Reduction of Resazurin by Glutathione activated by Sulfanes and Selenite

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Reduction of resazurin by glutathione is efficiently promoted by sulfanes (RSSSR and tetrathionate) or by traces of selenite: the effect can be explained by formation and redox cycling of persulfide (GSSH) and selenopersulfide (GSSeH), which appear to be significantly better reductants (antioxidants) than GSH.

Glutathione trisulfide, GSSSG, has long been known to propagate the reduction of cytochrome c by GSH.[1] The persulfide GSSH appears to be the active reductant in this system, and models envisaged to explain the redox cycle can be summarized by the reactions (1)–(3).[1,2]

$$GSSSG + GS^- \rightleftarrows GSS^- + GSSG \quad (1)$$

$$GSS^- + Fe^{III}\ Cytc \rightleftarrows GSS^{\cdot} + Fe^{II}\ Cytc \quad (2)$$

$$GSS^{\cdot} + GS^- + Fe^{III}\ Cytc \rightarrow GSSSG + Fe^{II}\ Cytc \quad (3)$$

Renewed attention has recently been paid to the redox chemistry of sulfanes,[2–4] particularly since persulfides are likely to be significantly better antioxidants in biological systems than thiols. Reduction of cytochrome c by GSH is activated also by the selenotrisulfide derivative of glutathione,[5] formed for instance by interaction of selenite with GSH [eqn. (4)].[6]

$$4GSH + SeO_3^{2-} \rightarrow GSSeSG + GSSG + 2OH^- + H_2O \quad (4)$$

The reductive catalytic activity of sulfanes in combination with GSH has so far only been demonstrated with cytochrome c as redox indicator. In the present communication we have tested resazurin (7-hydroxy-3*H*-phenoxazine-3-one *N*-oxide sodium salt) as electron acceptor. This dye changes colour from blue (λ_{max} = 600 nm) to pink (λ_{max} = 565 nm) upon reduction to resorufin.[7]

Fig. 1 shows time profiles for reduction of resazurin by GSH upon addition of various activating agents: $S_4O_6^{2-}$ (*a*), γ-irradiated cystamine (*b*), and SeO_3^{2-} (*c*). Reduction rates are collected in Table 1. Resazurin reduction is seen to be very slow in the control without activator. There was little change in the time profiles when going from aerobic to anaerobic conditions; an example is given in Fig. 1(*c*). Furthermore, none of the activators tested led to reduction of resazurin in absence of GSH. Absorption spectra were consistent with reduction of resazurin (RN→O) to resorufin (RN).

The rate of $S_4O_6^{2-}$-activated resazurin reduction (Table 1) is slower than that of cytochrome c reduction (3.9 μmol dm^{-3} min^{-1})[2] under comparable conditions; one reason may be that resazurin is an oxygen donor, whereas cytochrome c involves two 1 e^--reductions per cycle. We anticipate that $S_4O_6^{2-}$ interacts with GSH to form traces of the catalytically active persulfide.[2] As yet there is no proof, however, that the reaction cycle (1)–(2)–(3) proceeds *via* formation of reactive free-radical intermediates (GSS·). As already pointed out,[2] it is difficult to explain why the interaction of O_2 with GSS· fails to inhibit the reductive catalysis. In the case of resazurin we now propose an alternative reaction cycle which avoids the 'free radical problem' [eqns. (5), (6) and (1*a*)].

$$GSS^*H + RN{\rightarrow}O \rightarrow GSS^*OH + RN \quad (5)$$

$$GSH + GSS^*OH \rightarrow GSS^*SG + H_2O \quad (6)$$

$$GSH + GSS^*SG \rightleftarrows GSS^*H + GSSG \quad (1a)$$

Resazurin reduction as in Fig. 1(*a*) and (*c*) became slower above pH 7, in contrast to cytochrome c reduction which was speeded up at pH > 7;[2] therefore we believe that the reductant is GSSH in reaction (5) and GSS^- in reaction (2). The catalytic activity of persulfide is strongly enhanced when the sulfane sulfur (S*) is replaced by selenium (see below).

