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SULFANE-ACTIVATED REDUCTION OF CYTOCHROME *c* BY GLUTATHIONE

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The inorganic sulfane tetrathionate ($^{-}O_3SSSSO_3^{-}$) resembles glutathione trisulfide (GSSSG) in that it remarkably activates the reduction of cytochrome *c* by GSH, both under aerobic and anaerobic conditions. These observations can be explained by the formation of the persulfide GSS^{-}, due to nucleophilic displacements of sulfane sulfur. The GSS^{-} species has previously been proposed to act as a chain carrier in the catalytic reduction of cytochrome *c*, and perthiyl radicals GSS·, formed in the reduction step, were thought to recycle to sulfane *via* dimerization to GSSSSG.[2] The present study provides some arguments in favour of a chain mechanism involving the $GSS\cdot + GS^{-} \rightleftarrows (GSSSG)\cdot^{-}$ equilibrium and sulfane regeneration by a second electron transfer from $(GSSSG)\cdot^{-}$ to cytochrome *c*.

Thiosulfate sulfurtransferase (rhodanese) is shown to act as a cytochrome *c* reductase in the presence of thiosulfate and GSH, and again the generation of GSS^{-} can be envisaged to explain this result.

KEY WORDS: Cytochrome *c* reduction, glutathione free radicals, perthiyl radicals, rhodanese, tetrathionate, thiosulfate sulfurtransferase.

INTRODUCTION

Sulfanes like GSSSG, GSS^{-}, $^{-}O_3SSSO_3^{-}$ and $^{-}SSO_3^{-}$ appear to be of general biochemical importance in microorganisms, plants and animals, as indicated by the wide distribution of sulfurtransferases responsible for sulfane formation and interconversion. The ubiquity of sulfane metabolism in liver mitochondria has led to the suggestion that the function of sulfanes may be related to the electron transport chain and refinement of oxidative phosphorylation.[1] Trisulfides are actually known to catalyse the reduction of cytochrome *c* by glutathione, and the chain mechanism previously proposed is depicted in Scheme 1 by the reactions (1) to (4).[2] Perthiyl radicals (GSS·), occurring in the reaction (2), were thought to recycle to GSSSG *via* dimerization, reactions (3) and (4).

Perthiyl radicals like the product of reaction (2) are prominent intermediates formed by ionizing radiation in aqueous disulfide and thiol systems.[3-5] Stabilization of polysulfide radicals *via* formation of a three-electron bond between the terminal sulfurs $(-S.\therefore S:)^{-}$ has been proposed already in 1955.[6] But only recently further attention has been focused on redox processes involving trisulfides and perthiyl radicals[7-9]. Reduction of cytochrome *c* by GSH was shown to be stimulated efficiently by disulfides, as well as by disulfide proteins and thiols, which had been exposed to γ-radiation in aqueous solution.[9] These observations were explained by radiolytic generation of stable RSSSR and RSSSSR species, acting

#This paper is dedicated to Prof. Dr. Hans Mönig at the occasion of his 65th anniversary.

SCHEME 1
Chain mechanism for the catalytic reduction of cytochrome *c* by glutathione trisulfide

$GSSSG + GS^- \rightleftarrows GSSG + GSS^-$	(1)	*Initiation*[a]
$GSS^- + Fe(III)Cyt \rightleftarrows GSS\cdot + Fe(II)Cyt$	(2)	
$GSS\cdot + GSS\cdot \rightarrow GSSSSG$	(3)	*Propagation*[a]
$GSSSSG + GS^- \rightleftarrows GSSSG + GSS^-$	(4)	
$GSS\cdot + GS^- \rightleftarrows (GSSSG)^{\cdot -}$	(5)	*Propagation*[b]
$(GSSSG)^{\cdot -} + Fe(III)Cyt \rightarrow GSSSG + Fe(II)Cyt$	(6)	
$GSS^- + GS^- \rightleftarrows GSSG + S^{2-}$	(7)	*Termination*[a]

