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The glutathione free radical equilibrium, $GS^{\cdot} + GS^{-} \rightleftharpoons GSS^{\cdot -}G$, mediating electron transfer to Fe(III)-cytochrome *c*

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Abstract

The $GS^{\cdot} + GS^{-} \rightleftharpoons GSS^{\cdot -}G$ equilibrium (1) was reinvestigated as a function of pH and ionic strength, using pulse radiolysis to oxidize GSH to $GS^{\cdot}$. All radicals formed by water radiolysis can be converted to populate equilibrium (1), which presents an interesting chemical junction between an electron acceptor ($GS^{\cdot}$) and an electron donor ($GSS^{\cdot -}G$). The secondary decay of the $GS^{\cdot}/GSS^{\cdot -}G$ couple into the reducing carbon-centred radical $G(C_{\alpha}^{\cdot})SH$, as observed in alkaline solution [10] (Grierson et al., *Int. J. Radiat. Biol.* 62 (1992) 265), seems to be of minor importance in neutral solution. Reduction of Fe(III)-cytochrome *c* at pH 6.8, after pulse radiolytic generation of the $GS^{\cdot}/GSS^{\cdot -}G$ couple in absence of oxygen, proceeds with half-lifes in the order of 100 to 200 μs. From the concentration dependent rate and efficiency of reduction it is concluded that $GSS^{\cdot -}G$ is the reducing entity, $k(GSS^{\cdot -}G + Fe(III)Cyt\ c) \approx 6 \times 10^{7}\ M^{-1}\ s^{-1}$. The efficiency of reduction reaches about 72% (of $GS^{\cdot}$) for γ-radiolysis of deaerated solutions containing GSH and Fe(III)-cytochrome *c* at pH 6.8, even though the reverse reaction (−1) is favoured at this pH (where only few % of $GS^{\cdot}$ equilibrate to $GSS^{\cdot -}G$). Reduction is less efficient under pulse radiolysis conditions due to competing radical–radical termination (e.g. $GS^{\cdot} + GS^{\cdot} \rightarrow GSSG$). In presence of oxygen the efficiency of reduction is even higher, 95% for γ-radiolysis, and the rate of reduction indicates that $O_2^{\cdot -}$ is the reductant. Reversible formation of thiyl peroxy radicals ($GS^{\cdot} + O_2 \rightleftharpoons GSOO^{\cdot}$) seems to be overruled, via equilibrium (1), by irreversible electron transfer from $GSS^{\cdot -}G$ to O_2, for which a rate constant of $5.1 \times 10^{8}\ M^{-1}\ s^{-1}$ was estimated. The kinetics of copper-catalyzed reduction of Fe(III)-cytochrome *c* by GSH were investigated by stopped-flow techniques. The results presented indicate that the Cu(I)-thiolate complex is the reducing entity. It is concluded that Cu(II) does not interact with GSH to form the unbound $GS^{\cdot}$ radical, and that reduction in this case is not mediated by equilibrium (1).

Keywords: Copper–GSH complex; Cytochrome *c*; Disulfide radical anions; Electron transfer; Glutathione free radicals; Pulse radiolysis; Thiyl free radicals

1. Introduction

The redox chemistry of thiols and disulfides is of interest because many biological phenomena

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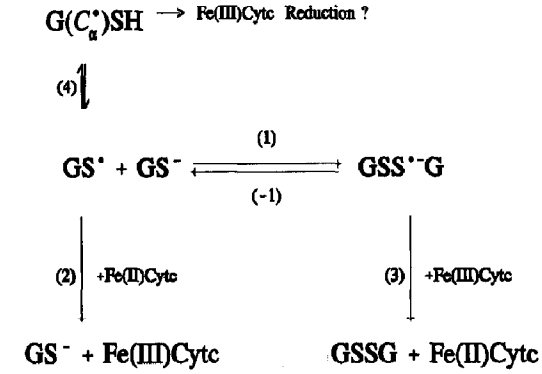


Scheme 1. Cytochrome *c* oxidation/reduction mediated by glutathione-derived free radical intermediates (GSH = γ-Glu–Cys–Gly).

such as radioprotection, transition metal activity, metabolism of xenobiotics and electron transport, may involve redox-active sulfur functions [1–4]. It is well-established that one-electron oxidation of the labile mercapto group leads to an equilibrium between free thiyl radicals and disulfide radical anions [4–8], as shown for glutathione in Scheme 1 (reaction (1)). Semi-oxidized thiol intermediates RS$^\bullet$ are strong oxidants with a reduction potential of the order of $E^\circ(RS^\bullet + H^+/RSH) = 1.3$ V (vs. NHE) [8,9]; the conjugated RSS$^{\bullet-}$R intermediates, on the other hand, are very powerful reductants with $E^\circ(RSSR/RSS^{\bullet-}R) = -1.6$ V, as compared to $E^\circ(RSS^{\bullet-}R/2RS^-) = 0.6$ V [8,9]. The equilibrium (1) therefore provides an interesting chemical junction between electron acceptor and donor function of semi-oxidized thiol. This is illustrated in Scheme 1 with cytochrome *c* as an one-electron donor/acceptor. Recently it was shown [10] that the thiyl radical derived from GSH also decays by an OH^--dependent intramolecular rearrangement to form a carbon-centred radical, G(C$_\alpha^\bullet$)SH at the glutamyl C$_\alpha$-position (see Scheme 1, reaction (4)). The G(C$_\alpha^\bullet$)SH radical was found to be a reducing species, as evidenced by reaction with 4-nitro-acetophenone (PNAP) to yield PNAP$^{\bullet-}$.

