

Glutathione Peroxidase-Like Activity of Simple Selenium Compounds. Peroxides and the Heterocyclic N-Oxide Resazurin Acting as O-Atom Donors

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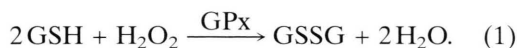
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Glutathione Peroxidase, Peroxide Reduction, Selenol Oxidation, Resazurin, Thiyl Radical

Selenite and selenocystamine [(CyaSe)₂] efficiently activate the decomposition of H₂O₂ by GSH and by other thiols, as demonstrated using a leuco crystal violet POD-based H₂O₂ assay which is applicable (unlike other assays) also in presence of thiols. The GPx-like activities were estimated to be 3.6 and 2.7 μmol H₂O₂/min per μmol SeO₃²⁻ and (CyaSe)₂, respectively. Both selenium compounds also activate reduction of the heterocyclic N-oxide resazurin (RN→O) to resorufin (RN) by GSH; H₂O₂ competes with reduction of this dye. GSSeH and CyaSeH, formed by interaction of GSH with SeO₃²⁻ and (CyaSe)₂, respectively, are likely to be the active reductants. CyaSeH, generated γ-radiolytically from (CyaSe)₂, exhibits an absorption peak at 243 nm and is removed by H₂O₂ with a rate constant of 9.7 × 10² M⁻¹ s⁻¹, and slightly slower by hydroperoxides. We have no evidence for one-electron interactions between GSSeH or CyaSeH and H₂O₂, with formation of free radical intermediates, as previously proposed in the case of selenium-activated reduction of cytochrome *c* by GSH (Levander *et al.*, Biochemistry **23**, 4591–4595 (1973)). Our results can be explained by O-atom transfer from the substrate to the active selenol group, RSeH + H₂O₂ (RN→O) → RSeOH + H₂O (RN), and recycling of RSeOH to RSeH (+ H₂O) by GSH, analogous to the selenenic acid pathway of GPx. The substrate specificity appears to be different, however, in that GPx is unable to catalyse RN→O reduction, and GSSeH hardly catalyses the decomposition of cumene- or *t*-butyl-hydroperoxide; CyaSeH, on the other hand, is active also with the hydroperoxides. RN→O is reduced to RN also by certain oxidizing free radicals, e.g. by the thiyl CyaS•; O-atom transfer may in this case lead to the generation of reactive oxyl radicals.

Introduction

Selenium, though very toxic at high levels (Painter, 1941; Moxon and Rhian, 1943; Martin, 1973; Shamberger, 1983a), is an essential trace element, used for instance as functional group (selenocysteine) in the antioxidative enzyme glutathione peroxidase (Rotruck *et al.*, 1973; Flohé, 1982) which catalyses the reaction (1):



It is the two-electron covalent chemistry of selenium which enables O-atom transfer from the peroxide (Frausto and Williams, 1991), without formation of free radicals as in the deleterious Fenton reaction [Fe(II) + H₂O₂ → Fe(III) + OH⁻ + •OH]. Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one), a seleno-organic drug with GPx-like activity,

has been studied extensively in the last decade as a promising non-toxic anti-oxidant with anti-inflammatory properties (Sies, 1994; Schewe, 1995). Great interest is currently also paid to the possible anticarcinogenic effects of selenium (Shamberger, 1983b) and its chemopreventive role in carcinogenesis (Ip, 1986; Vadhanavikit *et al.*, 1993). Regarding biologically important reactions involving selenium, there is however still a need of extensive studies of the mechanisms of such reactions in simple model systems (Kice *et al.*, 1980; Kice, 1981; Douglas, 1987).

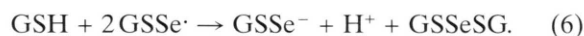
Reactions of selenite and alkyl-diselenides (RSeSeR) with thiols efficiently activate catalytic electron transfer to acceptors like cytochrome *c* (Levander *et al.*, 1973), methylene blue (Rhead and Schrauzer, 1974) and methemoglobin (Masukawa and Iwata, 1977), and reduction of oxygen (Tsen and Tappel, 1958; Seko *et al.*, 1989). Thus it appears possible that reduction of H₂O₂, as in reaction (1), can be activated also by these com-

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pounds. Reaction of selenite with thiols leads to seleno-trisulfides which react further, particularly at pH $\approx$ 7, to form unstable selenopersulfide derivatives, e.g. of glutathione (Ganther, 1968 and 1971):