[a] Proposed by Massey et al.[2].
[b] Proposed in this study.

as proposed by Massey *et al.*[2] (Scheme 1). The radiolytic stimulation of reductive catalytic activity of disulfides and thiols may be of fundamental importance in radiobiology, for instance in the "chemical repair" of free radical damage to biopolymers. It was also pointed out that the lasting stimulatory activity of irradiated disulfide-proteins on cytochrome *c* reduction by GSH might enable identification of protein-rich foodstuff treated by γ-radiation. A pulse radiolysis study[8] revealed that certain RSS· species can equilibrate according to reaction (5) in Scheme 1 to form trisulfide anion radicals, and that they are capable of oxidizing ascorbate (AH^-), and of interacting with oxygen to form inorganic sulfate as one final product:

$$RSS\cdot + AH^- \rightarrow RSSH + A^{\cdot -} \quad (8)$$

$$RSS\cdot + O_2 \rightarrow RSSOO\cdot \rightarrow \text{products } (SO_4^{2-} \text{ etc}) \quad (9)$$

In the case of penicillamine the rate constants $k_8 = 4.1 \times 10^6\ M^{-1}\ s^{-1}$ and $k_9 = 5.1 \times 10^6\ M^{-1}\ s^{-1}$ have been measured.[8]

The present results show that the inorganic sulfanes tetrathionate and thiosulfate are also capable of stimulating cytochrome *c* reduction by GSH. Furthermore we suggest an alternative pathway for the recycling of GSSSG, which is represented in Scheme 1 by the reactions (5) to (6).

MATERIALS AND METHODS

The following commercial products were applied as received: cytochrome *c* (Fe(III)Cyt, horse heart) and rhodanese (thiosulfate sulfurtransferase, EC 2.8.1.1, bovine liver) from Sigma Chemie; L-glutathione (GSH and GSSG) and Cu,Zn-superoxide dismutase (SOD, EC 1.15.1.1, bovine erythrocytes) from Serva Feinbiochemica; ethylenediamine tetraacetic acid disodium salt (EDTA) and tetrathionate ($Na_2O_6S_4 \cdot 2H_2O$) from Fluka; and thiosulfate ($Na_2O_3S_2 \cdot 5H_2O$) from Aldrich. Other chemicals such as phosphate buffers were of highest purity available.

Solutions were freshly prepared for each experiment using redistilled water, and anaerobic conditions were obtained by flushing of the components to be mixed for about 30 min with N_2. The three components were rapidly mixed in the optical cell (1 cm), in most cases in the order: [I] (cytochrome *c*) + [II] (GSH) + [III] (tetra-