Fe(II)–cytochrome *c* is readily oxidized by GS$^\bullet$ (see Scheme 1) with a rate constant of $k_2 = 2.5 \times 10^8$ M^{-1} s^{-1}. However, the yield of reaction (2) decreases drastically above pH 6 [11], due to the competing reaction (1) coupled to equilibrium (5):

$$GSH \rightleftharpoons GS^- + H^+ \quad (pK_5 = 9.2) \qquad (5)$$

Reaction (3) has to our knowledge not yet been investigated, although it is thermodynamically feasible, considering the highly negative potential of the GSSG/GSS$^{\bullet-}$G couple in comparison to E°(Fe(III)Cyt*c*/Fe(II)Cyt*c*) = 0.27 V. Fe(III)–Cytochrome *c* is reduced by thiols generally [1], but the reduction is strongly inhibited by EDTA [12] since transition-metal impurities apparently catalyze the oxidation of thiols. It has been assumed [4] that Cu(II) can interact with thiols to generate RS$^\bullet$ species, thus Cu(II) might indeed be expected to initiate reduction, e.g. by the reaction pathway (1) → (3) in Scheme 1. Equilibrium (1), as a link between $1e^-$-oxidation and $1e^-$-reduction, could in our opinion be of general importance in a variety of biological redox processes. In the absence of substrates like cytochrome *c*, equilibrium (1) is rapidly established [6] and easily described as a function of pH:

$$\frac{[GSS^{\bullet-}G]}{[GSS^{\bullet-}G] + [GS^\bullet]} = \left(1 + \frac{1 + 10^{pK_5(I) - pH}}{K_1[GSH]_{tot}}\right)^{-1} \qquad (6)$$

The simplified Brønsted–Debye equation can be applied to allow for the effect of ionic strength (I) on the reverse reaction (5):

$$pK_5(I) = pK_5(0) - 1.02\sqrt{I}\,.$$

The GSS$^{\bullet-}$G intermediate is detectable by its strong absorption around 420 nm [5–7], after pulse radiolysis of GSH solutions. The equilibrium constant $K_1 = 3.9 \times 10^3$ M^{-1} has been estimated from the kinetics at pH 11.7, $k_1 = 6.2 \times 10^8$ M^{-1} s^{-1} and $k_{-1} = 1.6 \times 10^5$ s^{-1} [6]. We have now reinvestigated the equilibrium (1) as a function of pH and ionic strength, and have demonstrated that glutathione derived free radicals lead to fast and efficient reduction of Fe(III)–cytochrome *c* both under aerobic and anaerobic conditions. In the context we have also investigated the copper-catalyzed reduction of Fe(III)–cytochrome *c* by GSH.

2. Materials and methods

Reaction (1) can be initiated conveniently by radiolysis of aqueous solutions of GSH. Reactive water radiolysis products are formed at known yields, G-values (μM/Gy), and in homogeneous distribution within about 10^{-8} s after ionization [8,13,14]:

$$H_2O \xrightarrow{\text{radiation}} {}^{\cdot}OH(0.28) + {}^{\cdot}H(0.06) + e^-_{aq}(0.28) \quad (7)$$

Solutions were deaerated by gentle bubbling (> 30 min) with N_2 or N_2O. In both environments $GS^{\cdot}$ is generated at a yield of up to 0.62 μM/Gy [7,13], see also Section 3.1, via reactions (7) to (12):

$${}^{\cdot}OH + GSH \longrightarrow H_2O + GS^{\cdot} \quad \text{(and other products)} \quad (8)$$

$$e^-_{aq} + GSH \longrightarrow SH^- + G^{\cdot} \quad \text{and} \quad GS^- + H^{\cdot} \quad (9)$$

$$H^{\cdot} + GSH \longrightarrow H_2 + GS^{\cdot} \quad \text{and} \quad H_2S + G^{\cdot} \quad (10)$$

$$G^{\cdot} + GSH \longrightarrow GH + GS^{\cdot} \quad (11)$$

$$e^-_{aq} + N_2O + H^+ \longrightarrow N_2 + {}^{\cdot}OH \quad (12)$$

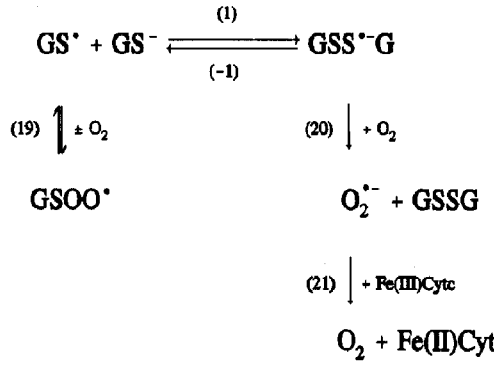
In N_2O-saturated solution (23 mM N_2O) the reaction sequence (9) to (11) is replaced by the reactions (12) and (8). Reactions were also initiated by $^{\cdot}CO_2^-$ radicals, generated at a yield of 0.62 μM/Gy by radiolysis of N_2O-saturated solutions of $HCOO^-$ [13,15]:

$${}^{\cdot}OH, \; H^{\cdot} + HCOO^- \longrightarrow H_2O, \; H_2 + {}^{\cdot}CO_2^- \quad (13)$$

$${}^{\cdot}CO_2^- + GSH \longrightarrow HCOO^- + GS^{\cdot} \quad (14)$$

or by $Br_2^{\cdot -}$, the product of $^{\cdot}OH$-induced oxidation of Br^-. In some experiments GSH solutions were saturated with N_2O/O_2 mixtures at molar ratios of 4:1 or 20:1. Under these conditions reaction (12) still applies but $GSS^{\cdot -}G$ and $H^{\cdot}$ react with O_2 to form $O_2^{\cdot -}$, and $GS^{\cdot}$ equilibrates with O_2 to form $GSOO^{\cdot}$ [13], see Scheme 2.

The pulse radiolysis facility of the Paterson Institute for Cancer Research [16] was used with optical detection of the transients; absorbed doses (Gy) were determined by SCN^--dosimetry as previously [7]. γ-Radiolysis experiments were performed with a Co-60 source (Gammacell 220; Atomic Energy of Canada Ltd.) at a dose rate of 11 Gy/min and dose control by Fricke-dosimetry [17]. Irradiation was in both cases at ambient temperature (20 to 22°C). The stopped-flow measurements (Section 3.4) used a SFA-12 "Rapid Kinetics Stopped-Flow Accessory" (Hi-Tech Scientific Ltd.) coupled with a UV–Vis spectrophotometer (Shimadzu Corporation).



Scheme 2. Fe(III)–cytochrome c reduction mediated by glutathione-derived free radical intermediates in the presence of oxygen.

