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Reduction of Cytochrome *c* by Thioglycolate: Participation of Catalysts[1]

DEMETRI M. KOKKINAKIS[2] AND JOHANNES EVERSE

Department of Biochemistry, Texas Tech University Health Sciences Center, Lubbock, Texas 79430

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The reduction of cytochrome *c* by thioglycolic acid was found to be extremely sensitive to metal catalysis. The rate of the uncatalyzed reaction was negligible and independent of pH, indicating that thioglycolic acid cannot reduce cytochrome *c* directly. Both copper and iron act as catalysts with copper being superior to iron. The metal-catalyzed reaction appears to be independent of pH and the presence of oxygen but is sensitive to the presence of chelating agents. The reduction of cytochrome *c* by thioglycolic acid is also catalyzed by impurities present in oxidized glutathione. The rate of this reaction is sensitive to changes in pH and oxygen concentration but insensitive to changes in ionic strength. Chelating agents have no effect on the rate of this reaction. The data, therefore, suggest that the reduction of cytochrome *c* by thioglycolic acid can proceed via distinct mechanisms which are dependent on the nature of the catalyst.

About a decade ago Bäuerlein and his colleagues (*1–3*) observed that the reduction of a heme compound by a thiol under appropriate conditions can promote the formation of pyrophosphate bonds in solutions of inorganic phosphate and phosphate derivatives. Thus, the reduction of hemin by thioglycolic acid in the presence of ADP and inorganic phosphate yielded 0.16 mol of ATP, together with pyrophosphate and $(ADP)_2$, per mole of reduced hemin. To the best of our knowledge, this is the first time that phosphorylation has been shown to occur coupled to the reduction of a heme compound in a soluble system.

At about the same time Painter and Hunter (*4, 5*) published a series of articles indicating that a high degree of phosphorylation of ADP is obtained when cytochrome *c* is reduced by glutathione in the presence of large amounts of oxidized glutathione. These observations could not be confirmed at a later date. An inquiry by one of us (J. E.) revealed, however, that at about the same time significant changes were implemented in the procedure for the commercial preparation of oxidized glutathione. Hence, there could have been a change in the nature of the impurities present in oxidized glutathione, which could explain the discrepancy.

It has been well established that thiol derivatives in the form of nonheme iron proteins play an important but undefined role in the mitochondrial electron transport chain (*6–8*). These proteins undergo oxidation–reduction reactions in con-

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[2] Present address: Dept. of Biochemistry, Northwestern University Medical School, 303 E. Chicago Ave., Chicago, IL 60611

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junction with the other components of the electron transport chain and probably form an integral part of the chain.

Based on the above-mentioned observations we thought it of interest to pursue a detailed investigation into the mechanism of the reduction of cytochrome *c* by thiols. In previous reports from this laboratory we have discussed the reactivity of various thiols toward cytochrome *c* and the catalytic effects exerted by trisulfides (*9*), as well as the kinetic mechanism of the cytochrome *c* reduction by glutathione (*10, 11*). In the later communications we discussed the pH dependence of the reaction at constant ionic strength, and concluded that cytochrome *c* is reduced by a thiol complex rather than by the free thiol anion. The simplest interpretation was formation of a thiolcytochrome *c* complex with an intramolecular electron exchange as the rate-determining step.

In this communication we report on the pH, ionic strength, and metal ion dependence of the cytochrome *c* reduction by thioglycolic acid and glutathione. Thioglycolic acid was chosen in this study simply because its purity is readily ascertained.

METHODS AND MATERIALS

All buffers were prepared from stock solutions of 0.5 *M* Tris, and 0.5 *M* Tris–sulfate, that had been previously treated to remove metal contaminants. Solutions to be purified from metal contamination were passed through a Chelex-100 (Bio-Rad Lab.) column (75 × 5 cm); the column was then washed with an equal volume of distilled water and both the eluates were combined. The molarity of the buffer solutions, prepared by appropriate dilutions of the stock solutions, was determined from their conductivity.

Horse heart ferricytochrome *c* (Sigma Chemical Co., type III) was dissolved in 1 m*M* Tris–sulfate buffer,[3] pH 7.0, to a final concentration of 200 μM and was dialyzed versus the same buffer supplemented with 1% (w/v) Chelex-100. Solutions of cytochrome *c* of the desired concentration, buffer molarity, and pH were made by diluting the dialyzed solution of cytochrome *c* with the stock solution of Tris and/or Tris–sulfate and with distilled water. Ferricytochrome *c* concentrations were determined by measuring the change in absorbance at 550 nm that results from a full reduction of cytochrome *c* with sodium ascorbate ($\Delta\epsilon_{550}$ = 18,500 M^{-1} cm^{-1}) (*12*).