GSSe⁻ has been proposed to be the active electron transfer catalyst in the case of cytochrome *c* reduction by GSH (Levander *et al.*, 1973):



This reaction scheme is analogous to that for reduction of cytochrome *c* by GSH with GSSG as catalyst (Massey *et al.*, 1971), i.e. GSSG and GSS⁻ substituted for GSSeSG and GSSe⁻ in the reaction chain (5)-(6)-(3). As yet there is no proof however for the generation of free radical intermediates (GSSe[·] or GSS[·]) in reactions catalysed by GSSe⁻ or GSS⁻; it is difficult for instance to explain why the reaction of O₂ with GSS[·] fails to inhibit the recycling of persulfide *via* reaction (6) in the case of GSS⁻-catalysed reduction of cytochrome *c* (Prütz, 1993).

An alternative reaction scheme, which involves GSSeH as chain carrier but avoids the 'free radical problem', has recently been proposed to explain catalytic reduction of the heterocyclic N-oxide resazurin (RN→O) to resorufin (RN) in GSH/SeO₃²⁻ systems, i.e. the reactions cycle (7)-(8)-(3) (Prütz, 1994):



H₂O₂ inhibited RN→O reduction, suggesting a competitive chain reaction (9)-(8)-(3),



the sum of which matches the GPx-reaction (1). We now present further evidence that catalytic reduction of H₂O₂ and hydroperoxides by GSH can be activated both by selenite and selenocystamine.

Materials and Methods

Chemicals

The following commercial products were used as received: selenocystamine·2HCl ((CyaSe)₂), cysteamine·HCl (CyaSH), DL-dithiothreitol (DTT), DNA (from salmon testes), glutathione peroxidase (GPx, from bovine erythrocytes, EC 1.11.1.9) resorufin (RN = 7-hydroxy-3H-phenoxazine-3-on Na-salt), and deferoxamine mesylate from Sigma Chemie; *t*-butyl-hydroperoxide, cumene-hydroperoxide, cystamine·2HCl, leuco crystal violet (CVH = Tris-(4-dimethylamino-phenyl)-methane), crystal violet (CV⁺ = Tris-(4-dimethylamino-phenyl)-carboniumchloride) from Aldrich; bovine serum albumine (BSA), EDTA-Na-salt, Gly-Gly-Gly, GSH, GSSG, peroxidase (POD, from horseradish, EC 1.11.1.7), N-acetyl-L-cysteine (NACySH), resazurin (RN→O = 7-hydroxy-3H-phenoxazine-3-on-N-oxide Na-salt), NADH, dodecylsulfate-Na-salt (SDS) and Tris from Serva Feinbiochemica; H₂O₂ 30% (perhydrol, stabilized with ammonium nitrate) from Fluka; KI, HCOONa and SeO₃Na₂·5H₂O from Merck. All solutions were prepared with redistilled water.

Stopped-flow experiments

Stopped-flow measurements were performed with a SFA-12 "Rapid Kinetics Stopped-Flow-Accessory" (Hi-Tech Scientific Ltd.) using a flow-through mixing cell of 1 cm optical path, coupled with a UV-Vis spectrophotometer (Shimadzu Corp.).

Conditions for investigating interactions of selenium compounds with GSH in absence or presence of H₂O₂ are given in Scheme 1. Solutions were freshly prepared for each experiment, and anaerobic conditions were obtained (if desired) by flushing the components gently with high purity N₂, before filling the (gastight) syringes of the stopped-flow system. Stopped-flow was also applied to investigate reduction of RN→O, and reactions of the selenol CyaSeH with H₂O₂ and RN→O.

Determination of H₂O₂

Iodometry was used to calibrate H₂O₂ stock solutions. Consumption of H₂O₂ on incubating the

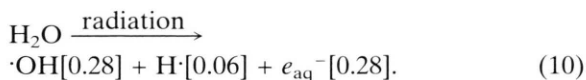
Component A	Component B
0.2 mM SeO_3^{2-} or (CyaSe) ₂ 10–50 mM Buffer ± H_2O_2 and/or $\text{RN} \rightarrow \text{O}$ ± Additive	20 mM GSH 10–50 mM Buffer ± Chelator ^a

Scheme 1. Typical conditions used to initiate Gpx-like reactions of simple selenium compounds. The components **A** and **B** were air-saturated (unless otherwise stated) and mixed 1:1 (v/v) under thermostatically controlled conditions at 20 °C. ^a Tests were made ± chelators, EDTA (20 mM) or deferoxamine (1 mM), to assure that catalysis was not due to transition metal impurities; Cu^{2+} for instance, an efficient catalyst of cytochrome *c* reduction by GSH (Prütz *et al.*, 1994), was found to be unable to catalyse $\text{RN} \rightarrow \text{O}$ reduction by GSH.

