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Kinetics and Mechanism of the Reduction of Horse Heart Ferricytochrome *c* by Glutathione†

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ABSTRACT: A detailed investigation of the reduction of cytochrome *c* by glutathione has shown that the reaction proceeds through several steps. A rapid combination of the reducing agent with the cytochrome leads to the formation of a glutathione–cytochrome intermediate in which the glutathione most likely interacts with the edge of the heme moiety. The electron transfer takes place in a subsequent slower step. Since cytochrome *c*(III) exists in two conformational forms at neutral pH [Kujundzic, N., & Everse, J. (1978) *Biochem. Biophys. Res. Commun. 82*, 1211], the reduction of cytochrome *c* by glutathione may be represented by

$$\text{cyt } c(\text{III}) + \text{GS}^- \underset{}{\overset{K_1}{\rightleftharpoons}} \text{cyt } c(\text{III}) \ldots \text{GS}^- \xrightarrow{k_1} \text{products}$$

$$\text{cyt } c^*(\text{III}) + \text{GS}^- \underset{}{\overset{K_2}{\rightleftharpoons}} \text{cyt } c^*(\text{III}) \ldots \text{GS}^- \xrightarrow{k_2} \text{products}$$

At 25 °C, pH 7.5, and an ionic strength of 1.0 (NaCl), $k_1 = 1.2 \times 10^{-3}$ s^{-1}, $k_2 = 2.0 \times 10^{-3}$ s^{-1}, $K_1 = 2.9 \times 10^3$ M^{-1}, and $K_2 = 5.3 \times 10^3$ M^{-1}. The reaction is catalyzed by trisulfides, and second-order rate constants of 4.55×10^3 and 7.14×10^3 M^{-1} s^{-1} were obtained for methyl trisulfide and cysteine trisulfide, respectively.

Several years ago Dickerson et al. (1971) and Takano et al. (1973) elucidated the spatial structure of both the ferri- and ferrocytochrome *c* by X-ray diffraction. According to these studies, the heme group lies in a crevice of globular protein and is covalently bound to the protein by thioether bridges formed between the porphyrin ring and two cysteine residues in the peptide chain. The iron atom is positioned in the plane of the porphyrin ring with the fifth and sixth coordination sites occupied by a ring nitrogen atom of histidine-18 and the sulfur atom of methionine-80, respectively. An important feature of the structure is that one edge of the porphyrin ring is exposed to the surface of the protein.

A broad spectrum of oxidizing and reducing agents has been used to probe the reactivity of cytochrome *c* in the hope that its behavior in such reactions might advance our understanding of its function in vivo. Several detailed proposals regarding

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the events that take place during the course of the oxidation–reduction reactions have been made as a result of these studies. In general, however, two ways have been described in which an electron could reach the iron atom of the cytochrome. Electron transfer can occur via a "remote" pathway, a term which apparently describes all possible reactions that are initiated at the periphery of the protein. Alternatively, the reduction could occur via a group or reagent that inserts itself into the heme crevice and in doing so may or may not displace one of the protein ligands of the iron atom. Both of these mechanisms could be operative depending on the conditions and on the nature of the reactants. The reduction of ferricytochrome *c* by $Ru(NH_3)_6^{2+}$ (Ewall & Bennett, 1974) and $Fe(EDTA)^{2-}$ (Hodges et al., 1974) were suggested to be outer sphere reactions, with the reductants attacking at the exposed heme edge. The exposed heme edge was also suggested as the site of action in the oxidation of ferrocytochrome *c* by tris(1,10-phenanthroline)cobalt(III) (McArdle et al., 1974) as well as in the cytochrome self-exchange reaction (Sutin, 1972). A direct attack of the reductant on the heme iron atom was proposed for the reduction of cytochrome *c* by chromous ions and by dithionite (Creutz & Sutin, 1973; Yandell et al., 1973). The reaction was plausibly interpreted as being rate limited by the opening of the heme crevice and/or a substitution of the protein ligand by the reactant at the iron atom. The actual electron transport is reportedly rapid. It should be noted, however, that this interpretation has been disputed by Lambeth & Palmer (1973) and by Miller & Cusanovich (1975).