GSSSG, commonly present as a contaminant in commercial samples of GSSG,[1,5] is formed also by γ-irradiation of

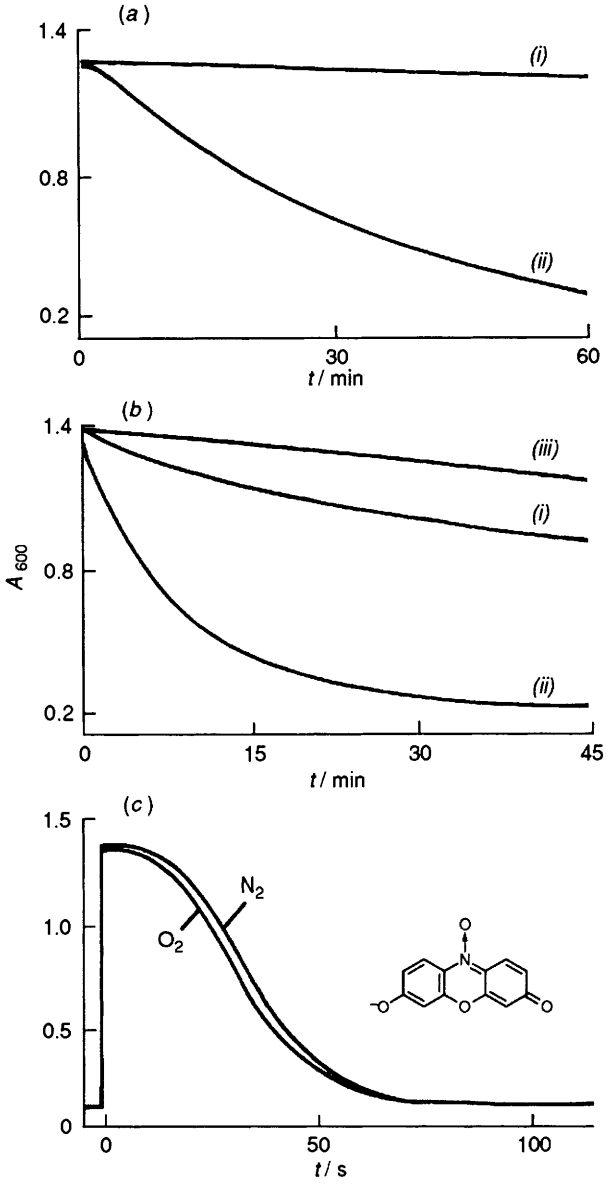

Fig. 1 Time profiles of 600 nm absorbance changes showing reduction of resazurin (50 μmol dm^{-3}) by GSH {5 mmol dm^{-3} [10 mmol dm^{-3} for (*c*)]} upon addition of various activators, in phosphate buffer (25 mmol dm^{-3}, pH 6.5), NaCl (25 mmol dm^{-3}) and EDTA (9 mmol dm^{-3}). (*a*) Activation by $S_4O_6^{2-}$ [0 (*i*) and 1 mmol dm^{-3} (*ii*)]; (*b*) activation by γ-irradiated cystamine, 0.5 mmol dm^{-3} cystamine in N_2O-saturated 5 mmol dm^{-3} phosphate buffer (pH 6.8) was ^{60}Co-γ-irradiated (200 Gy) in absence (*i*) and presence (*ii*) of 12 mmol dm^{-3} KBr, and diluted 1:4 in the final reaction mix, (*iii*) unirradiated control (± Br^-); (*c*) activation by SeO_3^{2-} (50 μmol dm^{-3}) in oxygenated (O_2) and deoxygenated (N_2) solution, using a stopped-flow system, the resazurin structure is shown.

Table 1 Rates of reduction of resazurin by GSH in presence of various additives

Additives[a] (μmol dm^{-3})	Reduction rate[b]/ μmol dm^{-3} min^{-1}
Control	0.05
$S_4O_6^{2-}$ (1000)	1.27
Cystamine (125)	0.17
Cystamine (125), γ-irradiated	0.84
Cystamine (125) + KBr (3000), γ-irrad.	4.76
SeO_3^{2-} (2.5)	7.00
SeO_3^{2-} (50)	74.6
SeO_3^{2-} (50) + SOD (5)	76.1

[a] Basal medium and irradiation conditions as in Fig. 1. [b] Rates were estimated using $\Delta\varepsilon_{600} = 2.66 \times 10^4$ dm^3 mol^{-1} cm^{-1} for the difference between the absorption coefficients of RN→O and RN. Maximum rates are given in cases where reduction occurred after a lag phase [Fig. 1(*c*)].