Horse heart Fe(III)–cytochrome *c* (Fe(III) Cytc) from Sigma (Type C-7752), L-glutathione (GSH and GSSG) and β-nicotinamide adenosine dinucleotide disodium-salt $3H_2O$ (NADH) from Serva, and ethylenediaminetetraacetic acid disodium-salt (EDTA) from Fluka were used without further purification. EDTA was added at a ratio of [EDTA]/[GSH] < 1 to suppress catalytic oxidation of GSH by adventitious transition-metals (see Section 1); higher EDTA concentrations did not further reduce metal catalysis but would have led to interference with reaction (8), as can be seen from published reaction rate constants [14]. The yield of Fe(III)–cytochrome *c* reduction was determined from the change in optical absorption at 550 nm, by comparison with $^{\cdot}CO_2^-$ or $O_2^{\cdot -}$-induced reduction [18] at a yield of 0.62 μM/Gy, by irradiating Fe(III)–cytochrome *c* solutions containing HCOONa (0.1 M); in the N_2O-saturated system all water radicals are

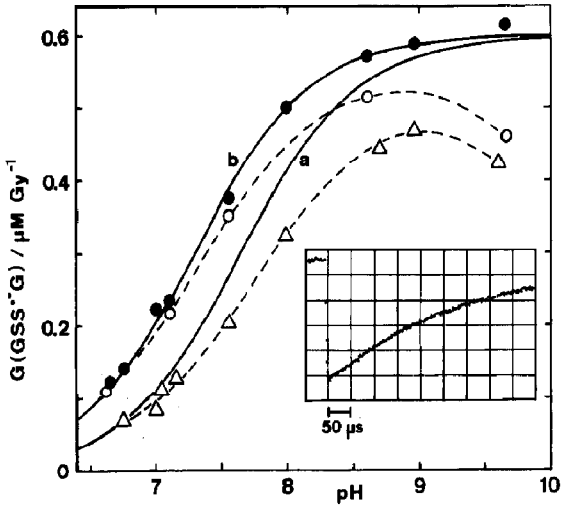


Fig. 1. Initial yield of $GSS^{\cdot-}G$ as a function of pH, obtained by pulse radiolysis of unbuffered N_2O-saturated aqueous solutions of 8 mM GSH: (△) no salt added, (○) in presence of 0.2 M NaCl, (●) in presence of 0.2 M HCOONa. Doses of 0.4 to 1 Gy per pulse were applied, the pulse length was 1 to 2 μs. G-values were determined from the 420 nm absorbance (10 cm cell) by extrapolating decay kinetics to "zero" time, with $\epsilon_{420}(GSS^{\cdot-}G) = 8000\ M^{-1}\ cm^{-1}$ [6]. Inserted is a time profile of the 410 nm transmission change for system (●) at pH 8.97 after a 0.93 Gy pulse. Drawn curves were calculated from equation (6) for conditions as applied in the experiment, (a) at $I = 0.008$, and (b) at $I = 0.208$, using $pK_5 = 9.2$, $K_1 = 3.5 \times 10^3\ M^{-1}$ and a total yield of $G(GSS^{\cdot-}G) + G(GS^{\cdot}) = 0.62\ \mu M\ Gy^{-1}$.

transformed into $^{\cdot}CO_2^-$, reactions (7), (12) and (13), in the O_2-saturated system both e_{aq}^- and $^{\cdot}CO_2^-$ lead to generation of $O_2^{\cdot-}$.

3. Results and discussion

3.1. The $GS^{\cdot} + GS^- \rightleftharpoons GSS^{\cdot-}G$ equilibrium

Figure 1 shows the pH-dependent yield of $GSS^{\cdot-}G$ upon pulse radiolysis of N_2O-saturated solutions containing 8 mM glutathione under various conditions (symbols and broken curves). Formation of the $GS^{\cdot}$ species (see Section 2) and its equilibration by reaction (1) occurs within a few μs; a typical time profile, showing the change in optical transmission due to the rapid formation and slow decay of $GSS^{\cdot-}G$, is inserted in Fig. 1. Yields of $GSS^{\cdot-}G$ were estimated from its absorption at 410 to 420 nm, where $GS^{\cdot}$ is transparent. The solid curves were calculated from eq. (6) for ionic strengths of 0.008 (a) and 0.208 (b), as applied in the experiments (△) and (○, ●), respectively. Yields of $GSS^{\cdot-}G$, i.e. the G-values in μM/Gy, are reasonably well described by equation (6) around pH 7, for all conditions applied. The $^{\cdot}OH$-induced $GSS^{\cdot-}G$ formation (△, ○) is less efficient in alkaline solution, consistent with previous observations [19], which have shown that $^{\cdot}OH$ can also react with the thiolate GS^- to form carbon-centred intermediates which do not lead to $GSS^{\cdot-}G$. Equation (6) gives, however, a satisfactory description, over the whole pH range, of the data (●) obtained with $^{\cdot}CO_2^-$ as oxidant, reaction (14); similar results were obtained with $Br_2^{\cdot-}$. The total yield $G(GSS^{\cdot-}G) + G(GS^{\cdot}) = 0.62\ \mu M\ Gy^{-1}$, used in calculating the curves **a** and **b**, assumes that all water radicals formed in reaction (7) are eventually fed into equilibrium (1). The equilibrium constant $K_1 = 3.5 \times 10^3\ M^{-1}$, applied to fit the experimental yields in Fig. 1 (●), is slightly lower than the previous value ($3.9 \times 10^3\ M^{-1}$) estimated from the kinetics at pH 11.7 [6]. Only a lower limit of the rate constant for reaction (14) could be estimated, $k_{14} > 2 \times 10^7\ M^{-1}\ s^{-1}$ at pH 7, from the rate of $GSS^{\cdot-}G$ formation.