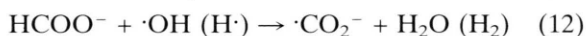
mixed components A and B (Scheme 1) could, however, not be determined with common spectroscopic H_2O_2 assays such as permanganate, iodometry or 4-amino-antipyrin/POD, due to interference by GSH. We have applied, with slight modification, the POD leuco crystal violet (CVH) assay (Mottola *et al.*, 1970), which was found to be applicable also in presence of GSH. After incubation, 20 µl of the mixture [A+B] (Scheme 1) was quickly combined with 600 µl CVH [0.5 mM in 10 mM HCl] and 380 µl POD [200 U/ml] to give the blue protonated dye CVH^{2+} (within seconds); 2 ml buffer + solubilizer [100 mM phosphate (pH 6.8) + 0.1 M SDS] was then added to give the stable violet form CV^+ , the absorption of which was measured at 590 nm. Calibration, i.e. the above procedure without SeO_3^{2-} in the component A, gave an absorbance of $A_{590} = 0.266$ (1 cm cell) per mM H_2O_2 in component A (which may be converted to a virtual absorption coefficient of $\epsilon_{590} = 80000 \text{ cm}^{-1} \text{ M}^{-1}$ of H_2O_2 in the final mixture). The calibration was linear in $[\text{H}_2\text{O}_2]$ up to about 5 mM H_2O_2 in component A; comparison with the absorption of authentic CV^+ indicated that the CVH assay involves a simple 1:1 CV^+ per H_2O_2 stoichiometry.

γ -Radiolysis

γ -Radiolysis was applied to initiate free radical reactions by the water radiolysis products. Equation (10) shows the primary free radical species and radiolytic yields [$\mu\text{M}/\text{Gy}$]:



The reducing radical anions $\text{O}_2^{\cdot-}$ and $\cdot\text{CO}_2^-$ were generated by γ -irradiation of aerated or N_2O -saturated formate (40–200 mM) solutions, where all products of reaction (10) are converted into the respective radical anion (see e.g. Prütz, 1993; Prütz *et al.*, 1994):



The selenyl radical $\text{CyaSe}\cdot$, for example, was generated by reducing $(\text{CyaSe})_2$ with e_{aq}^- (Tamba and Badiello, 1973):



Since $\text{CyaSe}\cdot$, in absence of further additives, can be assumed to recombine to $(\text{CyaSe})_2$, the reactions (14) and (15) also provide a convenient means of generating the selenol CyaSeH . A ^{60}Co - γ -source (Atomic Energy of Canada Ltd.) producing a dose rate of 11 Gy/min was applied.

Results

Reaction of GSH with SeO_3^{2-} in absence and presence of H_2O_2

GSSeSG , the product of reaction (2), has been shown to be remarkably stable at initial concentrations $\text{GSH}/\text{SeO}_3^{2-} < 4$ (Ganther, 1968 and 1971). The sequential stages of reaction (2) have not been resolved in detail in the case of GSH; it is likely that a complex, pH-dependent three-stage mechanism applies as observed with 1-butanethiol (Kice *et al.*, 1980, Kice, 1981). The scenario becomes even more complicated under catalytic conditions ($\text{GSH}/\text{SeO}_3^{2-} \gg 4$) applied in this study. Fig. 1 gives, as an example, absorption spectra at various stages, taken from stopped-flow time profiles at $\text{GSH}/\text{SeO}_3^{2-} = 100$ and pH 6.5, in absence of H_2O_2 . Four consecutive processes are discernible, particularly from the trace at 270 nm (inset). The primary spectrum (at 6.7 s) can be attributed to GSSeSG and its precursors (e.g. $\text{GSSe}(\text{O})\text{SG}$ and GSSeOH), the secondary spectrum (at 28 s) is in our opinion mainly due to GSSeH formed in reaction (3). GSSeH then eliminates elemental