It appears that the detailed mechanism of cytochrome *c* reduction has thus far been investigated with compounds that are of limited biological interest. It would therefore be of significant interest to examine the reactivity of cytochrome *c* with several naturally occurring reducing agents. A preliminary report that deals with the comparative reactivity of several naturally occurring thiols as a reductant for cytochrome *c* has recently appeared from this laboratory (Ginsburgh & Everse, 1978). In this paper we wish to report the results of a more detailed investigation of the kinetics of the reduction of ferricytochrome *c* by reduced glutathione.

Materials and Methods

Horse heart ferricytochrome *c* (Sigma Chemical Co., type III) was used without further purification except when otherwise noted. Ferricytochrome *c* concentrations were determined by measuring the absorbance at 550 nm that results from a full reduction of the cytochrome *c* with dithionite ($\Delta\epsilon_{550} = 1.85 \times 10^4$ M^{-1} cm^{-1}) (Margoliash & Frohwirt, 1959). Buffered cytochrome *c* solutions were always prepared about 30 min before use, in order to obtain conformational equilibrium. Reduced glutathione was purchased from Calbiochem.

Methyl trisulfide was purchased from Eastman Kodak Chemical Co. Stock solutions of methyl trisulfide were prepared just before use by dissolving the proper amounts of methyl trisulfide in absolute ethanol. The concentrations were adjusted such that the ethanol concentration in the final solutions did not exceed 1%. Cysteine trisulfide was prepared by the method of Fletcher & Robson (1963), except that *N*-acetylcysteine was used as the starting material. Stock solutions were prepared by dissolving the proper amount of cysteine trisulfide in 0.1 M HCl.

All other chemicals were of analytical grade.

The reduction of ferricytochrome *c* by glutathione was studied by mixing the proper quantity of buffered solutions of the reactants in a stoppered spectrophotometer cell. Kinetic measurements were recorded on a Beckman spectrophotometer Model 24, by following the change in absorbance at 550 nm. Catalytic effects of the trisulfides were studied by following the kinetics on an Aminco-Morrow stopped flow spectrophotometer equipped with an Aminco DASAR system. Values for the observed rate constants were determined from a weighed least-squares analysis of the time dependence of log $(A_\infty - A_t)$, in which A_∞ and A_t represent the absorbancies of the solutions at 550 nm at times ∞ and t, respectively. A computer program for two relaxations was used for the calculations. The pH measurements were performed with a Beckman digital pH meter, Model 3500. The solutions were buffered with Tris-HCl, and the ionic strength was adjusted with NaCl.

Theoretical Interpretation of Kinetic Data

Electron transfer that occurs via an attack of the reducing species at the exposed edge of the heme moiety of cytochrome *c*, as proposed by Ewall & Bennett (1974) and by Hodges et al. (1974), follows the mechanism shown in eq 1 in which the

$$\text{cyt } c(\text{III}) + GS^- \rightleftharpoons \text{cyt } c(\text{III})\ GS^- \rightarrow \text{products} \quad (1)$$

intermediate form represents the complex formed between glutathione and cytochrome *c* before the transfer of electrons has occurred. In an earlier communication (Kujundzic & Everse, 1978), we reported some preliminary observations concerning the reduction of ferricytochrome *c* by reduced glutathione. We demonstrated that the reaction follows biphasic kinetics and concluded that our results were consistent with the existence of two conformational forms of cytochrome *c*, namely, cytochrome *c* and cytochrome *c**. The conversion of cyt *c* to cyt *c** occurs by deprotonation ($pK \sim 7$), followed by a slow conformational change in the protein structure. On the basis of these results, eq 1 should be rewritten as shown in eq 2, where cyt *c*(III) ... GS^- and cyt *c*(III)* ... GS^-

$$\text{cyt } c(\text{III}) + GS^- \overset{K_1}{\rightleftharpoons} \text{cyt } c(\text{III}) \ldots GS^- \overset{k_1}{\rightleftharpoons} P$$

$$\text{cyt } c(\text{III})^* + GS^- \overset{K_2}{\rightleftharpoons} \text{cyt } c(\text{III})^* \ldots GS^- \xrightarrow{k_2} P \quad (2)$$

represent the related intermediates.