Thioglycolic acid (Sigma Chemical Co., grade I) was distilled twice under vacuum and stored at −20°C for as long as 2 months without any significant loss of the sulfhydryl group activity as demonstrated by the dithiodipyridine test (*13*).

Oxidized glutathione disodium salt (Sigma Chemical Co., grade IV) was dissolved in 1 m*M* Tris–sulfate buffer, pH 7.0, to a final concentration of 60 mg/ml, and was passed through a Chelex-100 column (30 × 2 cm). Elution of the disulfide

[3] The addition of Tris-base to a fixed concentration of Tris–sulfate allows one to prepare a series of buffers of different pH but constant ionic strength. The molarity of Tris–sulfate buffers therefore reflects the concentration of the anion.

was monitored by its uv absorption at 273 nm. Eluates containing the oxidized glutathione were combined and diluted to the desired concentration with Tris–sulfate buffer of the appropriate concentration and pH.

Assay Method

Thioglycolic acid solutions (2.94 m*M*) were made fresh every 30 min from a stock solution of 150 m*M* thioglycolic acid in water adjusted to pH 7.0 with Tris base. Stock solutions of thioglycolic acid were prepared fresh every day from the purified thiol. Solutions of cytochrome *c* (80 μM) were prepared at least 2 hr prior to their use in order to achieve conformational equilibrium. Oxidized glutathione, *ortho*-phenanthroline, EDTA, copper sulfate, and ferrous ammonium sulfate solutions were prepared in glass-distilled water at concentrations 21 times higher than the desired final concentration.

The reduction of cytochrome *c* by thioglycolic acid was studied by mixing 1 ml of the thiol solution with 0.1 ml of the metal, oxidized glutathione, or other supplement. The reaction was initiated by the addition of 1 ml cytochrome *c* solution. Kinetic measurements were recorded on a Beckman spectrophotometer Model 134 by following the change in absorbance at 550 nm. Ferricytochrome *c* concentrations were calculated from the change in the absorption using 18,500 $cm^{-1}\ M^{-1}$ as the difference of the molecular absorptivity index between reduced and oxidized cytochrome *c*. Semilogarithmic plots of the declining concentrations of cytochrome c^{III} versus time were made for each case and pseudo-first-order rate constants were calculated from the half times of the reactions whenever plots were found to be linear.

Anaerobic experiments were conducted in an anaerobic cell (*14*). Because of the limitations of the cell the volume of the cytochrome *c* solution had to be reduced. The concentrations of the reagents were, therefore, changed accordingly. In the anaerobic experiments any activators or inhibitors were added to the chamber containing the thiol.

RESULTS

The kinetic patterns for the reduction of cytochrome *c* by excess thioglycolic acid depend on the nature of the catalysts employed to facilitate the reaction. In the absence of any catalyst (Fig. 1) the reduction of cytochrome *c* is first order with respect to the concentration of the ferric form of the cytochrome. Copper salts in catalytic amounts have a pronounced effect in accelerating the reaction without altering its kinetic behavior (Fig. 2). Small changes in the rate but not in the order of the reaction are observed with pH and with the exclusion of oxygen in both the uncatalyzed and copper-catalyzed systems. However, these changes are too small to account for a major pH or oxygen effect on the reactivity of cytochrome *c* or on the concentration and composition of the reducing species.

The kinetic patterns of the iron-mediated reduction of cytochrome *c* are shown in Fig. 3. Deviation from first-order kinetics are observed near neutral pH but not under alkaline conditions. Although initial rates are independent of pH, the

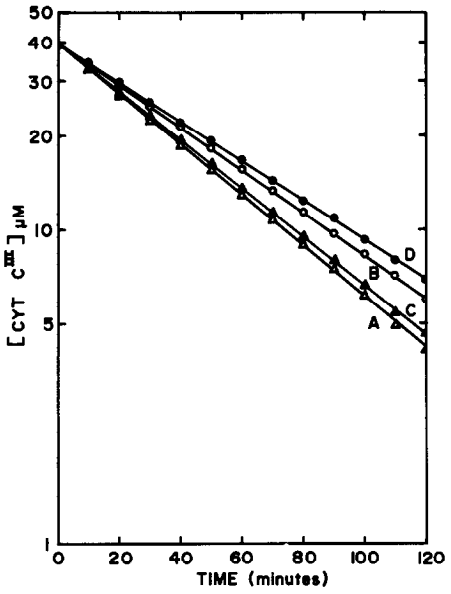

FIG. 1. Reduction of cytochrome *c* by thioglycolate in the absence (A,B) and presence (C,D) of oxygen. Cytochrome *c*, 40 μM; thioglycolate, 1400 μM; buffer, 0.02 *M* Tris–sulfate at pH 7.0 (A,C) and pH 8.0 (B,D).