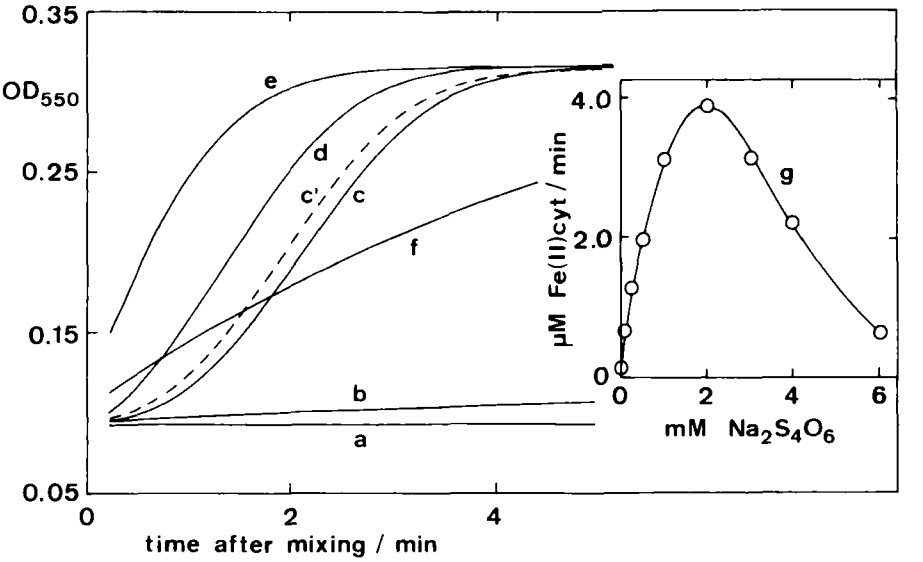

FIGURE 1 Time profiles and concentration dependence of tetrathionate-activated reduction of cytochrome *c* by GSH at 20°C. The results were obtained by rapid mixing of equal volumes of the following buffered (0.1 M phosphate, pH 6.8) components: [I] 36 μM Fe(III)Cyt. *c* + 12 mM EDTA + buffer, [II] 16 mM GSH + 12 mM EDTA + buffer, [III] 3 mM $Na_2S_4O_6$ (and other concentrations). Solutions were air-saturated, except the broken curve.
Identification: (a) control without GSH in [II]; (b) control without $Na_2S_4O_6$ in [III]; (c) [I] + [II] + [III]; (c') as c but anaerobic conditions; (d) [II] + [III] incubated 0.5 min before adding [I]; (e) [II] + [III] incubated 5 min before adding [I]; (f) [II] + [III] incubated 40 min before adding [I]; (g) cytochrome *c* reduction rate at 1.2 min after mixing as in (c) versus [$Na_2S_4O_6$] in the component [III].

thionate or thiosulfate ± rhodanese). Reduction of cytochrome *c* was monitored at 550 nm ($\Delta\lambda$ = 0.4 nm), using a Perkin Elmer spectrophotometer, and reduction rates were estimated assuming $\Delta\epsilon$ = 20,000 M^{-1} cm^{-1} (cf. ref. 9) for the difference in molar extinction coefficients between reduced and oxidized cytochrome *c*. The cell was thermostated, commonly at 20°C.

RESULTS

Time profiles for the reduction of cytochrome *c* by GSH and tetrathionate are shown in Figure 1. Tetrathionate alone (curve a) was completely inactive, and GSH alone (curve b) exhibited only a very low activity in the presence of EDTA, which was applied as previously[2,9,10] to suppress catalytic reactions of adventitious transition metal ions. Fast reduction of cytochrome *c* occurred in the presence of both GSH and tetrathionate (curve c), and in analogy to reaction (1) in Scheme 1 we assume that the system is activated by formation GSS^-, due to nucleophilic displacements of sulfane sulfur, followed by the reactions (2) to (7). A variety of such displacement reactions are possible,[11] as depicted by the following examples:

$$GS^- + {}^-O_3SSSSO_3^- \rightleftarrows GSSSO_3^- + {}^-SSO_3^- \quad (10)$$

$$GS^- + GSSSO_3^- \rightleftarrows GSSG + {}^-SSO_3^- \quad (10a)$$

$$GS^- + GSSSO_3^- \rightleftarrows GSS^- + GSSO_3^- \quad (10b)$$

Cytochrome *c* reduction was accomplished within few minutes, but with an initial lag phase (Figure 1c) which indicates that the above reactions generate GSS^- slowly and in low concentration. The system could in fact be pre-activated by incubating tetrathionate with GSH for a few minutes before adding cytochrome *c* (Figure 1d and e). Longer incubation led however to a loss of the tetrathionate activity (Figure 1f), consistent with the final depletion of GSS^- by the terminating reaction (7). The initial step in the time profile obtained after 5 min incubation (Figure 1e) indicates that the reaction (2) is fast; only a low steady-state concentration of GSS^- (< 10 μM) seems yet to be formed as the coupled equilibria (10) to (10b) and (7) proceed, in competition with other sulfur displacement reactions.

Cytochrome *c* reduction was inhibited at high tetrathionate concentrations (Figure 1g). The sulfur displacement reactions, with the examples (10) to (10b) given, imply that at least two GSH molecules are consumed by each tetrathionate molecule. Activation of the system apparently requires the condition $[GSH] \gg [S_4O_6^{2-}]$, and we have in fact recognized that the activity of tetrathionate fades almost completely at $[GSH]/[S_4O_6^{2-}] < 2$. A possible explanation for the activity loss under the latter conditions is that tetrathionate also removes GSS^-, like it removes GS^-.