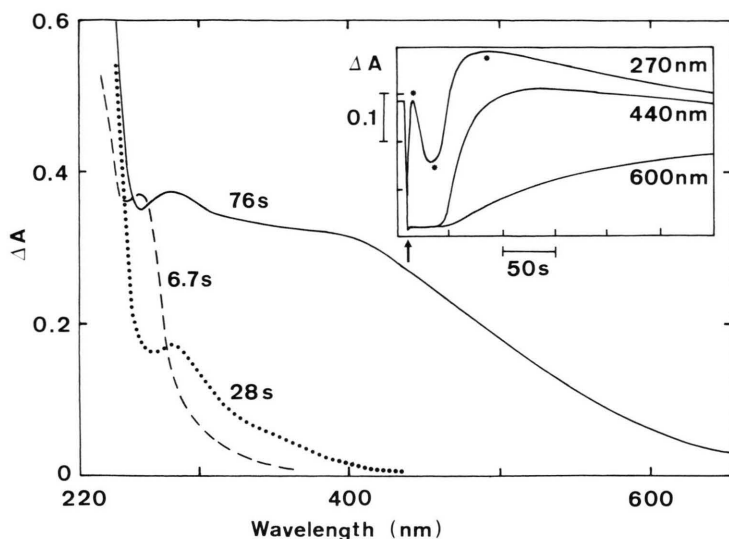


Fig. 1. Stopped-flow spectra and time profiles of absorbance changes induced by interaction between selenite and glutathione. The results were obtained for the conditions given in Scheme 1 in absence of H_2O_2 , using 50 mM phosphate (pH 6.5), 50 mM NaCl (as additive) and 25 mM EDTA. Spectra at 6.7, 28 and 76 sec were taken from stopped-flow ($\uparrow$) time profiles, such as those shown in the inset, at the times marked (*).

Se° , reaction (4), with a broad visible absorption (spectrum at 76 s) which continues to change in a time scale of minutes (see 270 and 600 nm traces) due to colloid formation. We have confirmed that the kinetics change dramatically when going to acid solutions (Ganther, 1971). The elimination of Se° was most pronounced around pH 6.5, and efficiently inhibited at pH > 7, probably due to deprotonation promoting the reverse reaction (4).

In a first attempt to demonstrate reaction (9) we have inspected its competition with the Se° -eliminating reaction (4). Fig. 2 shows that Se° -elimination at pH 6.4, detected by the build-up of 400 nm absorption, is progressively retarded on

increasing the H_2O_2 concentration. According to our model, reaction (4) is suppressed in presence of H_2O_2 due to redox cycling of GSSeH through reactions (9), (8) and (3); the delayed onset of Se° -elimination marks the depletion of H_2O_2 . From the time dependence (Fig. 2) we conclude that roughly 1 minute is required to remove 0.5 mM H_2O_2 , thus the activity could be in the order of 5 $\mu\text{mol H}_2\text{O}_2/\text{min}$ per $\mu\text{mol SeO}_3^{2-}$ applied to initiate catalysis. More accurate estimates are given below.

Interactions of resazurin and H_2O_2 with selenium compounds

Resazurin ($\text{RN} \rightarrow \text{O}$) changes colour from blue (absorption peaks at 600 and 380 nm) to pink (absorption peak at 565 nm) upon reduction to resorufin (RN) (DeBaum and de Stevens, 1951) [spectra and the structure of $\text{RN} \rightarrow \text{O}$ are shown below, Fig. 6]. Heterocyclic N-oxides like $\text{RN} \rightarrow \text{O}$ are of interest also because they may interact with selenium compounds like peroxides, by O-atom transfer (Prütz, 1994). The data presented in Fig. 3 show that reduction of $\text{RN} \rightarrow \text{O}$ by GSH is activated both by SeO_3^{2-} and $(\text{CyaSe})_2$ and is inhibited in both cases by H_2O_2 . $(\text{CyaSe})_2$ -activated reduction of $\text{RN} \rightarrow \text{O}$ by GSH was inhibited with similar efficiency also by *t*-butyl- and cumene-hydroperoxide, but SeO_3^{2-} -activated reduction was hardly inhibited by these two hydroperoxides. In