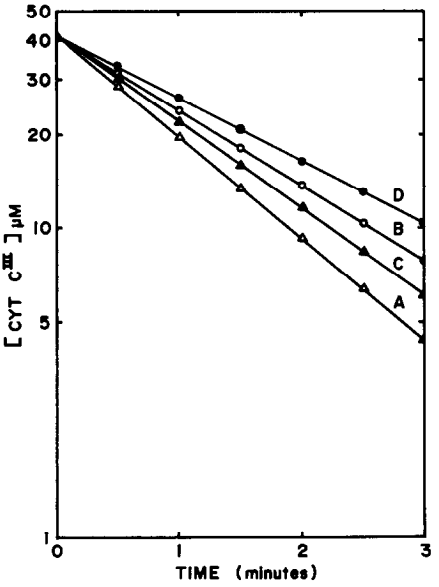

FIG. 2. Catalytic effect of Cu^{2+} on the reduction of cytochrome *c* by thioglycolate in the absence (A,B) and presence (C,D) of oxygen. Cytochrome *c*, 40 μM; thioglycolate, 140 μM; $CuSO_4$, 1 μM; buffer, 0.02 *M* Tris–sulfate at pH 7.0 (A,C) and pH 8.0 (B,D).

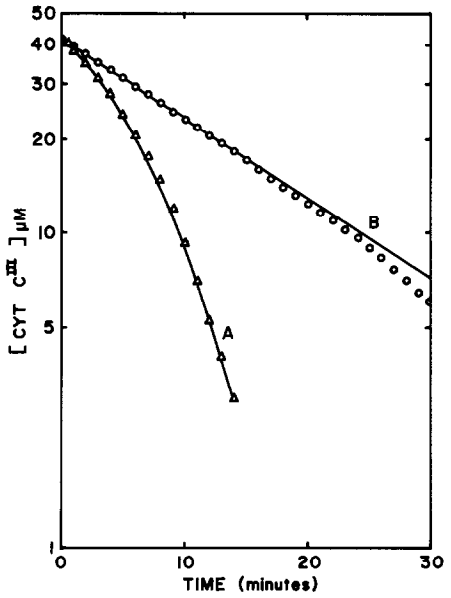

FIG. 3. Catalytic effect of Fe^{2+} on the reduction of cytochrome *c* by thioglycolate. Identical curves were obtained in the presence and absence of oxygen. Cytochrome *c*, 40 μM; thioglycolate, 1400 μM; $Fe_2(NH_4)_2\ SO_4$, 8 μM; buffer, 0.05 *M* Tris–sulfate at pH 7.0 (A) and pH 8.0 (B).

autocatalytic character of the reactions performed in neutral or acid pH results in an overall faster reduction of cytochrome *c* with decreasing pH. The presence of oxygen has no effect on the rate or the kinetic behavior of the iron-mediated reduction of cytochrome *c*, at least at the low concentrations of iron tested ($<8\ \mu M$).

Figure 4 shows the effect of oxidized glutathione on the reduction of cytochrome *c* by thioglycolic acid. In the presence of oxygen this reaction is biphasic. Analysis of the biphasic kinetics reveals the existence of a fast but short-lived rate superimposed on a slower first-order rate (Fig. 4B). The extent of the fast reaction depends on the pH and the concentration of the oxidized glutathione (Table 1) and becomes unnoticeable near neutral pH even at extremely high concentrations of the catalyst. The removal of the oxygen from the reaction mixtures results in increased rates at all pH values tested and abolishes the biphasic character of the reaction observed at alkaline pH values. It is interesting to note that the rate of the anaerobic system is equal to the rate of the fast reaction observed under aerobic conditions.