Previous[9] and the present experiments (Figure 1c/c′) reveal that the sulfane-activated reduction of cytochrome *c* is hardly affected by oxygen. In view of reaction (9), the propagating reaction (3) was expected to be very sensitive to oxygen. The reaction (5),[8] which in our opinion could be involved in the propagation (Scheme 1), would certainly compete more favourably with reaction (9). Since $(GSSSG)^{\cdot -}$ presumably behaves like $(GSSG)^{\cdot -}$ as an electron donor[12] we propose that cytochrome *c* is reduced by the reaction (6). Interaction of oxygen with $(GSSSG)^{\cdot -}$ would yield $O_2^{\cdot -}$ radicals, which readily reduce cytochrome *c*[13] in a by-pass of reaction (6). It was not possible, however, to demonstrate that $O_2^{\cdot -}$ is involved in the aerobic system (Figure 1c) by applying SOD as scavenger, because SOD was found to activate cytochrome *c* reduction by GSH (in the absence of tetrathionate), even in anaerobic solution. This effect of SOD will be described elsewhere in more detail.

When changing from pH 6.8 to pH 7.4 phosphate buffer, other conditions as in Figure 1c, the reduction of cytochrome *c* was speeded up by a factor of about 2.6, consistent with previous results on reductive activation of GSH by organic sulfanes.[9] The system was also very temperature-sensitive; thus the reduction rate increased by a factor of about 3.3, when going from 20 to 38°C.

The data shown in Table 1 reveal that thiosulfate, i.e. the product of the reactions (10) and (10a), does not reduce cytochrome *c*, nor does it activate reduction of cytochrome *c* by GSH. However, when rhodanese was added a remarkable cytochrome *c* reductase activity of this enzyme was observed, in the presence of both GSH and thiosulfate.

TABLE I
Rhodanese activating the reduction of cytochrome *c* by GSH and thiosulfate

Additives: components [II] and [III][a]	Reduction Rate[b] (μM Fe(II)Cyt/min)	
	[III] without Rhodanese	[III] with Rhodanese
[II] 0 mM GSH [III] 3 mM $Na_2S_2O_3$	0	0
[II] 16 mM GSH [III] 0 mM $Na_2S_2O_3$	0.15	1.00
[II] 16 mM GSH [III] 3 mM $Na_2S_2O_3$	0.15	4.25

[a] Conditions as in Figure 1(a-c), except that $Na_2S_4O_6$ in the component [III] was replaced by $Na_2S_2O_3$ ± 38 U/ml rhodanese (~ 5 μM).
[b] Reduction rate 1 min after mixing the components [I], [II] and [III].

DISCUSSION

The above results provide arguments in favour of a chain mechanism involving the reactions (5) and (6) in the propagation step (Scheme 1). The reaction (8) actually implies that RSS· is a stronger oxidant than the ascorbate radical $A^{\cdot -}$. Since the cytochrome *c* reduction potential (E_7°(Fe(III)Cyt/Fe(II)Cyt) = 0.27 V) is of the same order as that of the ascorbate radical ($E_7^\circ(A^{\cdot -}, H^+/AH^-) = 0.30$ V),[14] we anticipate that the reverse of reaction (2) predominates, and that the concentration of GSS· is too low to enable a successful competition of reaction (3) with the alternative reaction (5) at high GSH concentrations (as applied in Figure 1). Reaction (5), on the other hand, pulls the equilibrium (2) to the right by transforming GSS· into $(GSSSG)^{\cdot -}$ radicals, and the latter species can be expected to behave like the disulfide radical anion[5,12] as a powerful reductant. The electron transfer reaction (6), now proposed to propagate the reaction chain, simultaneously regenerates GSSSG and causes reduction of two cytochrome *c* molecules per reaction cycle (Scheme 1). Also the inferior effect of oxygen, as discussed above, is more consistent with the propagating reaction sequence (5)-(6) than with the sequence (3)-(4).