We conclude that equation (6) is appropriate to estimate the equilibrium concentration $[GSS^{\cdot-}G]$ for reaction (1) under the conditions (pH 6.8) applied in Sections 3.2 and 3.3, and that $^{\cdot}CO_2^-$, in contrast to $^{\cdot}OH$, quantitatively oxidizes GSH/GS^- to $GS^{\cdot}$ even in alkaline solution. It should be noted that formation of $GSS^{\cdot-}G$ from $GS^{\cdot}$ does not necessarily proceed via the coupled equilibria (1) and (5). Particularly at $pH \ll pK_5$ we have also to envisage the sequence of reactions (1a) and (1b):

$$GS^{\cdot} + GSH \rightleftharpoons GSS^{\cdot}HG \qquad (1a)$$

$$GSS^{\cdot}HG \rightleftharpoons GSS^{\cdot-}G + H^+ \qquad (1b)$$

At equilibrium the set of reactions (1a) and (1b) is equivalent to that of reactions (1) and (5), since $K_1K_5 = K_{1a}K_{1b} = ([GSS^{\cdot-}G][H^+])/([GS^{\cdot}][GSH])$, however, the rates of equilibration may be different. With $k_1 = 6.2 \times 10^8\ M^{-1}\ s^{-1}$ and $[GS^-] \approx 30\ \mu M$ (at pH 6.8 and 8 mM GSH, Fig 1 (△)) the

reaction (1) should proceed with a half-life of $t_{1/2} = \ln 2/(k_1[GS^-]) \approx 37\ \mu s$, in contrast to that observed under these conditions, $t_{1/2} < 5\ \mu s$. Therefore we think that the faster reaction sequence (1a)–(1b) is relevant, at least at pH $\ll$ pK_5. A detailed investigation of the kinetics of reactions (1a) and (1b) was impossible with the time resolution available, since RSS$^{\cdot}$HR species are very short-lived, $t_{1/2} \ll 10^{-7}$ s [20].

In the low dose range 0.5–1.3 Gy ([GS$^{\cdot}$] + [GSS$^{\cdot-}$G] < 0.8 μM), the GSS$^{\cdot-}$G species decayed by first-order kinetics, with a relatively fast and practically dose-independent rate, e.g. $k = 3.3 \times 10^3\ s^{-1}$ for the trace at pH 9 inserted in Fig. 1. In these experiments we used specifically purified (research grade) N_2O, in order to minimize reactions of GSS$^{\cdot-}$G with O_2 contaminations, and we confirmed that repeated pulsing (to deplete O_2) had no effect on the GSS$^{\cdot-}$G decay rate. In alkaline solutions (pH 9) the first-order decay of GSS$^{\cdot-}$G led to simultaneous formation of a new transient absorbing around 280 nm with $G \times \epsilon_{280} = 2.4 \times 10^{-3}\ cm^{-1}\ Gy^{-1}$, as shown in Fig. 2 (○); this transient has recently been assigned to G(C$_\alpha^{\cdot}$)SH [10] and is represented in Scheme 1. At pH 6.8 and conditions as in Fig. 1 the GSS$^{\cdot-}$G decay was still first-order, $k \sim 1.5 \times 10^3\ s^{-1}$, with simultaneous formation of secondary products absorbing below 300 nm; however, there was no distinct absorbance peak above 240 nm and the absorption was much weaker (Fig. 2 (●)) than in alkaline solution. We conclude that reaction (4) is of minor importance in neutral solution, probably due to self-terminating second-order reactions such as (15), (16) and (17) [6,8]:

$$GSS^{\cdot-}G + GSS^{\cdot-}G \longrightarrow GSSG + 2GS^- \quad (15)$$

$$GS^{\cdot} + GSS^{\cdot-}G \longrightarrow GSSG + GS^- \quad (16)$$

$$GS^{\cdot} + GS^{\cdot} \longrightarrow GSSG \quad (17)$$

In the case of penicillamine [21] and cysteine [22] the RSS$^{\cdot-}$R species have been proposed to decay (at low dose rates) also by carbon–sulfur bond scission to form perthiyl radicals:

$$RSS^{\cdot-}R \longrightarrow RSS^{\cdot} + R^- \quad (18)$$

RSS$^{\cdot}$ species derived from amino-containing disulfides exhibit weak absorptions around 380 nm, but no such absorption was found in the case of GSSG [23], see also Fig. 2 (●). Nevertheless we think that reaction (18) is involved to a minor extent in the decay of the GS$^{\cdot}$/GSS$^{\cdot-}$G couple (see Section 3.2).

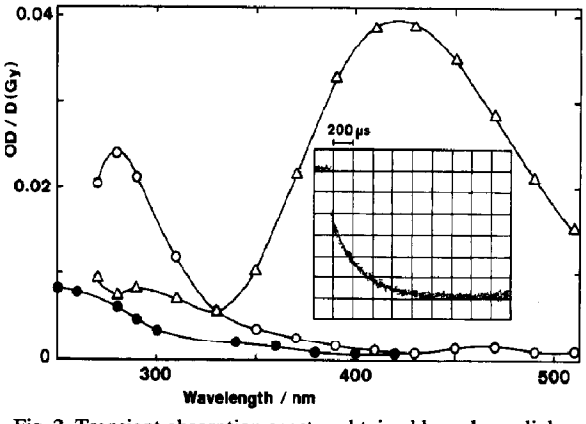


Fig. 2. Transient absorption spectra obtained by pulse radiolysis of N_2O-saturated aqueous solutions of glutathione (1.3 Gy per pulse, 10 cm optical path). Open symbols refer to a solution containing 8 mM GSH and 0.2 M KBr at pH 9, i.e. with $Br_2^{\cdot-}$ as oxidant (see Section 2), (△) 28 μs after the pulse, and (○) 1.3 ms after the pulse; the time profile showing the change in optical transmission in this system due to the secondary product formation at 280 nm. The spectrum (●) refers to a solution containing 2 mM GSH, 10 mM phosphate buffer (pH 6.8) and 0.2 M HCOONa, i.e. with $^{\cdot}CO_2^-$ as oxidant, 1.6 ms after the pulse.

3.2. *Fe(III)–cytochrome c reduction in N_2O-saturated GSH solutions*

Pulse radiolysis of N_2O-saturated solutions containing Fe(III)–cytochrome *c*, GSH, phosphate buffer (pH 6.8) and EDTA leads to relatively fast reduction of the cytochrome, as shown in Fig. 3 trace **a**. For the conditions applied, GS$^{\cdot}$ is generated by $^{\cdot}$OH and H$^{\cdot}$ radicals (see Section 2), and Fe(III)–cytochrome *c* reduction can be explained as depicted in Scheme 1. Trace **b** in Fig. 3 shows, as a reference, the reduction by $^{\cdot}CO_2^-$ at a yield of 0.62 μM/Gy. EDTA required to suppress spurious metal-catalyzed reduction of Fe(III)–cytochrome *c* by GSH (see Section 3.4) did not appear to interfere, since a variation of the EDTA concentration between 0.5 and 2 mM had no effect on the time profiles. The traces **c**

and **d**, refering to N_2O/O_2 systems, are discussed below (Section 3.3).