The effect of oxygen, buffer concentration (ionic strength), and pH on the rates of reduction of cytochrome *c* under various types of catalysis is summarized in Table 2. Rates are expressed as first-order rate constants and are calculated from the half-lives of linear first-order plots. In case of biphasic kinetics, such as the ones shown for the aerobic reduction of cytochrome *c* in the presence of oxidized glutathione, the initial fast reaction was ignored; and rates were calculated for the predominant slow first-order reaction. For the iron-induced autocatalytic patterns

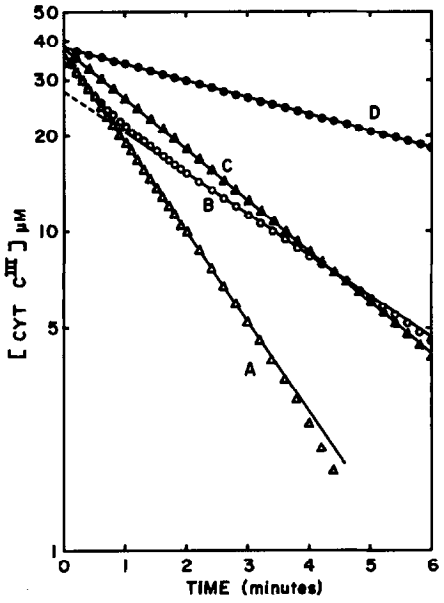

FIG. 4. Catalytic effect of oxidized glutathione on the reduction of cytochrome *c* by thioglycolate in the absence (A,C) and presence (B,D) of oxygen. Cytochrome *c*, 40 μM; thioglycolate, 1400 μM; GSSG, 2 mM; buffer, 0.02 M Tris–sulfate at pH 7.0 (C,D) and pH 8.0 (A,B).

the initial rates were measured and expressed as first-order rate constants assuming an initial first-order reaction.

Comparison of the first-order rate constants reveals that in the absence of any catalyst the reduction of cytochrome *c* by thioglycolic acid is not significant. This, along with the absence of a considerable pH effect, suggests that the thiolate ion has little or no capacity to reduce the heme iron directly. Copper and iron accelerate the reaction, but copper is by far a superior catalyst as compared to iron. The presence of oxygen and pH have little effect on the rates of the reactions

TABLE 1

EFFECT OF OXIDIZED GLUTATHIONE ON THE RATE AND KINETIC PATTERNS OF THE REDUCTION OF CYTOCHROME *c* BY THIOGLYCOLIC ACID

	pH 7.0		pH 8.0	
GSSG (mM)	Rate of reduction ($k_1 \times 10^6$ s^{-1})	Slow reaction (%)	Rate of reduction ($k_1 \times 10^6$ s^{-1})	Slow reaction (%)
0.0	144	100	183	100
0.5	321	100	412	98
1.0	550	99	962	85
2.0	1450	96	3850	82
4.0	5775	95	21000	80

TABLE 2

EFFECT OF OXYGEN, pH, AND BUFFER CONCENTRATION ON THE RATE OF THE REDUCTION OF CYTOCHROME *c* BY THIOGLYCOLIC ACID UNDER VARIOUS TYPES OF CATALYSIS

Catalyst	Buffer concentration (*M*)	pH	Rate ($+O_2$) ($k_1 \times 10^6$ s^{-1})	Rate ($-O_2$) ($k_1 \times 10^6$ s^{-1})
—	0.05	8.0	245 ± 90	284 ± 115
—	0.05	7.0	270 ± 92	290 ± 116
—	0.02	8.0	270 ± 99	307 ± 122
—	0.02	7.0	290 ± 91	315 ± 126
Cu (1 μM)	0.05	8.0	5,572 ± 260	5,590 ± 700
	0.05	7.0	6,409 ± 280	7,651 ± 1120
	0.02	8.0	9,095 ± 210	8,300 ± 810
	0.02	7.0	10,567 ± 250	12,842 ± 1220
Fe (8 μM)	0.05	8.0	988 ± 91	1,006 ± 120
	0.05	7.0	1,291 ± 110	1,503 ± 160
	0.02	8.0	1,950 ± 150	1,956 ± 185
	0.02	7.0	2,267 ± 180	2,445 ± 260
GSSG (2 mM)	0.05	8.0	4,339 ± 220	14,996 ± 1560
	0.05	7.0	1,969 ± 130	6,874 ± 765
	0.02	8.0	4,844 ± 250	17,077 ± 1610
	0.02	7.0	2,243 ± 145	7,652 ± 905

catalyzed by these two metals, and therefore these two factors have little effect on the concentration and nature of the reducing species during the reaction intervals. Increasing ionic strength has a negative effect on the rates of the metal-catalyzed reactions, which indicates that charged metal complexes but not the free metal ions participate in the reduction of the heme iron.