This mechanism leads to the expression for the observed rate constants shown in eq 3. Assuming glutathione to be a weak

$$k_{\text{obsd}(1,2)} = \frac{k_{1,2}K_{1,2}[GS^-]}{1 + K_{1,2}[GS^-]} \quad (3)$$

acid:

$$GSH \overset{K_a}{\rightleftharpoons} GS^- + H^+$$

Under conditions where [GSH] ≫ [cyt *c*], the glutathione anion concentration may be expressed as shown in eq 4, where

$$[GS^-] = \frac{K_a[GSH]_T}{K_a + [H^+]} \quad (4)$$

$[GSH]_T$ is the total glutathione concentration. Substituting eq 2 into eq 1, one obtains eq 5 or 6.

$$k_{\text{obsd}(1,2)} = \frac{k_{1,2}K_{1,2}K[GSH]_T}{K_a + [H^+] + K_{1,2}K_a[GSH]_T} \quad (5)$$

$$1/k_{\text{obsd}(1,2)} = \frac{K_a + [H^+]}{k_{1,2}K_{1,2}K_a}\frac{1}{[GSH]_T} + \frac{1}{k_{1,2}} \quad (6)$$

The second mechanism for the reduction of cytochrome *c*(III), proposed by Sutin et al. (Sutin & Yandell, 1972; Yandell et al., 1973; Creuts & Sutin, 1974), involves a direct attack by the reducing species on the heme iron. This

Table 1: First-Order Rate Constants for the Reduction of Ferricytochrome *c* with Glutathione at 25 °C[a]

[GSH] × 10^4 (M)	$k_{obsd(1)}$ × 10^3 (s^{-1})	$k_{obsd(2)}$ × 10^3 (s^{-1})
5.0	0.80	
7.5	1.10	
10.0	1.56	
12.5	1.84	0.61
25.0	2.99	0.79
33.3	3.58	0.86
50.0	5.70	1.24
100.0	7.44	1.32

[a] Ferricytochrome *c* concentration was 3.3 × 10^{-5} M in all experiments. Solutions were buffered at pH 7.5 with Tris-HCl. Final buffer concentration was 0.1 M. Ionic strength was 1.0, adjusted with NaCl.

mechanism requires the opening and closing of the heme crevice and possibly the dissociation and reassociation of one of the iron ligands. The mechanism may be represented by eq 7 where cyt *c*(III)# represents a form of ferricytochrome

$$\text{cyt } c(\text{III}) \underset{k_{-1}'}{\overset{k_1'}{\rightleftharpoons}} \text{cyt } c(\text{III})\#$$

$$\text{cyt } c(\text{III})\# + GS^- \xrightarrow{k_2'} P \qquad (7)$$

c that serves as an intermediate during the reaction.

When the usual steady-state approximation is made, this mechanism yields eq 8 for the observed pseudo-first-order rate

$$k'_{obsd} = \frac{k'_1 k'_2[GS^-]}{k'_{-1} + k'_2[GS^-]} \qquad (8)$$

constants which is of the same form as eq 3.

It is thus not possible to distinguish between the two mechanisms by using steady-state kinetic data if the conformational change in the cytochrome molecule (K'_1, eq 7) or the formation of the intermediate complex ($K_{1,2}$, eq 2) represents the rate-limiting step of the reaction. In such a case, the observed rate constants will become independent of the glutathione concentration at high concentrations of the reductant. However, by using pre-steady-state kinetics, a distinction between the two mechanisms can be made as demonstrated by the results reported in this paper.

