times 10^{11}\,M^{-1}sec^{-1}$ at 20° and pH 6 (8); H atoms, $1 \times 10^{10}\,M^{-1}\,sec^{-1}$ (9); COO^-, $5.8 \times 10^{10}\,M^{-1}\,sec^{-1}$ (10); $Fe(CN)_6{}^{4-}$, $2.6 \times 10^4\,M^{-1}\,sec^{-1}$ at 22° and pH 7 (11); $Fe^{II}cyt\ c$, $2 \times 10^4\,M^{-1}\,sec^{-1}$ (12); and chromium(II) in the presence of catalyzing anions (3). The reaction of ferricytochrome with $O_2{}^-$, $1.1 \times 10^5\,M^{-1}\,sec^{-1}$ (10), is relatively slow at the radical concentrations used, and consequently could proceed by either a remote or an adjacent pathway.

The reactions of $Fe^{III}cyt\ c$ with $Fe^{II}cyt\ c$ and with $Fe(CN)_6{}^{4-}$ have been rationalized in terms of an outer-sphere model, using steric considerations and the Marcus theory (13). In the context of an outer-sphere model, the reactivity order: $CO_2{}^- > SO_2{}^- > O_2{}^- > S_2O_4{}^{2-}$ might parallel the driving forces for the reductions.§

Adjacent attack pathway

We ascribe process **1** to the rupture of the bond between iron (III) and the sulfur atom of methionine-80 or to the opening of the heme crevice. The value of $k_1 = 30 \pm 5\ sec^{-1}$ determined in this work is in good agreement with $k_1 = 60 \pm$
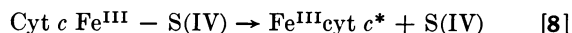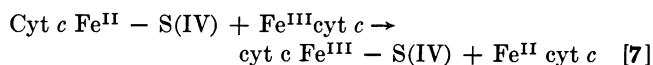
§ The most general rate law includes, of course, terms for the attack of dithionite and the dithionite radical on cytochrome c and its crevice-opened form. The limits $1.0 \times 10^6 \leq k_5 \leq 3 \times 10^7$ $M^{-1}\ sec^{-1}$ are consistent with our data. Further experiments directed toward defining k_5 more precisely, as well as an investigation of the temperature dependence of the reaction, are currently in progress and will be reported later.

The potentials for the reactions, $S_2O_4{}^{2-} + e \rightleftharpoons SO_2{}^- + S(IV)$ and $SO_2{}^- + e \rightleftharpoons S(IV)$, estimated from the $E°$ for the oxidation of dithionite to sulfite and the equilibrium constant for reaction 4 are -0.18 and $-0.74\ v$, respectively, at pH 7.

20 sec^{-1} found under similar conditions (25°, pH 6–7, 1 M ionic strength) for the reaction of ferricytochrome *c* with various anions (2) and with chromium(II) in chloride media (3). In terms of this interpretation, reaction 2 corresponds to the adjacent attack of dithionite on ferricytochrome *c* and requires the opening of the heme crevice. Once this opening has occurred, the dithionite can attack either the porphyrin in the *plane* of the ring system or the iron atom itself.

Because of the properties of $S_2O_4^{2-}$, we prefer the interpretation that reaction 2 corresponds to attack of dithionite on iron rather than on the porphyrin system. Direct porphyrin attack could be the preferred mode, however, for reducing agents having greater affinity for the porphyrin π system. The cytochrome *c* reductase (Fe^{II}cyt c_1) itself might be such a reducing agent in the form, for example, of an aromatic radical side-chain designed to stack parallel to the porphyrin plane. We stress that, despite a difference in the oxidizing site, the limiting rate constant (k_1) for parallel porphyrin attack would be identical to that for pathways involving iron binding, as, in both cases, the crevice-opening conformation change is rate determining.