The reduction of cytochrome *c* in the presence of oxidized glutathione appears to be very sensitive to the presence of oxygen and to pH changes but is unaffected

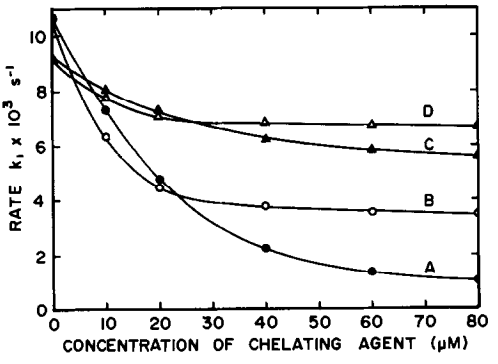

FIG. 5. Inhibition of copper-catalyzed reduction of cytochrome *c* by EDTA (A,C) and *o*-phenanthroline (B,D). Cytochrome *c*, 40 μM; thioglycolate, 1400 μM; $CuSO_4$, 1 μM; buffer, 0.02 *M* Tris–sulfate at pH 7.0 (A,B) and pH 8.0 (C,D).

TABLE 3

EFFECT OF CHELATING AGENTS ON THE RATE OF THE REDUCTION OF CYTOCHROME *c* BY THIOGLYCOLIC ACID

Catalyst	Chelating agent	Buffer concentration (*M*)	pH	Rate (O_2) ($k_1 \times 10^6$ s^{-1})
—	*o*-phenanthroline (20 μM)	0.05	8.0	145 ± 56
—		0.05	7.0	140 ± 56
—		0.02	8.0	170 ± 61
—		0.02	7.0	160 ± 67
—	EDTA (20 μM)	0.05	8.0	40 ± 18
—		0.05	7.0	48 ± 19
—		0.02	8.0	55 ± 21
—		0.02	7.0	61 ± 25
GSSG (2 m*M*)	*o*-phenanthroline (20 μM)	0.05	8.0	4508 ± 220
		0.05	7.0	2050 ± 150
		0.02	8.0	4812 ± 210
		0.02	7.0	1770 ± 130
GSSG (2 m*M*)	EDTA (20 μM)	0.05	8.0	4600 ± 230
		0.05	7.0	2100 ± 195
		0.02	8.0	4727 ± 220
		0.02	7.0	1910 ± 120

by changes in the ionic strength of the reaction medium. In this respect catalysis by oxidized glutathione is distinct from metal catalysis.

Chelating agents such as EDTA and *ortho*-phenanthroline inhibit the uncatalyzed and the metal-mediated reduction of cytochrome *c* by thioglycolic acid at concentrations as low as 20 μM. In the case of the iron-mediated reaction the inhibition by these agents is complete, and the rates are suppressed even below the levels of the uncatalyzed reactions. Unlike iron, the copper-mediated reduction of cytochrome *c* resists inhibition by chelating agents especially at alkaline pH values (Fig. 5). Rates decline 60 and 30% at pH 7 and 8, respectively, with the addition of 20 μM of the chelating agent, but further additions have little effect on the rates. In general EDTA is a better inhibitor than *ortho*-phenanthroline. Because most of the inhibition is observed at concentrations of the chelating agent well below the levels of cytochrome *c*, it is evident that the inhibition results from an interaction of the chelating agent with the metal ions rather than with the cytochrome *c*.

Chelating agents have no effect on the reduction of cytochrome *c* by thioglycolic acid in the presence of oxidized glutathione under all conditions tested (Table 3), suggesting that the involvement of metals in this type of catalysis may be negligible. However, further investigation is required to rule out the involvement of a metal complex stronger than the respective complexes of EDTA and *ortho*-phenanthroline.

DISCUSSION

The low rates of reduction of cytochrome *c* by thioglycolic acid under conditions of minimum metal contamination indicate that this thiol has little or no capacity to reduce the heme iron of cytochrome *c* directly. The same conclusion is derived for the thiolate ion of thioglycolic acid, since changes of the pH and therefore of the concentration of this ion do not significantly affect the rate of the reaction.