Results

Observed Rate Constants. We have previously shown (Kujundzic & Everse, 1978) that the reduction of ferricytochrome *c* by glutathione in neutral and slightly alkaline solutions proceeds as a biphasic reaction. The two pseudo-first-order rate constants for the reduction of 3.3 × 10^{-5} M ferricytochrome *c* with 5–100 × 10^{-4} M glutathione have now been determined at pH 7.5 and an ionic strength of 1.0 (NaCl) and the obtained values are presented in Table I. It is apparent that the two observed rate constants are not directly proportional to the glutathione concentration, but tend to a limiting value at high glutathione concentrations. This is illustrated in Figure 1, which shows that a plot of $1/k_{obsd}$ of the two phases vs. $1/[GSH]_T$ produces straight lines. Essentially the same results were obtained following a further purification of the cytochrome by gel filtration over Sephadex G-50.

Figure 2 shows the dependence of the observed rate constants on pH. Both rate constants increase with increasing pH and the changes in the rate constants as a function of pH appear to be similar for both phases within the pH range of 7 to 8.

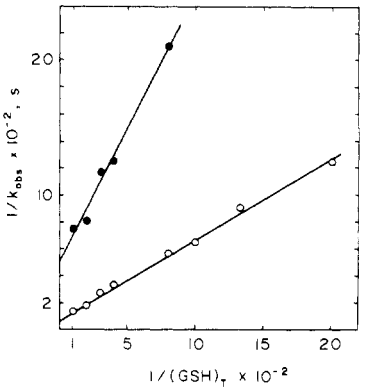


FIGURE 1: Plot, suggested by eq 6, of $1/k_{obsd1,2}$ vs. $1/GSH_T$. [Cyt *c*] = 3.3 × 10^{-5} M; pH 7.5 (Tris-HCl, 0.1 M); *I* = 1.0 (NaCl); λ 550 nm; *t* 25 °C: (○) initial phase; (●) slower phase.

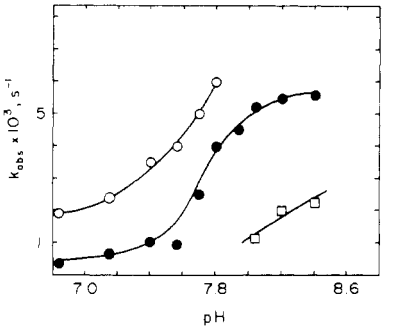


FIGURE 2: Effect of pH on the pseudo-first-order rate constants for the reduction of ferricytochrome *c* by glutathione. (○) Initial phase; (●) slower phase. After pH was increased above pH ~8, a new reaction appeared (□). [Cyt *c*] = 3.3 × 10^{-5} M; [GSH] = 3.3 × 10^{-3} M; *I* = 1.0 (NaCl); Tris-HCl, 0.1 M; λ 550 nm; *t* 25 °C.

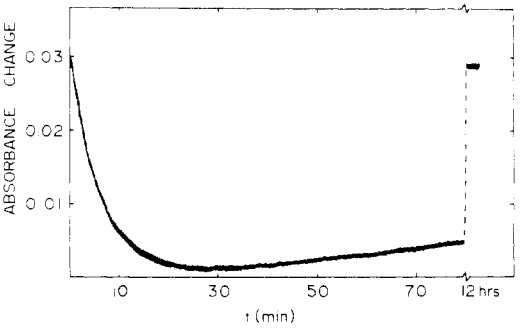


FIGURE 3: Change in optical density at the 339-nm isosbestic point of cyt *c*(III)/cyt *c*(II) spectra during the reduction of ferricytochrome *c* by glutathione. [Cyt *c*] = 3.3 × 10^{-5} M; [GSH] = 1.67 × 10^{-3} M; *I* = 1.0 (NaCl), pH 7.8 (Tris-HCl, 0.1 M); λ 550 nm; *t* 25 °C.

Formation of Intermediates during the Reaction. A few experiments were conducted in an attempt to demonstrate the existence of a cytochrome *c*–glutathione intermediate complex. For this purpose, the change in absorbancy was monitored during the reaction by using a wavelength that is an isosbestic point in the ferricytochrome *c*/ferrocytochrome *c* spectra, i.e., 339 nm (Margoliash & Frohwirt, 1959). Changes in the absorbance at this wavelength were observed during the reduction, which indicate the formation and subsequent breakdown of one or more intermediary complexes. The change in absorbancy at 339 nm that occurs during the reduction is shown in Figure 3.