Both the efficiency and the first order rates of reduction do not increase linearly with [Fe(III) Cytc], as can be seen from the data collected in Table 1. The efficiency was furthermore found to decrease upon increasing the dose per pulse, which indicates that the second-order decay reactions (15) to (17) compete with the electron transfer. In order to reduce $GSS^{\cdot-}G/GS^{\cdot}$ self-termination even further we also investigated the system (N_2-saturated) by low dose rate γ-radiolysis. From the initial slope of the dose–effect curve shown in Fig. 4, we can estimate an upper limit of 72% (0.45 μM Fe(II)Cytc/Gy) for the efficiency of reduction by $GS^{\cdot}$-derived species. A possible reason for the limited efficiency, even under conditions of very slow $GSS^{\cdot-}G/GS^{\cdot}$ self-termination, is the irreversible decay of $GSS^{\cdot-}G$ by reaction (18). Reduction of Fe(III)–cytochrome *c* actually continues slowly after γ-irradiation, as can be seen in Fig. 4 (insert). This

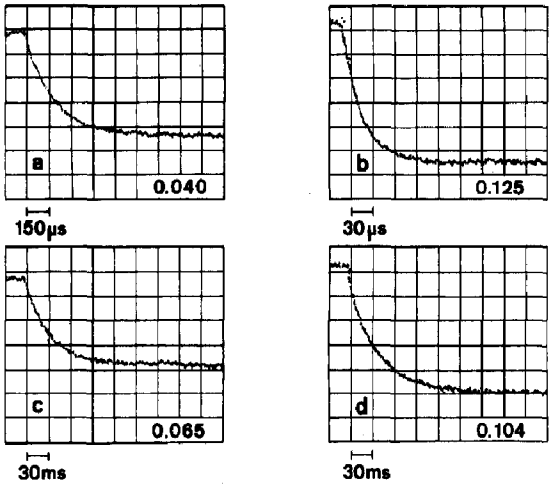


Fig. 3. Time profiles showing the change in optical transmission at 550 nm ($\Delta\lambda$ 5 nm) due to pulse radiolytically induced reduction of Fe(III)–cytochrome *c* (50 μM) in 10 mM phosphate buffer (pH 6.8), using 5.3 Gy per pulse and cells of 2.5 cm optical path. (**a**) N_2O-saturated solution containing 2 mM GSH and 1 mM EDTA; (**b**) N_2O-saturated solution containing 50 mM HCOONa, as reference for trace **a** (see Section 2); (**c**) same as trace **a** but N_2O/O_2-saturated solution (80% N_2O, 20% O_2); (**d**) same as trace **b** but O_2-saturated, as reference for trace *c* (see Section 2). GSH was injected into the final solution **a** or **c** immediately before pulse radiolysis. The numbers give the total change in 550 nm absorbance.

Table 1

Efficiencies and first-order rates for reduction of Fe(III)–cytochrome *c* by glutathione-derived free radical species [a]

[Fe(III)Cytc] (μM)	Dose (Gy)	Efficiency of reduction [b]	Rate [c] (s^{-1})
N_2O-saturated solutions			
0	4.8	–	3400 [e]
10	4.8	16%	3200
20	4.8; 10.0	26%; 18% [d]	3600
30	7.0	30%	3800
40	4.8	28%	4800
50	4.8	32%	5100
60	2.9; 7.0	41%; 30% [d]	5500
N_2O/O_2 (4:1) solution			
50	4.8	62%	29

[a] Results obtained from time profiles as in Fig. 3, by pulse radiolysis of solutions containing 2 mM GSH, 10 mM phosphate buffer (pH 6.8) and 0.5 to 1 mM EDTA.

[b] Yields estimated from 550 nm absorbance changes relative to $^{\cdot}CO_2^-$-induced reduction, or $O_2^{\cdot-}$-induced reduction (in the N_2O/O_2 system, see Section 2).

[c] Rates (k) ± 10% (S.D.).

[d] For the higher dose.

[e] $GSS^{\cdot-}G$ decay rate; reaction (4) (Scheme 1) is slower, $k_4 < 2000\ s^{-1}$, since second-order reactions (15) to (17) contribute to the decay of $GSS^{\cdot-}G$ at the dose applied in the above experiment (cf. Section 3.1 and [10]).

observation is consistent with reaction (18), since the perthiyl radical $GSS^{\cdot}$ formed is a precursor of higher sulfides (GSSSSG and GSSSG) which are known to catalyze the slow reduction of Fe(III)–cytochrome *c* by GSH [24,25]. That the yield of reduction falls off at higher doses (Fig. 4) can be explained by the counteracting reaction (2) (Scheme 1) which is progressively fed upon reduction of Fe(III)–cytochrome *c*.

The question now arises whether $GSS^{\cdot-}G$ or $G(C_\alpha^{\cdot})SH$ is the reducing entity (see Scheme 1). Under the conditions (pH 6.8, 1–2 mM GSH) applied in all experiments with Fe(III)–cytochrome *c*, less than 4% of $GS^{\cdot}$ equilibrates by reaction (1) to $GSS^{\cdot-}G$ (according to eq.6). Thus, considering the relatively high yields of reduction (Table 1), reaction (3) would to be rather efficient in pulling equilibrium (1) to the right-hand side. From Fig. 2 (●) it was concluded that the formation of $G(C_\alpha^{\cdot})SH$ is also inefficient at pH 6.8, probably due to competing $GS^{\cdot}/GSS^{\cdot-}G$ self-