Transition metals and also nonmetal contaminants present in commercial samples of oxidized glutathione (*15*) catalyze the reduction of cytochrome *c* by thioglycolic acid or by other sulfhydryl reagents (*16*). Metal and nonmetal catalysis were tested separately, and it was found that these two types of catalysis show different responses to pH, ionic strength, presence of oxygen or chelating agents. Metal catalysis, in general, is sensitive to ionic strength, but pH or the presence of oxygen has little effect on the rates of such reactions. In contrast, the presence of oxidized glutathione induces a reaction sensitive to pH and oxygen but independent of ionic strength. In all these aspects the cytochrome *c*–thioglycolic acid system resembles the glutathione–cytochrome *c* system described by Froede and Hunter (*16*).

In most reactions tested the rate of the reduction of the cytochrome *c* is first order with respect to the concentration of the oxidized form of the cytochrome. First-order kinetics indicate that the only variable in the rate law of the reaction

$$\text{rate} = k_{\text{obs}} [\text{cyt } c^{\text{III}}]$$

is the concentration of cytochrome *c*, while the concentration of the reductant is maintained at constant steady state levels throughout the reaction.[4] Deviations from the first-order kinetics observed in the cases of iron and oxidized glutathione-mediated reactions under acid and alkaline conditions, respectively, are rather complicated cases and cannot be discussed in the absence of additional information concerning the nature of the reducing species.

The addition of transition metals to solutions of sulfhydryl compounds results in the formation of complexes the composition and coordination of which depend largely on the metal itself and the relative concentration of the sulfhydryl compound (*17–20*). Copper and iron in the presence of excess sulfhydryl compounds form cupric and ferric complexes, respectively, in which one atom of metal is coordinated to two molecules of the sulfhydryl compound (*21–24*). These complexes undergo a redox cycle during which the thiol is oxidized to the respective disulfide while residual oxygen is consumed. In the case of the iron thioglycolate system the redox cycle has been studied in detail, and it has been demonstrated that the rate-determining step is the bleaching of the ferrithioglycolate complex (*25, 26*). This reaction yields ferrothioglycolate complexes which are labile to oxygen, and therefore their steady state concentration is negligible under aerobic conditions. The same situation is expected to be true in the case of the copper thioglycolate system, although supporting experimental evidence exists only for

[4] K_{obs} is a function of the concentration of the reducing species.

the cupric cysteinate complex (*23*). At metal concentrations as low as the ones employed for the catalysis of the reduction of cytochrome *c* by thioglycolic acid the reduction of the ferric or cupric thioglycolate complexes to the respective ferro and cuprous form and the consequent oxygen uptake are too slow to interfere with the reduction of cytochrome *c*. The same conclusion is derived from the insensitivity of the metal-catalyzed reactions to oxygen. A possible exception to this view could arise in the iron-catalyzed reduction of cytochrome *c* under acid conditions where the reduction of ferric complexes is accelerated (*26*). Such a possibility will be explored in a later communication.

Addition of EDTA or *ortho*-phenanthroline to the iron-mediated reaction completely reverses the effect of this metal. Since the inhibitory effect of the chelating agents is observed at concentrations much lower than those of cytochrome *c*, it becomes obvious that the chelating agent inhibits because it complexes with the metal and restricts the formation of the iron thioglycolate complexes. *ortho*-Phenanthroline forms very stable complexes with ferrous iron, having a pK of 21.4 (*27*). However, these complexes cannot reduce cytochrome *c* due to their high reduction potentials ($E_0 = +1.14$). On the other hand, EDTA forms stable complexes with ferric iron with a pK of 23.7 (*28*), but its reduction potential ($E_0 = 0.120$) does not permit a favorable interaction with thioglycolic acid ($E_0 = -0.14$). Hence, the iron complexes of EDTA and *ortho*-phenanthroline fail to act as electron bridges between the cytochrome *c* and the thioglycolic acid in spite of the fact that interactions between the active site of cytochrome *c* with the ferrous forms of these complexes is considered to be favorable (*29*).

More complicated patterns of inhibition by chelating agents are observed with the copper-mediated reduction of cytochrome *c*. Reduction of cytochrome *c* by cupric thioglycolate complexes is inhibited by chelating agents, but the inability of these agents to completely suppress the reaction indicates that species capable of reducing the heme iron are continuously generated in such a system. Cupric copper forms stable complexes with *ortho*-phenanthroline ($pK_2 = 15.82$), but the unsaturated nature of this ligand stabilizes the reduced form of the metal (*27*). Stabilization of the cuprous state and a possible increase of the rate of reduction of the cupric complexes by thioglycolic acid is supported by the fact that *ortho*-phenanthroline activates copper-mediated autoxidation of various sulfhydryl compounds (*30*). Cuprous complexes with *ortho*-phenanthroline (*31*) or thioglycolic acid, similar to those reported with cysteine (*18*), or even mixed complexes of these two reagents could participate in the reduction of cytochrome *c* and explain the failure of *ortho*-phenanthroline to completely inhibit the reduction of cytochrome *c*. The pH effect induced in the presence of chelating agents and the interference of oxygen in these reactions (unpublished observations) also support a possible involvement of cuprous copper (*18, 30*).