In order to establish the complexing site of glutathione with cytochrome, the spectrum of ferricytochrome *c* was measured immediately after the addition of glutathione, in the range of 680–710 nm. No change in spectrum was observed under those conditions. Furthermore, the rate of reduction of the

Table II: Rate and Equilibrium Constants for the Reaction of Cytochrome *c* with Glutathione[a]

$k_1 = 1.21 \times 10^{-2} \pm 0.19 \times 10^{-2}$ s^{-1}
$k_2 = 2.00 \times 10^{-3} \pm 0.22 \times 10^{-3}$ s^{-1}
$K_1 = 2.94 \times 10^{3} \pm 0.06 \times 10^{3}$ M^{-1}
$K_2 = 5.30 \times 10^{3} \pm 0.35 \times 10^{3}$ M^{-1}

[a] A pK_a value of 8.8 for GSH was used in these calculations (Jocelin, 1972).

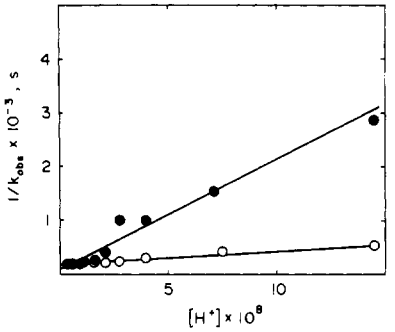


FIGURE 4: Plot, suggested by eq 9, of $1/k_{obsd1,2}$ vs. H^+ concentration. Conditions as in Figure 2.

cytochrome was the same whether the reaction was followed at 695 or at 550 nm.

Determination of Kinetic Constants. The values of k_1 and k_2, and K_1 and K_2 (eq 6), calculated from the intercepts and slopes of Figure 1 are listed in Table II.

Equation 5 may be transformed as shown in eq 9.

$$1/k_{obsd(1,2)} = \frac{1}{k_{1,2}K_{1,2}K_a[GSH]_T}[H^+] + \frac{1 + K_{1,2}[GSH]_T}{k_{1,2}K_{1,2}[GSH]_T} \quad (9)$$

When $1/k_{obsd(1,2)}$ was plotted as a function of the H^+ concentration, Figure 4 was obtained. Even though this figure does not provide enough data to calculate the rate and equilibrium constants, it is obvious that the relationship between $1/k_{obsd}$ and the H^+ concentration is linear, in agreement with eq 9.

Effect of Trisulfides. The effect of cysteine trisulfide on the reduction of ferricytochrome *c* by glutathione was first noted by Massey et al. (1971). These authors observed a significant enhancement of the reduction rate upon the addition of small amounts of trisulfide, and on this basis the action of the trisulfide was assumed to be catalytic. Similar observations were made in our laboratory (Ginsburgh & Everse, 1978). It appeared appropriate to investigate at this point whether or not the action of the trisulfides is truly catalytic.

It is evident from our previous observations (Kunjundzic & Everse, 1978) that at pH 7.8 ferricytochrome *c* exists for 90% in one conformational form. This pH was therefore selected to study the effect of trisulfides on the reduction rate. It was found that both cysteine trisulfide and methyl trisulfide substantially enhance the rate of the reduction.

The changes in k_{obsd} as a function of the trisulfide concentration are shown in Figure 5. It is known that trisulfides are not very stable at alkaline pH values and for this reason measurements were made in a stopped-flow apparatus immediately following the dilution of the stock solution of the trisulfide into buffer. Some lack of reproducibility was observed at the higher concentrations of the trisulfides.