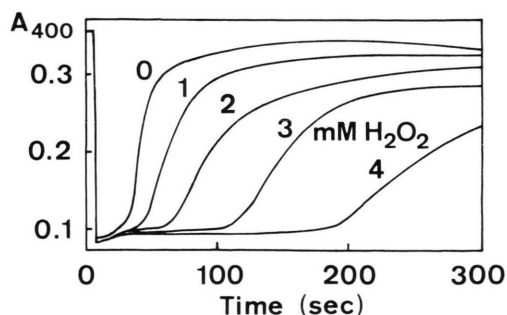


Fig. 2. Stopped-flow time profiles of 400 nm absorbance changes showing that H_2O_2 competes with Se° elimination upon interaction of SeO_3^{2-} with GSH. The results refer to the conditions given in Scheme 1, using 50 mM phosphate (pH 6.4) and without chelator in component B. Initial H_2O_2 concentrations in component A are indicated.

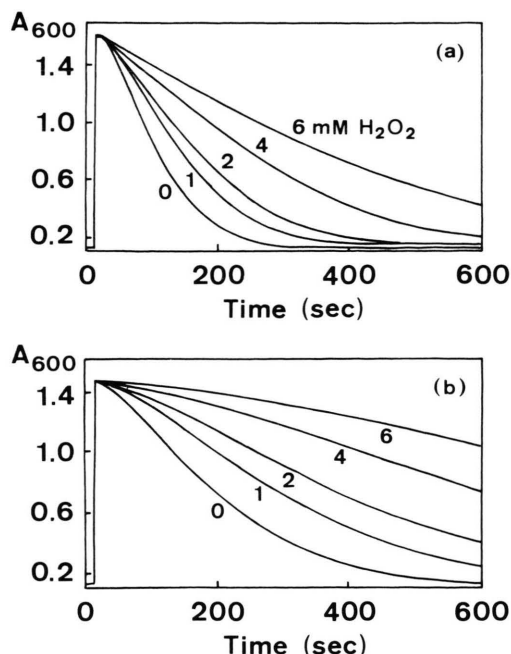


Fig. 3. Effect of H₂O₂ on the reduction of resazurin by GSH in presence of SeO₃²⁻ (a), or selenocystamine (b). Conditions as in Scheme 1, but component A containing both H₂O₂ and 100 μ M RN $\rightarrow$ O, and (a) 50 μ M SeO₃²⁻ in 50 mM Tris (pH 7.2), (b) 100 μ M (CyaSe)₂ in 50 mM phosphate (pH 6.8) without chelator in component B; H₂O₂ concentrations (in component A) are indicated. Reduction of RN $\rightarrow$ O to RN is monitored by the absorption change at 600 nm (Prütz, 1994; spectra are shown in Fig. 6). In the time scale of 10 min there was no RN $\rightarrow$ O reduction by GSH in absence of the Se-compounds, and no reduction by the Se-compounds in absence of GSH.

the context of specificity we have recognized that GPx is unable catalyse reduction of RN $\rightarrow$ O by GSH.

The inhibitory effect of H₂O₂ on the selenium-catalysed reduction of RN $\rightarrow$ O by GSH (Fig. 3) might tentatively be explained by reoxidation (RN + H₂O₂ $\rightarrow$ RN $\rightarrow$ O + H₂O). Addition of H₂O₂ to solutions of authentic RN gave however no evidence for such a reaction. The interaction of SeO₃²⁻ with GSH under aerobic conditions has been proposed to generate the O₂^{·-} radical (Seko *et al.*, 1989), a reducing entity which would be removed by H₂O₂. However, also this mechanism cannot explain the results in Fig. 3, because O₂^{·-} is unable to reduce RN $\rightarrow$ O; this was established by γ -radiolytic generation of O₂^{·-} (Materials and Methods) in the presence of RN $\rightarrow$ O.

When SeO₃²⁻ was preincubated with GSH for a few minutes before addition of RN $\rightarrow$ O (in absence of H₂O₂), the reduction of RN $\rightarrow$ O became much slower. This is probably due to a loss of the reductant GSSeH, proposed to act in reaction (7), by the reactions (4) and (16),



If H₂Se was the reducing entity, as has been suggested in the case of reduction of methylene blue (Rhead and Schrauzer, 1974) and oxygen (Seko *et al.*, 1989) in presence of SeO₃²⁻ and GSH, one would have expected faster rather than slower reduction of RN $\rightarrow$ O after preincubation of SeO₃²⁻ with GSH. This provides a further argument in favour of the reaction (7).