The effect of oxidized glutathione on the reduction of cytochrome *c* by sulfhydryl compounds has been described in the past (*16*). Oxidized glutathione itself has no effect as a catalyst (*15*), but products of the thermal decomposition of this reagent (*9*), probably trisulfides or sulfur in the zero valence state, can accelerate the reduction of cytochrome *c* even in the absence of metal catalysts. Samples of oxidized glutathione tested in this study have no capacity to reduce cytochrome *c*

by themselves, and the presence of thioglycolic acid is absolutely necessary for the reaction to occur. This behavior and the fact that the thioglycolic acid has little capacity to reduce cytochrome *c* suggest that a reaction between the thiol and the trisulfide could result in the formation of a steady state concentration of some powerful reducing species. Massey and collaborators (*15*) proposed the following scheme to explain the catalytic effect of the trisulfides:

$$RSSSR + RS^- \rightarrow RSS^- + RSSR \quad [1]$$

$$RSS^- + \text{Cyt } c^{III} \rightarrow RSS\cdot + \text{Cyt } c^{II} \quad [2]$$

$$2\,RSS\cdot \rightarrow RSSSSR \quad [3]$$

$$RSSSSR + RS^- \rightarrow RSSSR + RSS^- \quad [4]$$

The above mechanism explains the first-order kinetic patterns if reaction [2] is assumed to be the rate-limiting step. It also explains the pH effect if only the ionized thiol could activate the trisulfide (reaction [1]), but it fails to account for the oxygen effect and for the presence of biphasic kinetics observed under aerobic conditions and alkaline pH.

Oxidized glutathione at concentrations used to catalyze the reduction of the cytochrome has only a minimum effect on the rate of autoxidation of thioglycolic acid in the presence or absence of cytochrome *c*, thus indicating that the RSS^- species formed during the interaction of the trisulfide with the thiolate ion cannot be very sensitive to oxygen. Sensitivity to oxygen must be assigned to another intermediate of the catalytic cycle. A good possibility is the persulfide free radical ($RSS\cdot$), and the following scheme is proposed to explain the inhibition by oxygen:

$$RSSSR + RS^- \rightleftarrows RSS^- + RSSR \quad [5]$$

$$RSS^- + \text{Cyt } c^{III} \rightarrow RSS\cdot + \text{Cyt } c^{III} \quad [6]$$

$$RSS\cdot + RS^- \leftrightarrows RS\dot{S}SR \quad [7]$$

$$RS\dot{S}SR + \text{Cyt } c^{III} \rightarrow RSSSR + \text{Cyt } c^{II} \quad [8]$$

$$RSS\cdot + O_2 \leftrightarrows RSSO_2, \quad [9]$$

In this mechanism the persulfide free radical reacts with a thiolate ion to form a trisulfide with an extra electron. This radical reduces another molecule of cytochrome *c* and thus regenerates the catalyst. Reaction [9] requires that in the presence of oxygen the persulfide radical (or the trisulfide radical) reversibly reacts with oxygen to form a more stable oxygenated radical. Presumably the latter radical is unable to reduce cytochrome *c*. Reaction [9] would thus in effect lower the concentration of the active catalyst, which would explain the inhibition by oxygen.

The fact that the reduction of hemin by thioglycolic acid, as studied by Bäuerlein and his colleagues (*1–3*), actually may proceed via a metal–thiol complex suggests that this reaction may have been a better model of the interaction between a cytochrome and a nonheme iron protein than the authors originally anticipated. It also suggests that the phosphorylation that may occur during this

oxidation–reduction reaction (*1–3*) could be a model of the chemical mechanisms taking place during oxidative phosphorylation. Alternatively, the mechanism proposed in Eqs. [5]–[9] could also be a model of the mechanism involved in the passage of electrons into this or other cytochromes during mitochondrial electron transport. Further studies will have to reveal the merit of these models.