The first-order plot is linear for more than 90% of the reaction, and k_{obsd} increases linearly with increasing concentrations of the trisulfides over the concentration range from

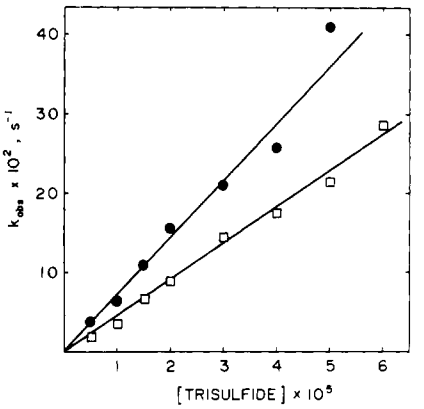


FIGURE 5: Plot of k_{obsd} vs. [trisulfide] for the reduction of ferricytochrome *c* by glutathione in the presence of cysteine trisulfide (●) and methyl trisulfide (□). [Cyt *c*] = 2.2×10^{-5} M; [GSH] = 2.2×10^{-3} M; pH 7.8 (Tris-HCl, 0.2 M); I = 1.0 (NaCl); λ 550 nm, *t* 25 °C.

5 to 60 μM. Trisulfides themselves do not reduce cytochrome *c*, as shown by the fact that the absorption of ferricytochrome *c* at 550 nm does not change upon the addition of a trisulfide in the absence of a reducing agent. Also no changes are observed in the absorption spectrum between 680 and 710 nm under the same conditions.

The reactions follow the rate law shown in eq 10,

$$\text{rate} = k_{obsd}[\text{cyt } c(\text{III})] \quad (10)$$

where k_{obsd} includes the trisulfide concentration which remains unchanged during the reaction. Therefore, eq 11 can be

$$k_{obsd} = k[\text{trisulfide}] + k_0 \quad (11)$$

proposed, where k_0 is the pseudo-first-order rate constant for the reduction of cytochrome *c* with glutathione under the same experimental conditions but in the absence of the trisulfides ($k_0 \sim 5 \times 10^{-3}$ s^{-1}). From the slopes of the curves in Figure 5, k was calculated as a second-order rate constant. The obtained values are 7.14×10^3 M^{-1} s^{-1} for the cysteine trisulfide, and 4.55×10^3 M^{-1} s^{-1} for the methyl trisulfide.

Discussion

Our kinetic data show that the rate of reduction of ferricytochrome *c* with glutathione reaches a limiting value at high concentrations of the reductant. An analogous type of kinetic behavior has been observed in reactions of ferricytochrome *c* with various anions (Sutin & Yandell 1972). These reactions were plausibly interpreted as a displacement of the methionine-80 sulfur from its coordination site followed by a binding of the anions to the iron. The rate constants for the opening of the heme crevice as the initial step of the reaction was estimated to be about 60 s^{-1}. Confirmation of this model was obtained by the reaction of ferricytochrome *c* with chromous ion as well as with dithionite, and this mechanism has been described as the "adjacent" mechanism (Yandell et al., 1973; Creutz & Sutin, 1973).

According to the "adjacent" mechanism, the limiting rate (k'_1) should be independent of the nature of the reductant. Our results show, however, that the maximum value for the rate of reduction of ferricytochrome *c* with glutathione is far less than 60 s^{-1} under conditions similar to those used in Sutin's experiments (I = 1.0). Furthermore, the absorption maximum of the ferricytochrome *c* at 695 nm has been generally associated with the integrity of the methionine-80 sulfur–iron bond (Wilson & Greenwood, 1971). The addition of ligands such as cyanide, azide, imidazole, or pyridine, which have been

found to displace methionine-80 sulfur, causes the immediate disappearance of this absorption maximum (Sutin & Yandell, 1972; Creutz & Sutin, 1974). In our experiments with glutathione as the reductant, no change in absorption was observed between 680 and 710 nm immediately following the addition of reduced glutathione. The rates of reduction of the cytochrome were identical when measured at 550 nm and at 695 nm. On this basis we assume that the methionine-80 sulfur–iron bond was not disturbed during the reaction and, consequently, that the site of compexing of glutathione with ferricytochrome is not likely at the heme iron.

The obtained association constants between the two conformational forms of ferricytochrome *c* and glutathione of 3×10^3 M^{-1} and 5×10^3 $M,^{-1}$, respectively, are in good agreement with other similar systems. For example, Stellwagen & Cass (1975) found that ferrocyanide binds to ferricytochrome *c* with an association constant of about 10^3 M^{-1}. They proposed that such complexation occurs at the exposed heme edge of the porphyrin ring. For the same system, an association constant of 2.4×10^3 M^{-1} was reported by Miller & Cusanovich (1975).