disulfides (and thiols).[3,8] As shown in Fig. 1(*b*) and Table 1 the reduction of resazurin by GSH is only slightly activated by unirradiated cystamine. The activity of cystamine is enhanced by irradiation in N_2O-saturated solution, *i.e.* with ·OH as oxidant, and more efficiently by irradiation in presence of bromide, *i.e.* with $Br_2^{\cdot-}$ as oxidant.† This result is consistent with pulse radiolysis observations which have shown that $Br_2^{\cdot-}$ is much more efficient than ·OH in generating the cystamine perthiyl radical CyaSS·, the precursor of trisulfide.[8] Displacement of sulfane sulfur between CyaSSSCya and GSH, as in reaction (1), may in this case generate both GSSH and CyaSSH as active reductant.

Selenite activates resazurin reduction by GSH much more dramatically than the other additives tested, independent of oxygen [Fig. 1(*c*)]. Complete reduction of resazurin was achieved even at $[RN{\rightarrow}O]/[SeO_3^{2-}] > 100$. These observations can be accommodated by assuming that the selenotrisulfide derivative of glutathione, generated in reaction (4), acts like GSSSG in the reaction cycle (5)–(6)–(1a); the turnover rate appears however to be much higher when the sulfane sulfur (S*) is replaced by Se. A lag phase is seen in the SeO_3^{2-}-activated reduction [Fig. 1(*c*)], consistent with a relatively slow production of GSSeSG, as indicated also by stopped-flow investigations of the individual steps of reaction (4) in absence of resazurin (to be published elsewhere). We have confirmed that SeO_3^{2-} activates cytochrome c reduction by GSH;[5] however, there was hardly any lag phase in this case, again indicating a different mechanism of cytochrome c reduction as compared to resazurin reduction.

In absence of additives like resazurin the SeO_3^{2-}-activated oxidation of GSH requires oxygen for the regeneration of SeO_3^{2-};[9] also formation of $O_2^{\cdot-}$ has been envisaged.[10] SeO_3^{2-}-activated reduction of resazurin by GSH, on the other hand, is oxygen-independent [Fig. 1(*c*)], and superoxide dismutase (SOD) did not inhibit reduction (Table 1). Hence it can be concluded that $O_2^{\cdot-}$ is not the electron donor in our system. The proposed reaction chain (5)–(6)–(1a) does in fact not require oxygen. A high reductive activity of selenite–GSH was also found using nitro blue tetrazolium (NBT) as electron acceptor; tetrathionate, on the other hand, did not activate NBT reduction by GSH. This reveals that GSSeH is a more powerful reducing agent than GSSH.

The SeO_3^{2-}-activated reduction of resazurin by GSH was progressively inhibited by H_2O_2 at $c > 0.5$ mmol dm^{-3}, and we have unequivocal evidence that this is due to a competitive catalytic reduction of H_2O_2, most likely due to reaction (7),

$$GSSeH + H_2O_2 \rightarrow GSSeOH + H_2O \quad (7)$$

and recycling of GSSeOH to GSSeH as depicted for persulfide by reactions (6) and (1a). The overall reaction catalysed by GSSeH ($2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$) actually corresponds to that of the seleno-enzyme glutathione peroxidase.[11,12]

Interactions of selenite with thiols are of relevance to the biochemical, toxicological and nutritional properties of selenium.[11,12] The present results reveal that selenite-catalysed oxidation of GSH, which is thought to be a deleterious process *in vivo*,[9,10] concomitantly activates reductive chain reactions with selenopersulfide as chain carrier. The catalytic reduction of H_2O_2 provides an interesting example of antioxidative capability of selenite in combination with thiols.

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Footnote

† The ·OH radical is the main oxidizing intermediate formed by γ-irradiation of N_2O-saturated water: $H_2O + \gamma \rightarrow e_{aq}^- + {\cdot}OH + H^{\cdot}$, followed by $e_{aq}^- + N_2O + H^+ \rightarrow {\cdot}OH + N_2$. In presence of Br^-, the ·OH radical is removed by formation of $Br_2^{\cdot-}$.[8]

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