Equilibration as in reaction (5) prevails in the case of penicillamine.[8] The situation appears to be more complex, however, for GSH since the glutathione trisulfide anion might preferably decay, as in the case of cysteine,[8] by the reaction (5a):

$$(GSSSG)^{\cdot -} \rightleftarrows GSS^- + GS\cdot \qquad (5a)$$

This reaction directly regenerates GSS^-, and feeds the well-known equilibrium (12), $K_{12} = 3.9 \times 10^3\ M^{-1}$:[15]

$$\begin{array}{ccc} GS\cdot + GS^- & \xrightleftharpoons{(12)} & (GSSG)^{\cdot -} \\ (11)\ \downarrow + \text{Fe(II)Cyt} & & (13)\ \downarrow + \text{Fe(III)Cyt} \\ GS^- + \text{Fe(III)Cyt} & & GSSG + \text{Fe(II)Cyt} \end{array}$$

As pointed out previously,[5,12,16] the equilibrium (12) presents an interesting chemical junction between electron acceptor (GS·) and electron donor ($(GSSG)^{\cdot -}$) function of this thiol-disulfide system (comparable to p-and n-type semiconductors).

By pulse and γ-radiolysis of solutions containing GSH and cytochrome *c* at pH 6.8 (Prütz, Butler and Land, unpublished results) we have shown that the oxidizing species GS· depletes almost quantitatively by reducing cytochrome *c* *via* the reaction pathway (12)–(13), with $k_{13} \approx 10^8\ M^{-1}\ s^{-1}$, even under conditions $[GSS^{\cdot -}]/[GS\cdot] = [GS^-] \times K_{12} \ll 0.1$. The reaction sequence (5a)–(12)–(13) eventually leads to the same result as the sequence (5)–(6)–(1). Reoxidation of cytochrome *c* by GS·, reaction (11),[17] may however be relevant after accumulation of [Fe(II)Cyt] > [Fe(III)Cyt].

It has been suggested that GSSSG-catalysed cytochrome *c* reduction might be involved in the process of oxidative phosphorylation.[2] In the present study we suggest that free radicals like $(GSSSG)^{\cdot -}$ and $(GSSG)^{\cdot -}$ are involved in the propagation step as $1e^-$-donors. The reduction potentials for the species occurring e.g. in the reaction (12), $E°(RS\cdot/RS^-) \sim 0.8\ V$ and $E°(RSSR/(RSSR)^{\cdot -}) = -1.6\ V$,[12] are certainly beyond the normal range of biochemical $1e^-$-redox reactions. Sulfanes seem however capable of activating the high potential $1e^-$-donor/acceptor equilibria (5), (5a) and (12), in the present system *via* reaction (2). This is in our opinion a possible function of sulfanes which may be of more general importance in biological systems.

The data given in Table 1 show that thiosulfate sulfurtransferase, more commonly known as rhodanese, exhibits a substantial cytochrome *c* reductase activity in the presence of thiosulfate and GSH. One of the functions ascribed to rhodanese is the protection of mitochondrial components from the toxic action of cyanide,[1] by catalysis of sulfur transfer from thiosulfate to cyanide. It is known, however, that rhodanese also can catalyse the reductive dismutation of thiosulfate by GSH to sulfite and sulfide.[1,18] Our results suggest that this reaction proceedes *via* formation of persulfide, i.e the active species in reaction (2),

$$S_2O_3^{2-} + GSH \xrightarrow{cat} SO_3^{2-} + GSS^- + H^- \qquad (14)$$

$$GSS^- + GSH \rightleftarrows GSSG + HS^- \qquad (7)$$

It is not known whether rhodanese is involved in mitochondrial electron transport, but in view of the present results we think that attention should be paid to this possibility.