The change in absorbance at the isosbestic points may be due to the difference between the absorbance of the formed complex and that of the products. The very slow reversible absorbance change, that occurs only at the 339-nm isosbestic point but not at 526.5 or 542 nm, may be explained by the formation of GSSG. An eventual reoxidation of the cytochrome was excluded because a fully reduced spectrum of the cytochrome was found after 12 h. It appears, therefore, that the product of the reduction is a complex between the reduced cytochrome and glutathione and that this complex very slowly dissociates to form cytochrome *c*(II) and GSSG:

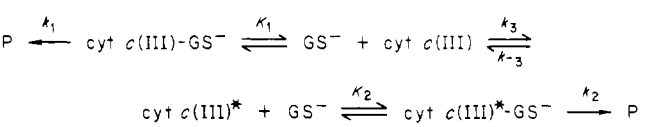
$$2 \text{ cyt } c(\text{II})\text{–GS} \rightarrow 2 \text{ cyt } c(\text{II}) + \text{GSSG}$$

Further experiments are currently in progress to elucidate the events that take place in our system before and after the electron is transferred.

A heme-linked ionization with a p$K \sim 9$ is quite well described. This ionization is followed by a conformational change in the protein structure, forming a "high pH form" of the cytochrome. On the basis of NMR studies of the methionine-80 methyl resonance, Gupta & Koenig (1971) suggested that the high pH form of the ferricytochrome *c* has a different ligand, possibly Lys-79 N instead of Met-80 S. This suggestion was supported in several other studies (Schejter & George, 1964; Greenwood & Palmer, 1965; Schechter & Saludjian, 1967; Stellwagen, 1968; Sreenathan & Taylor, 1971).

Another heme-linked ionization of ferricytochrome *c* with a p$K = 7$, also followed by a conformational change, was adopted from pH-jump experiments (Aviram & Krauss, 1974). A similar protonation has been noted by Miller & Cusanovich (1975). Recently the existence of two conformers has been demonstrated at neutral pH and very low ionic strength. However, the interconversion between the conformers has been attributed to an ionic strength effect rather than to pH (Goldkorn & Schejter, 1977a,b). Because the ionization of the cytochrome with respect to p$K = 7$ as well as its related conformational change in the protein structure has not yet been well established and because of its possible biological importance, our attention in this work was particularly attracted to the reactivity of these two forms towards reduced glutathione. Our system may be expressed as shown in Scheme I where P represents the reduced forms of the cytochrome *c*. For k_1 and $k_2 \ll k_3$ and k_{-3}, two parallel paths of the reduction are predicted. Our values of 1.2×10^{-2} s^{-1} for k_1 and 2.0×10^{-3} s^{-1} for k_2 suggest that the interconversion between the two conformational forms should be slower than 2×10^{-4} s^{-1} since no evidence for a third relaxation process was observed under our experimental conditions. For the reduction of ferricytochrome *c* with ascorbate and dithionite at $I = 0$, pH 7, rate constants of 1×10^{-2} s^{-1} and 4×10^{-2} s^{-1} were found, respectively, which should be comparable to our k_3 and k_{-3} values (Goldkorn & Schejter, 1977a,b). Under our experimental conditions, $I = 1.0$, pH 7.5, it is apparent that the interconversion of cyt *c*(III)* to cyt *c*(III) is a much slower process. Perhaps the formation of the complexes with glutathione stabilizes the conformational forms, resulting in a decreased rate of interconversion. When ferricytochrome *c* buffered at pH 7.8 is mixed with glutathione buffered at pH 7.0 (final pH 7.5), a monophasic reaction occurs for about 90% of the total reaction, which shows that no interconversion occurs during the time of the reduction. This result supports the conclusion that the rate of interconversion between the two forms of cytochrome *c* is very slow in the presence of glutathione. Unfortunately, this phenomenon can only be studied over a relatively short pH range (pH $\sim$ 7–8). When the pH is increased above 7.8, it seems that a new reaction appears (squares in Figure 2). This may be attributed to the deprotonation with a p*K* of about 9, discussed earlier, and a subsequent formation of the high pH form of the cytochrome. Also some lack of reproducibility was observed in this pH range. On the other hand, when the pH was decreased below pH 6.8, the reaction tends to become partly zero order.