REFERENCES

1. E. BÄUERLEIN, M. KLINGENFUSZ, AND TH. WIELAND, *Eur. J. Biochem.* **24,** 308–312 (1971).
2. E. BÄUERLEIN, *Hoppe-Seyler's Z. Physiol. Chem.* **354,** 827–828 (1973).
3. E. BÄUERLEIN, "Glutathione" (L. Flohe, H. C. Benöhr, H. Sies, H. D. Waller, and A. Wendel, Eds.), pp. 44–55. Georg Thieme, Stuttgart, 1974.
4. A. A. PAINTER AND F. E. HUNTER, *Biochem. Biophys. Res. Commun.* **40,** 360–368, 369–377, 378–386 and 387–395 (1970).
5. A. A. PAINTER AND F. E. HUNTER, JR., *Science* **170,** 552–553 (1970).
6. T. OHNISHI AND M. PRING, "Dynamics of Energy-Transducing Membranes" (L. Ernster, R. W. Estabrook, and E. C. Slater, Eds.), pp. 169–180. Elsevier, Amsterdam, 1974.
7. T. OHNISHI, *Eur. J. Biochem.* **64,** 91–103 (1976).
8. H. BEINERT, "Iron-Sulfur Proteins" (W. Lovenberg, Ed.), Vol. III, pp. 61–100. Academic Press, New York, 1977.
9. C. L. GINSBURGH AND J. EVERSE, *Bioorg. Chem.* **7,** 481–492 (1978).
10. N. KUJUNDZIC AND J. EVERSE, *Biochem. Biophys. Res. Commun.* **82,** 1211–1216 (1978).
11. J. EVERSE AND N. KUJUNDZIC, *Biochemistry* **12,** 2668–2673 (1979).
12. E. MARGOLIASH AND N. FROHWIRT, *Biochem. J.* **71,** 570–572 (1959).
13. D. R. GRASSETTI AND J. F. MURRAY, *Arch. Biochem. Biophys.* **119,** 41–49 (1967).
14. A. LAZAROW AND S. J. COOPERSTEIN, *Science* **120,** 674–675 (1954).
15. V. MASSEY, C. H. WILLIAMS, JR., AND G. PALMER, *Biochem. Biophys. Res. Commun.* **42,** 730–738 (1971).
16. H. C. FROEDE AND F. E. HUNTER, JR., *Biochem. Biophys. Res. Commun.* **38,** 954–961 (1970).
17. L. MICHAELIS AND M. P. SCHUBERT, *J. Amer. Chem. Soc.* **52,** 4418–4426 (1930).
18. W. STRICKS AND I. M. KOLTHOFF, *J. Amer. Chem. Soc.* **73,** 1723–1727 (1951).
19. I. M. KLOTZ, G. H. CZERLINSKI, AND H. A. FIESS, *J. Amer. Chem. Soc.* **80,** 2920–2923 (1958).
20. M. P. SCHUBERT, *J. Amer. Chem. Soc.* **54,** 4077–4085 (1932).
21. N. TANAKA, I. M. KOLTHOFF, AND W. STRICKS, *J. Amer. Chem. Soc.* **77,** 1996–2004 (1955).
22. Q. FERNANDO AND H. FREISER, *J. Amer. Chem. Soc.* **80,** 4928–4931 (1958).
23. D. CAVALLINI, D. DEMARCO, S. DUPRE, AND G. ROTILIO, *Arch. Biochem. Biophys.* **130,** 354–361.
24. D. L. LEUSSING AND I. M. KOLTHOFF *J. Amer. Chem. Soc.* **75,** 3904–3911 (1953).
25. D. L. LEUSSING AND L. NEWMAN, *J. Amer. Chem. Soc.* **78,** 552–555 (1956).
26. H. LAMFROM AND S. O. NIELSEN, *J. Amer. Chem. Soc.* **79,** 1966–1970 (1957).
27. C. V. BANKS AND R. I. BYSTROFF, *J. Amer. Chem. Soc.* **81,** 6153–6158 (1959).
28. S. S. JONES AND F. A. LONG, *J. Phys. Chem.* **56,** 25–33 (1952).
29. S. WHERLAND AND H. B. GRAY, *Proc. Nat. Acad. Sci.* **73,** 2950–2954 (1976).
30. K. KOBASHI, *Biochim. Biophys. Acta* **158,** 239–245 (1968).
31. B. R. JAMES AND R. J. P. WILLIAMS, *J. Chem. Soc.* **1961,** 2007–2013 (1961).