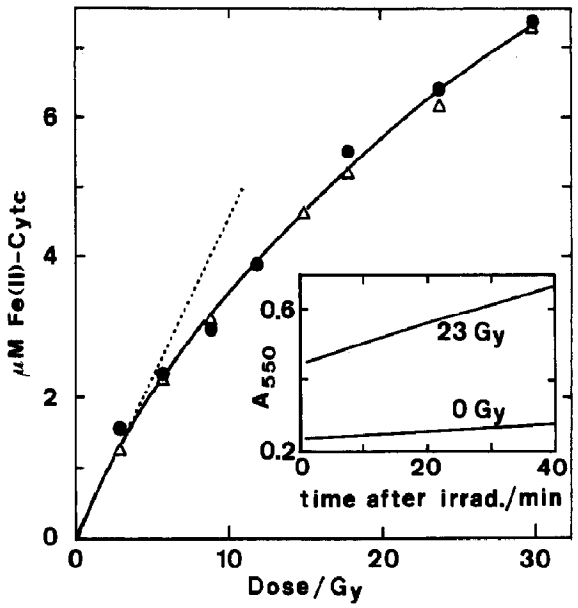


Fig. 4. Reduction of Fe(III)–cytochrome *c* by glutathione-derived free radicals as a function of dose, applied to generate GS·. The results were obtained by (Co-60) γ-radiolysis of N_2-saturated solutions containing 1 m*M* GSH, 0.2 m*M* EDTA and 10 m*M* phosphate (pH 6.8), with $^{\cdot}CO_2^-$-induced reduction (see Section 2) as reference: (△) [Fe(III)Cytc] = 20 μ*M*, (●) [Fe(III)Cytc] = 30 μ*M*. The initial slope indicates a yield of 0.45 Fe(II)Cyt*c*/Gy. Inserted are time profiles showing the slow reduction in the unirradiated system (●) and the faster reduction proceeding after γ-irradiation. All data points were extrapolated to "zero" time; the time lapse between adding GSH to the solutions and measurements of A_{550} was generally less than 5 min, even for the highest dose.

terminations. One argument against G(C·$_\alpha$)SH being the main reducing entity is given by the concentration dependence of the reduction rates. These rates are higher than the GSS·$^-$G decay rate in the absence of Fe(III)–cytochrome *c* and approach the GSS·$^-$G decay rate only at lowest concentrations (Table 1). With G(C·$_\alpha$)SH as electron donor the reduction should proceed after the decay of GSS·$^-$G, as in the case of PNAP at pH 8.4 to 10 [10], where reduction was slower than the GSS·$^-$G decay by reaction (4). Also the concentration and dose dependent efficiency of reduction (Table 1) is more consistent with GSS·$^-$G being the electron donor. GS· removal by reaction (17) is fast ($2k_{17} = 3.4 \times 10^9\ M^{-1}\ s^{-1}$ [20]), and reduction by the reaction sequence (1) → (3) is therefore favoured at high [Fe(III) Cyt*c*] and at low doses, as indicated in Table 1. G(C·$_\alpha$)SH removal, on the other hand, is much slower ($2k \approx 3 \times 10^7\ M^{-1}\ s^{-1}$ [10], i.e. $t_{1/2} \approx 10$ ms at 5 Gy), thus the decay of this species would hardly compete with electron transfer in the 0.2 ms time scale (cf. Fig. 3a).

In the case of PNAP the reduction by G(C·$_\alpha$)SH was also demonstrated by pulse radiolysis of argon-saturated solutions containing GSSG and *t*-butanol at pH 9 [10]. In this system ·OH is scavenged by *t*-butanol and e_{aq}^- adds to the disulfide, initiating the sequence of reactions (−1) and (4) (Scheme 1); replenishment of GSS·$^-$G by reaction (1) is negligible since no GSH was added. In a similar experiment, i.e. by γ-radiolysis of N_2-saturated solutions containing GSSG (1 m*M*), *t*-butanol (1 *M*), EDTA (0.2 m*M*) and Fe(III)–cytochrome *c* (30 μ*M*) at pH 6.8 (10 m*M* phosphate), we have also observed reduction at a yield of 0.19 μ*M* Fe(II)Cyt*c*/Gy. However, reduction of Fe(III)–cytochrome *c* at the same yield was obtained when the above system was N_2O-saturated, i.e. when e_{aq}^--induced generation of G(C·$_\alpha$)SH was prevented by reaction (12). Radicals formed by reaction of ·OH with *t*-butanol apparently interfere in this system, thus no evidence can be presented for reduction by G(C·$_\alpha$)SH under the conditions applied.

Reduction rates were found to increase only smoothly with the Fe(III)–cytochrome *c* concentration (Table 1). At low concentrations the GSS·$^-$G/GS· self-termination (see above) presents a limiting factor. The concentration dependent rate of reduction at higher [Fe(III)Cyt*c*] enabled only a rough estimate of $k_3 \approx 6 \times 10^7\ M^{-1}\ s^{-1}$.

Although the present study was aimed at demonstrating the reductive interaction of GSS·$^-$G with Fe(III)–cytochrome *c*, we have also tested other electron acceptors such as O_2 (Section 3.3), PNAP and tetrathionate at pH ≈ 6.8. The first order rates of reduction of these substrates were much faster, under the conditions applied, than that of the natural decay of GSS·$^-$G into secondary species, thus GSS·$^-$G rather than G(C·$_\alpha$)SH was again likely to be the reductant. Reduction by G(C·$_\alpha$)SH via reaction (4) may be significant only at pH ≫ 7 or at very low substrate concentrations.

To sum up, reduction of Fe(III)–cytochrome *c* by glutathione-derived radicals at pH 6.8 appears mainly to involve $GSS^{\cdot-}G$, with reaction (3) pulling the equilibrium (1) efficiently to the right (see Scheme 1), even though the back reaction (−1) is favoured in neutral solution. The decay of the $GS^{\cdot}/GSS^{\cdot-}G$ couple to form the reducing $G(C_{\alpha}^{\cdot})SH$ species [10] seems to be significant only well above pH 7. Dissociation of $GSS^{\cdot-}G$ to form the perthiyl radical $GSS^{\cdot}$ seems to be another (minor) reaction route. Interest has recently been focused on the redox chemistry of $RSS^{\cdot}$ species [25,26], which might be common companions of thiyl radicals. $GSS^{\cdot}$ seems to be an intermediate also in sulfane-activated reduction (without irradiation) of Fe(III)–cytochrome *c* by GSH [24,27].

It is not yet known whether the equilibrium (1) is a fundamental reaction, involved e.g. in gating certain biological electron transfer processes, but we think that attention should also be paid to this possibility. Equilibrium (1) may also be relevant in bioreductive processes, such as activation of quinones and xenobiotics by GSH [28–30]. With respect to the scenario in aerobic biological systems the interactions of oxygen with the equilibrium (1) are of importance.