Scheme I



It has been reported (Froede & Hunter, 1970) that oxidized glutathione (GSSG) enhances the rate of reduction of ferricytochrome *c* by reduced glutathione. However, Massey et al. (1971) showed that the stimulatory effect of the GSSG on the reduction of ferricytochrome *c* by GSH may be due to S^0-containing impurities present in the GSSG. They also showed that the main impurity is glutathione trisulfide (GSSSG), and the active species involved in the rapid reduction of the cytochrome is probably the persulfide (GSS^-). A similar mechanism was proposed with selenium as a catalyst of the reduction (Levander et al., 1973). Our data show that the reduction of cytochrome *c* with reduced glutathione in the presence of trisulfides is a first-order reaction, indicating a catalytic character of the trisulfides. However, at this point it is not possible to conclude whether the reaction proceeds via the formation of a tertiary complex or whether the cytochrome *c* is reduced by GSS^- with a subsequent recombination of the trisulfides.

It should be noted that it is not possible to ascertain at the present time that the obtained rates of reduction of cytochrome *c* as reported in this paper are not subject to some form of catalysis. For example, if the reduced glutathione were contaminated with a small amount of a trisulfide, one would obtain an overestimation in the observed rate constants and, consequently, the pronounced mechanism would be oversimplified. That the presence of such a catalyst is a distinct possibility is illustrated by the fact that an estimated concentration of about 2 μM trisulfide would be required to give the maximum rate constants given in Table II. Values approaching these rate constants are obtained when glutathione concentrations in excess of 10 mM are used. It is obviously very difficult to rule out the presence of such small quantities

of impurities in the glutathione used in our experiments. Nevertheless, experiments are presently in progress to ascertain whether or not the reduction of cytochrome *c* by glutathione as described in this paper is subject to the action of one or more catalytic species present in our reagents.

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Messenger Ribonucleic Acid Metabolism in Mammalian Mitochondria: Relationship between the Decay of Mitochondrial mRNA and Their Poly(A)†

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ABSTRACT: The mitochondrial poly(adenylic acid)-containing mRNAs in mouse ascites cells pulsed with radioactive precursors contain 35–55 nucleotide long poly(adenylic acid) sequences. These sequences are shortened with age in cells chased with cold medium. The possible relationship between the decay rates of mRNA and their poly(adenylic acid) sequences has been investigated by using mitochondrial RNA and protein synthesis inhibitors. The pattern of mRNA decay as determined by a new solid-phase-bound complementary DNA procedure indicates the presence of two classes of poly(adenylic acid) containing mRNA in mammalian mitochondria: one decaying with a $t_{1/2}$ of 45 min and the other class with a $t_{1/2}$ of 210 min. Inhibitors such as ethidium bromide and puromycin which accelerate the decay of mitochondrial mRNA also cause an enhanced decay of poly(adenylic acid) sequences. These results have been interpreted as evidence supporting the involvement of poly(adenylic acid) sequences in the mRNA stability.

Since the first observation by Lim & Canellakis (1970) on the occurrence of 3′-poly(A)[1] in globin mRNA, such sequences have been detected in a great many eukaryotic mRNAs. (For reviews, see Darnell et al., 1973; Greenberg, 1975; Lewin, 1975.) Despite intensive efforts, however, the precise functional and/or regulatory role of mRNA-associated poly(A) remains somewhat unclear. Recent experiments by Huez et al. (1974), Sheiness et al. (1975), and Nudel et al. (1976) appear to show a close relationship between the size of 3′-poly(A) tail and the turnover rate of mRNA molecule, im-

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[1] Abbreviations used: poly(A), poly(adenylic acid); poly(U), poly(uridylic acid); oligo(dT), oligo(deoxythymidylic acid); cDNA, complementary DNA; EDTA, ethylenediaminetetraacetate; $NaDodSO_4$, sodium dodecyl sulfate.

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