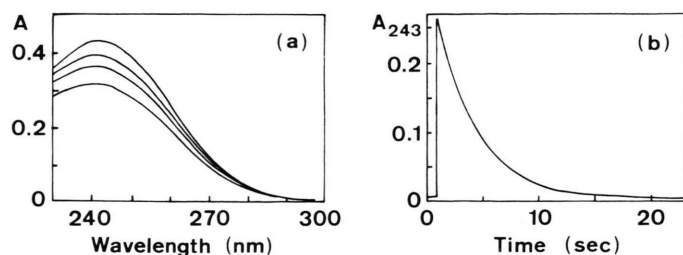


Fig. 4. Spectrum of CyaSeH (a), and kinetics of removal of CyaSeH by H₂O₂ (b). The results were obtained after 160 Gy γ -irradiation of a deaerated (N₂-saturated) solution containing 400 μ M (CyaSe)₂, 20 mM phosphate (pH 7.5), 0.2 M HCOONa and 400 μ M EDTA (see Materials and Methods). The spectra (a), recorded against the unirradiated solution, were taken (downward) at 0.5, 20, 40 and 60 min after γ -irradiation. The stopped-flow time profile (b) was obtained by rapid mixing of the above system with a deaerated 600 μ M H₂O₂ solution, within 4 min after γ -radiolysis.

When $(\text{CyaSe})_2$ was preincubated with GSH for a few minutes before addition of $\text{RN} \rightarrow \text{O}$ (in absence of H_2O_2), the reduction of $\text{RN} \rightarrow \text{O}$ became faster. Generation of the selenol CyaSeH , which is considered to be the active reductant in this system, is apparently relatively slow. In order to demonstrate the reaction of CyaSeH with $\text{RN} \rightarrow \text{O}$ and H_2O_2 we have generated CyaSeH γ -radiolytically (Materials and Methods). CyaSeH , with an absorption peak at 243 nm, is fairly stable under anaerobic conditions, as shown in Fig. 4a. This result is consistent with previous data obtained with selenocystine after incubation with GSH (Dickson and Tappel, 1969). In air-saturated solution CyaSeH is decomposed within about 10 minutes, with formation of secondary products absorbing in the UV. The stopped-flow time profile in Fig. 4b shows the fast removal of CyaSeH by H_2O_2 under anaerobic conditions. The reaction kinetics were mono-exponential and first-order in $[\text{H}_2\text{O}_2]$, and similar results were obtained upon mixing CyaSeH with *t*-butyl- or cumene-hydroperoxide solutions. Rate constants are given in Table I, they are notably higher than the rate of reaction of the ebselen selenol with H_2O_2 , $k = 0.47 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$ (Morgens-tern *et al.*, 1992). $\text{RN} \rightarrow \text{O}$ was also reduced by CyaSeH , however, the reaction was slow (10 min time scale), and due to the natural decay of CyaSeH it was not possible to give an accurate estimate of the rate constant.

The catalytic activity of simple selenium compounds

The results presented in Fig. 5 show the decomposition of H_2O_2 by GSH in presence of SeO_3^{2-} and $(\text{CyaSe})_2$, respectively, as detected with the CVH/POD assay (Materials and Methods). Catalytic activities, estimated from initial slopes in Fig. 4 are collected in Table II, together with val-

Table I. Rate constants of reaction of CyaSeH with H_2O_2 and hydroperoxides.

Peroxide	$k(\text{CyaSeH} + \text{ROOH})^a$ ($\text{M}^{-1} \text{ s}^{-1}$)
H_2O_2	9.7×10^2
<i>t</i> -Butyl-OOH	1.2×10^2
Cumene-OOH	2.5×10^2

^a Rate constants estimated from time profiles as in Fig. 4b under anaerobic conditions.