3.3. Interaction of oxygen with the $GS^{\cdot} + GS^{-} \rightleftharpoons GSS^{\cdot-}G$ equilibrium

Trace **c** in Fig. 3, with $O_2^{\cdot-}$-induced reduction (trace **d**) as reference, and the data in Table 1 show that the efficiency of reduction of Fe(III)–cytochrome *c* (50 μM) is much higher in pulse irradiated solutions containing both N_2O (80%) and O_2 (20%) than in the corresponding N_2O-saturated system, though the reduction is much slower. In γ-radiolysis experiments and conditions as in Fig. 4, we also observed an increase in the reduction yield from about 70% to 95% when going from the N_2O- to the N_2O/O_2-system.

In the latter environment the $GS^{\cdot}$ species is still formed at a yield of 0.62 μM/Gy (see Section 2), since reaction (12) overrules the generation of $O_2^{\cdot-}$ by addition of e_{aq}^{-} to O_2. Oxygen can, however, interact with $GS^{\cdot}$ and $GSS^{\cdot-}G$, as depicted in Scheme 2 by the reactions (19) and (20). The rate constant k_{19} is probably of the order of $10^9\ M^{-1}\ s^{-1}$ [31,32] (with certain discrepancies in the literature data), and we have now estimated a value of $k_{20} = 5.1 \times 10^8\ M^{-1}\ s^{-1}$ from the decay of $GSS^{\cdot-}G$ ($t_{1/2} \approx 5\ \mu s$) in a (4:1) N_2O/O_2-saturated solution containing GSH (8 mM) at pH 9. According to eq. (6), reaction (1) produces a ratio of $GSS^{\cdot-}G/GS^{\cdot} = 0.03$ at equilibrium, for the conditions applied in Fig. 3c. Therefore reaction (20) was expected to be unimportant in comparison to reaction (19), which generates the oxidizing $GSOO^{\cdot}$ radical [31,32]. In order to explain the high efficiency of reduction in the N_2O/O_2 system we have to take into account the reversibility of reaction (19). Oxygen seems to pull the coupled equilibria (19) and (1) successfully into the (irreversible) reaction (20), even in neutral solution where $GSS^{\cdot-}G$ formation is unfavourable. The subsequent reduction of Fe(III)–cytochrome *c* by $O_2^{\cdot-}$, reaction (21), has been investigated in detail; the slow rate of reduction, $k = 0.58 \times 10^6\ M^{-1}\ s^{-1}$ obtained from the first-order rate in Table 1 (last line), is consistent with previous estimates of $k_{21} = 0.26$ to 3.1 $\times 10^6\ M^{-1}\ s^{-1}$ (the rate depending on ionic strength) [18,33].

By pulse radiolysis (5 Gy) of (4:1) N_2O/O_2-saturated solutions containing just GSH (2 mM) at pH 6.8 (10 mM phosphate) we have detected the intermediate absorbing around 550 nm which has been assigned to $GSOO^{\cdot}$ [31], but this species decayed exponentially with a half-life of 45 μs. Within this time a long-lived product was formed which can be assigned to $O_2^{\cdot-}$ by its absorption with a peak at 245 nm. Using the extinction coefficient of $\epsilon_{245}(O_2^{\cdot-}) = 2350\ M^{-1}\ cm^{-1}$ [34] it could be estimated that $GS^{\cdot}$ led to the generation of $O_2^{\cdot-}$ with an efficiency of at least 60%, consistent with the yield of Fe(III)–cytochrome *c* reduction in the N_2O/O_2 system (Table 1). From the fast rate of formation of $O_2^{\cdot-}$ it could be concluded that the secondary $G(C_{\alpha}^{\cdot})SH$ species was not involved. A more detailed description of $O_2^{\cdot-}$ generation by thiol-derived free radicals will be given elsewhere.

Many quinones and redox-active drugs exhibit standard one-electron reduction potentials near that for oxygen [35], thus it is reasonable to assume (cf. Section 3.2) that, as in the case of

oxygen, the reduction of such compounds can be mediated by the glutathione free radical equilibrium (1). Thiyl radicals are not only formed by ionizing radiation (Section 2) but also as thiol metabolites by peroxidases [36,37] and possibly by reductive interactions of thiols with certain xenobiotics [28]. Our knowledge of the fate of RS$^{\cdot}$ species in aerobic biological systems is however rather limited. Considerable interest has been focused in recent years on the GSOO$^{\cdot}$ species [13,31,32,38], which might be a toxic metabolite. The above results show however that oxygen can efficiently deplete the equilibrium (19) through reaction (1) to generate GSSG and $O_2^{\cdot-}$ (Scheme 2). A high yield of disulfide has also been found after γ-radiolysis of N_2O/O_2-saturated solutions of other thiols, e.g. 2-mercaptoethanol at pH 5.7 [39]. The reductive activation of oxygen, i.e. reaction (20), may indeed be more important than reaction (19), and should therefore not be neglected when developing scenarios for thiyl radicals in aerobic biological systems. Very commonly it is assumed that thiyl radicals simply disappear to give disulfide, reaction (17), even in biological systems. In view of the high reactivity of RS$^{\cdot}$ and of the conjugated RSS$^{\cdot-}$R species, particularly with oxygen, it appears rather unlikely that reaction (17) is of importance *in vivo*.

3.4. Copper-catalyzed reduction of Fe(III)–cytochrome c by GSH

Transition metal ions like Cu^{2+} have been suggested to interact with dithiothreitol to form the corresponding thiyl radical, HS–R–S$^{\cdot}$, which was thought to be capable of transferring an electron for instance to O_2 [40]. More recent results have shown that the dithiothreitol species HS–R–S$^{\cdot}$ rapidly deprotonates in neutral solution to form a cyclic disulfide radical anion [41], which in this case is likely to be the reducing entity. Since the fast copper-catalyzed reduction of Fe(III)–cytochrome *c* by GSH might involve the equilibrium (1), we have analysed the kinetics of such systems (in the absence of EDTA) by rapid-mix and stopped-flow techniques.