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References

1. R.J. Huxtable (1986) Sulfane metabolism. In *Biochemistry of Sulfur*, Plenum Press, New York, pp. 124–129.
2. V. Massey, C.H. Williams and G. Palmer (1971) The presence of S°-containing impurities in commercial samples of oxidized glutathione and their catalytic effect on the reduction of cytochrome *c*. *Biochemical and Biophysical Research Communications*, **42**, 730–738.
3. J.W. Purdie, H.A. Gillis and N.V. Klassen (1973) The pulse radiolysis of penicillamine and penicillamine disulfide in aqueous solution. *Canadian Journal of Chemistry*, **51**, 3132–3142.
4. A.J. Elliot, R.J. McEachern and D.A. Armstrong (1981) Oxidation of amino-containing disulfides by $Br_2^{\cdot -}$ and ·OH. A pulse radiolysis study. *Journal of Physical Chemistry*, **85**, 68–75.

5. K.-D. Asmus (1983) Sulfur-centered free radicals. In *Radioprotectors and Anticarcinogens* (eds. O.F. Nygaard and M.G. Simic), Academic Press, New York, pp. 23–42.
6. F. Fairbrother, G. Gee, and G.T. Merrall (1955) The polymerization of sulfur. *Journal of Polymer Sciences*, **16**, 459–469.
7. Z. Wu, T.G. Back, R. Ahmad, R. Yamdagni and D.A. Armstrong (1982) Mechanism of reduction of bis(2-hydroxyethyl) trisulfide by e_{aq}^- and $\cdot CO_2^-$. *Journal of Physical Chemistry*, **86**, 4417–4422.
8. S.A. Everett, C. Schöneich, J.H. Stewart and K.-D. Asmus (1992) Perthiyl radicals, trisulfide radical ions, and sulfate formation. A combined photolysis and radiolysis study on redox processes with organic di-and trisulfides. *Journal of Physical Chemistry*, **96**, 306–314.
9. W.A. Prütz (1992) Catalytic reduction of Fe(III)-cytochrome *c* involving stable radiolysis products derived from disulphides, proteins and thiols. *International Journal of Radiation Biology*, **61**, 593–602.
10. H.C. Froede and F.E. Hunter (1970) Catalytic effect of GSSG on reduction of cytochrome *c* by GSH – possible model for facilitation of electron transfer and energy conservation by sulfonium ion formation. *Biochemical and Biophysical Research Communications*, **38**, 954–961.
11. A.J. Parker and N. Kharasch (1959) The scission of the sulfur-sulfur bond. *Chemical Reviews*, **59**, 583–628.
12. D.A. Armstrong (1990) Application of pulse radiolysis for the study of short-lived sulphur species. In *Sulfur-Centered Reactive Intermediates in Chemistry and Biology, NATO ASI Series Vol. 197 A* (eds. C. Chatgilialoglu and K.-D. Asmus), Plenum Press, New York and London, pp. 121–134 and 341–351.
13. W.H. Koppenol and J. Butler (1984) The radiation chemistry of cytochrome *c*. *Israel Journal of Chemistry*, **24**, 11–16.
14. T. Iyanagi, I. Yamazaki and K.F. Anan (1985) One-electron oxidation-reduction properties of ascorbic acid. *Biochimica Biophysica Acta*, **806**, 255–261.
15. M.Z. Hoffman and E. Hayon (1973) Pulse radiolysis study of sulfhydryl compounds in aqueous solution. *Journal of Physical Chemistry*, **77**, 990–996.
16. W.A. Prütz, J. Butler, E.J. Land and A.J. Swallow (1986) Unpaired electron migration between aromatic and sulfur peptide units. *Free Radical Research Communications*, **2**, 69–75.
17. L.G. Forni and R.L. Willson (1986) Thiyl free radicals and the oxidation of ferrocytochrome *c*. *Biochemical Journal*, **240**, 905–907.
18. A.B. Roy and P.A. Trudinger (1970) Rhodanese. In *The Biochemistry of Inorganic Compounds of Sulphur*, The University Press, Cambridge, pp. 190–197.

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