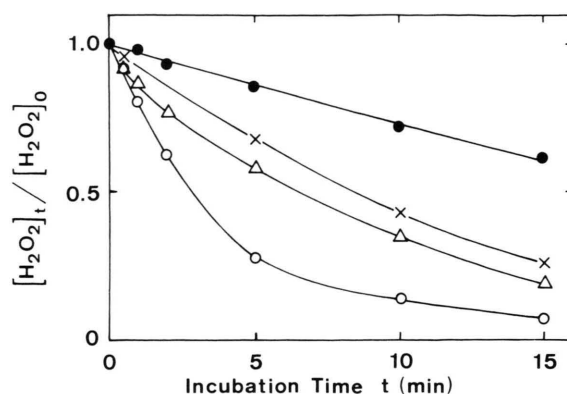


Fig. 5 Decomposition of H_2O_2 by incubation with GSH in presence of SeO_3^{2-} or selenocystamine. The results refer to incubation at 20°C of the components shown in Scheme 1, using 4 mM H_2O_2 , 10 mM phosphate (pH 6.8), 0.1 M NaCl (as additive), without chelator in component B. H_2O_2 was determined by the CVH/POD assay (Materials and Methods): (●) control without Se-compound, (○) 200 μM SeO_3^{2-} ; (×) as (○) but SeO_3^{2-} added to component B (Scheme 1) and incubated 10 min before mixing with H_2O_2 (component A without SeO_3^{2-}); (Δ) 200 μM $(\text{CyaSe})_2$.

Table II. Catalytic decomposition of H_2O_2 by thiols in presence of selenium activators.

No.	Activator (200 μM) ^a	Thiol (20 mM)	Additive	Activity ^b
1	$(\text{CyaSe})_2$	GSH	–	2.6
2	$(\text{CyaSe})_2$	GSH	deaerated	2.8
3	SeO_3^{2-}	GSH	–	3.6
4	SeO_3^{2-}	GSH	deaerated	3.4
5	SeO_3^{2-}	GSH	1 mM GSSG	3.4
6	SeO_3^{2-}	GSH	1 mM NADH	3.5
7	SeO_3^{2-}	GSH	0.1 g/L DNA	3.6
8	SeO_3^{2-}	GSH	0.1 g/L BSA	3.9
9	SeO_3^{2-}	GSH	0.2 mM Hg^{2+}	1.3
10	SeO_3^{2-}	GSH	0.2 mM Cu^{2+}	1.1 ^c
11	SeO_3^{2-}	DDT	–	17.2
12	SeO_3^{2-}	CyaSH	–	$\approx 15^d$

^a 20 μM in nos. 11 and 12.

^b Activities were estimated from initial rates (conditions as in Fig. 5), subtracting the rate of spontaneous H_2O_2 decomposition by the thiol alone. Numbers given are $\mu\text{mol H}_2\text{O}_2/\text{min}$ per μmol activator applied to initiate catalysis. Stated are concentrations before mixing components A and B (Scheme 1).

^c Cu^{2+} activates spontaneous H_2O_2 decomposition by GSH, but reduces the activity of selenite.

^d Spontaneous H_2O_2 decomposition by cysteamine is relatively fast.

ues obtained in various environments and with other thiols. In the absence of GSH there was no detectable loss of H_2O_2 upon addition of SeO_3^{2-} or $(\text{CyaSe})_2$, even at concentrations equal to $[\text{H}_2\text{O}_2]$. Decomposition of H_2O_2 by GSH in presence of selenocystine has previously been proposed to involve an O-atom transfer from H_2O_2 to the diselenide (Caldwell and Tappel, 1965), our results are however not consistent with such a reaction in the case of $(\text{CyaSe})_2$, and they also exclude a direct interaction between H_2O_2 and SeO_3^{2-} . The CVH/POD assay is not applicable to hydroperoxides; the effects of hydroperoxides on $\text{RN} \rightarrow \text{O}$ reduction (see above) certainly indicate differences in the specificity of SeO_3^{2-} - and $(\text{CyaSe})_2$ -activated reduction of hydroperoxides, but unfortunately we cannot present numbers for the activities.

The GPx-catalysed decomposition of H_2O_2 by GSH, when examined with the CVH/POD assay at conditions similar to those in Table II, gave an activity of about 9000 $\mu\text{mol H}_2\text{O}_2/\text{min}$ per $\mu\text{mol Se}$ provided by the protein. This demonstrates the remarkably high turnover rate of GPx as compared to the data in Table II. The GPx-like activity induced by $(\text{CyaSe})_2$ (Table II, no. 1) is actually similar to that of diphenyl diselenide but higher than that of ebselen (Wilson *et al.*, 1989), previously estimated from GSSG formation rates using the glutathione reductase-NADPH assay; ebselen may however be more specific with hydroperoxides formed in biomembranes and lipoproteins (Schewe, 1995). A comparison of the activities induced by $(\text{CyaSe})_2$ and SeO_3^{2-} (Table II, nos. 1 and 3) indicates that the latter is the more powerful system. It must be emphasized, however, that GSSeH is less stable than CyaSeH. The GPx-like activity induced by selenite was in fact much lower when SeO_3^{2-} was preincubated few minutes with GSH prior to addition of H_2O_2 (cf. Fig. 5 ($\times$) and ($\circ$)), resembling the behaviour found with $\text{RN} \rightarrow \text{O}$ as substrate (see above). This leads to the conclusion that also H_2O_2 decomposition does not involve Se^0 and H_2Se , the products of reactions (4) and (16). Addition of GSSG, which should reverse reaction (16), had no effect on the activity of the GSH/ SeO_3^{2-} system (Table II, no. 5).