The results shown in Fig. 5 reveal that the first-order rates of Fe(III)–cytochrome *c* reduction are independent of both Fe(III)cytochrome *c* and GSH concentration, but proportional to the Cu(II) concentration. For the conditions applied, [GSH] ≫ [Fe(III)Cyt*c*] > [Cu(II)], the time profiles for all data in Fig. 5 could be described satisfactorily by a simple rate equation,

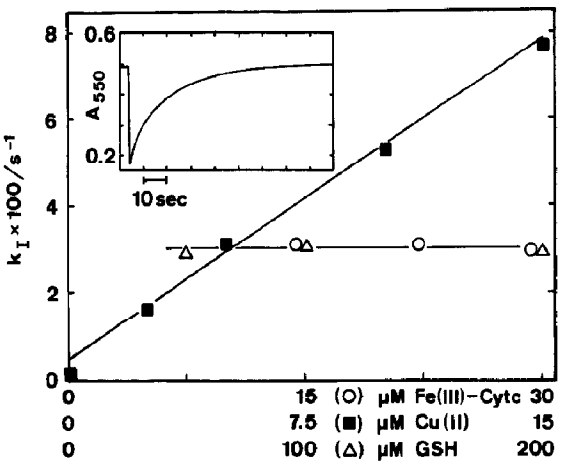


Fig. 5. Copper-catalyzed reduction of Fe(III)–cytochrome *c* by GSH. Kinetics of reduction versus the concentrations [Fe(III)Cyt*c*], [Cu(II)] and [GSH], respectively. The results were obtained with a stopped-flow system, by mixing GSH solutions with solutions containing Fe(III)–cytochrome *c*, Cu(II) and 10 m*M* phosphate buffer (pH 6.8) at 20°C. Final concentrations: (○) 0.2 m*M* GSH, 5 μM $CuCl_2$ and [Fe(III)Cyt*c*] varied, (■) 0.2 m*M* GSH, 22.5 μM Fe(III)–cytochrome *c* and [Cu(II)] varied, (△) 30 μM Fe(III)–cytochrome *c*, 5 μM $CuCl_2$ and [GSH] varied. Inserted is a time profile of the 550 nm absorbance change for the experiment (■) at 10 μM Cu(II).

$$\frac{c(t)}{c(0)} = e^{-k_x[\mathrm{Cu(II)}]t} \qquad (22)$$

with c = [Fe(III)Cyt*c*] and $k_x = 5 \times 10^3\ M^{-1}\ s^{-1}$. There was no difference in the time profiles when going from aerobic to anaerobic systems, thus oxygen seems not to be involved (apart from reoxidation of Fe(II)–cytochrome *c* within about one hour). The reduction kinetics were very similar to those in Fig. 5 also when Cu(II) was added to GSH solutions prior to rapid mixing with Fe(III)–cytochrome *c* at corresponding final concentrations of the components.

Cu(II)–thiolate complexes decay rapidly to produce disulfide and Cu(I)–thiolate complexes

[42,43], the decay probably occurring in two steps via formation of an intermediate,

$$Cu(II)GSH_x \overset{GSH}{\rightleftharpoons} Cu(I)GSH_y\text{–}GS^{\cdot} + H^+ \quad (23)$$

$$2Cu(I)GSH_y\text{–}GS^{\cdot} \xrightarrow{GSH} 2Cu(I)GSH_y + GSSG \quad (24)$$

$$Cu(II)GSH_x + Cu(I)GSS^{\cdot-}G \xrightarrow{GSH} 2Cu(I)GSH_y + GSSG \quad (24a)$$

The intermediate may contain copper in an indeterminable valence state [43], or it may contain a thiyl radical, bound to Cu(I)–thiolate in the form of a disulfide anion $Cu(I)GSS^{\cdot-}G$ [8,44]. The alternative reaction (24a) would involve an electron transfer from the $GSS^{\cdot-}G$ ligand to Cu(II), as observed in azurins [45].

Reduction of Fe(III)–cytochrome *c* (Fig. 5) can be explained, without postulating the formation of the free $GS^{\cdot}$ radical, by the fast generation of $Cu(I)GSH_y$, reactions (23) to (24a), followed by the slow rate-determining reaction (25):

$$Cu(I)GSH_y + Fe(III)Cyt\,c \xrightarrow{GSH} Cu(II)GSH_x + Fe(II)Cyt\,c \quad (25)$$

$Cu(II)GSH_x$, regenerated in reaction (25), is immediately transformed back to $Cu(I)GSH_y$ by the reactions (23) to (24a). The rate equation (22) indeed suggests that Fe(III)–cytochrome *c* reduction proceeds at a constant concentration of the reductant, here $[Cu(I)GSH_y]$, which is probably close to the initial [Cu(II)]. Therefore we propose that $k_x = k_{25}$, with $k_{25} > 5 \times 10^3\ M^{-1}\ s^{-1}$ if $[Cu(I)GSH_y] < [Cu(II)]$. The value of k_{25} is higher than $k(Cu(I) + Fe(III)Cyt\,c) = 1.5 \times 10^3\ M^{-1}\ s^{-1}$ [46], obtained by pulse radiolysis of solutions containing 0.1 *M* HCOONa to generate Cu(I) [reaction (13) followed by $Cu(II) + {}^{\cdot}CO_2^- \rightarrow Cu(I) + CO_2$]. The difference in the above rate constants is most likely due to differences in the ionic strength and of the Cu(I) ligands; we have recognized that k_{25} becomes much lower in the presence of 0.1 *M* NaCl, consistent with a salt effect on the rate of reaction of the negatively charged $Cu(I)GSH_y$ complex at the positively charged the surface of Fe(III)–cytochrome *c*. The above results might also be explained by formation of unbound $GS^{\cdot}$ in reaction (23), followed by the reactions (1) and (3). However, the latter two reactions are fast (Section 3.2) and it is difficult to explain the effects of Cu(II) and GSH concentration on the rates of reduction (Fig. 5) by assuming that reaction (23) is the rate-determining step.

Further evidence against formation of unbound $GS^{\cdot}$ radicals in reaction (23) was obtained by mixing Cu(II) (10 to 100 μM) with GSH (100 to 500 μM) and NADH (200 μM) under aerobic or anaerobic conditions. It would be expected that if $GS^{\cdot}$ were to be formed in this system, it would oxidize NADH [47]. No oxidation of NADH was observed.

The conclusion from all these results is that Cu(II), under the conditions applied, does not interact with GSH to form the free $GS^{\cdot}$ radical, and that the copper-catalyzed reduction of Fe(III)–cytochrome *c* by GSH is not mediated by equilibrium (1).

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