For dithionite attack on the iron, the immediate product should be a sulfite–Fe^{II}cyt *c* complex. The stopped-flow traces we obtained provide no evidence for accumulation of the Fe(II)–S(IV) intermediate required for this type of inner-sphere mechanism. Also the spectrum of the reduction product obtained within 1 min of mixing is that of ferrocytochrome *c*. These observations do not, however, necessarily argue against our interpretation, since the loss of S(IV) [presumably O-bonded as sulfite to Fe(II)] is expected to be very rapid. Thus the rate of release of X from X–Fe^{II}cyt *c* is 8.8×10^{-4} sec^{-1} at 25° when X = CO (14), 4.27×10^{-3} sec^{-1} at 25° when X = CN^- (14), and 1.08×10^{-1} sec^{-1} at 21° when X = imidazole (15). As this order probably parallels the affinity of Fe^{II}cyt *c* for X, and the affinity of Fe^{II}cyt *c* for oxygen-bonded ligands is expected to be small, rapid dissociation of sulfite–Fe^{II}cyt *c* is not surprising. Further, by analogy with the high spin, labile hydroxy complexes of Fe^{II}cyt *c* formed in alkaline solution, the sulfite complex should be high spin and, consequently, labile. Evidence for the sulfite intermediate is, however, provided by the behavior of the reaction in the high cytochrome *c*, excess dithionite, region. Thus, the increased rate observed under these conditions can be rationalized by postulating the rapid oxidation of the sulfite intermediate by ferricytochrome *c* before its dissociation.

$$\text{Cyt } c \text{ Fe}^{II} - \text{S(IV)} + \text{Fe}^{III}\text{cyt } c \rightarrow$$
$$\text{cyt } c \text{ Fe}^{III} - \text{S(IV)} + \text{Fe}^{II} \text{ cyt } c \quad [7]$$

$$\text{Cyt } c \text{ Fe}^{III} - \text{S(IV)} \rightarrow \text{Fe}^{III}\text{cyt } c^* + \text{S(IV)} \quad [8]$$

The above scheme can account for the increased rates provided reactions 7 and 8 are sufficiently rapid.

Since the adjacent attack mode appears to afford a viable mechanism for reductions of Fe^{III}cyt *c in vitro*, the question of its utilization *in vivo* arises. If the rate of the crevice-opening conformation change, 60 sec^{-1}, is appropriate for this process in mitochondria, the possibility of electron transfer by adjacent attack of Fe^{II}cyt c_1 (the cytochrome *c* reductase)

on Fe^{III}cyt *c* cannot be ruled out. In steady-state studies of respiring rat-liver mitochondria, Chance *et al.* (16) found the turnover number of cytochrome *c* to be 36 sec^{-1}, which is not inconsistent with a rate-determining opening of the heme crevice. More recently, Wagner *et al* (17) report a rate constant of 30–50 sec^{-1} for the Fe^{III}cyt *c* + Fe^{II}cyt c_1 reaction in pigeon-heart mitochondria. If an adjacent attack mode operates *in vivo*, its use could be a consequence of the specificity it offers over remote attack pathways. Selectivity is important in controlling the respiratory rate and in retaining the sequence of the mitochondrial redox chain with the end of efficient ATP production. Even in the absence of the operation of an adjacent attack mechanism, the heme-crevice opening step could be critical to the electron transfer rate in another way. Crevice opening and Fe-S bond rupture, producing five coordinate iron(III), may trigger conformation changes that render the Fe^{III}cyt *c* more susceptible to reduction. The nature of these conformation changes is suggested by the results of structural studies of Dickerson *et al.* which show that chloride and water occupy the heme pocket in ferricytochrome *c* (1), but that these are replaced by phenylalanine-82 in the reduced form (18). This conformation change [perhaps accompanied by the coordination of the chloride to the iron-(III)] may be dynamically coupled to the heme-crevice opening process in ferricytochrome *c*. The increased hydrophobic character of the region neighboring the crevice-opened iron (III) would render the site more reducible. There are thus several ways in which a crevice-opening conformation change could contribute to the selectivity necessary for efficient operation of the respiratory chain.

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