Selenium-activated reduction of H_2O_2 is unaffected by oxygen (Table II, nos. 1 and 3 compared with 2 and 4), as previously recognized also in the

case of SeO_3^{2-} -activated reduction of $\text{RN} \rightarrow \text{O}$ (Prütz, 1994). Interactions of O_2 with selenium derivatives of thiols described in literature (Tsen and Tappel, 1958; Dickson and Tappel, 1969; Seko *et al.*, 1989) are apparently much slower than the catalytic reactions under discussion. As already mentioned, GSH has been proposed to interact with SeO_3^{2-} under aerobic conditions to generate $\text{O}_2^{\cdot-}$ (Seko *et al.*, 1989). However, since H_2O_2 removal in the present systems is oxygen-independent it appears unlikely that H_2O_2 decomposition was due to the Haber-Weiss reaction, $\text{H}_2\text{O}_2 + \text{O}_2^{\cdot-} \rightarrow \text{O}_2 + \text{OH}^- + \cdot\text{OH}$. Further arguments against free radical mechanisms, as alternatives to the O-atom transfer reactions (7) and (9) are presented below (Discussion).

Decomposition of H_2O_2 in the GSH/ SeO_3^{2-} system is not impeded by additives like NADH, DNA or BSA (Table II, nos. 6 to 8), but is inhibited by Cu^{2+} and Hg^{2+} ions (Table II, nos. 9 and 10); both metal ions also inhibited reduction of $\text{RN} \rightarrow \text{O}$. The inhibitory effect of Cu^{2+} is in stark contrast to its stimulatory effect on selenium catalysed reduction of methylene blue (MB) (Rhead and Schrauzer, 1974). The reason for this difference may be that the reduction proceeds by O-atom transfer in the case of H_2O_2 and $\text{RN} \rightarrow \text{O}$, and by electron transfer in the case of MB. Hg^{2+} is commonly known to be an antagonist of selenium catalysed reactions (Levander *et al.*, 1973; Rhead and Schrauzer, 1974; Masukawa and Iwata, 1977; Shamberger, 1983c), probably due to formation of inactive selenium-mercury complexes. Mixing of the components A and B (Scheme 1) in the presence of SeO_3^{2-} and Hg^{2+} (without H_2O_2) actually led to immediate formation of stable tawny compounds, indicating that Hg^{2+} rapidly interacts with the products of reactions (2) to (4).

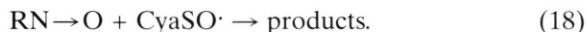
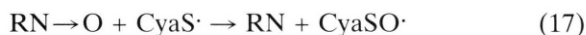
Of the few thiols tested, DTT is the most efficient donor of SeO_3^{2-} -activated H_2O_2 decomposition (Table II, no. 11). Also CyaSH, a poor donor for GPx (Flohé, 1982), is more efficient than GSH in the SeO_3^{2-} system (Table II, no. 12).

Free radical interactions with resazurin

In the context of the O-atom transfer reaction (7), we have considered the possibility that $\text{RN} \rightarrow \text{O}$ may behave as an O-atom donor also in reactions with certain free radicals. γ -Radiolysis of

a N_2 -saturated solution containing $\text{RN} \rightarrow \text{O}$ and formate in presence of either $(\text{CyaSe})_2$ or $(\text{CyaS})_2$ revealed a stark contrast in the behaviour of these two compounds. The selenyl radical $\text{CyaSe}^\cdot$ generated upon irradiation (Materials and Methods) did not react with $\text{RN} \rightarrow \text{O}$. The thiyl radical $\text{CyaS}^\cdot$, on the other hand, generated analogous to reactions (14) and (15) by reduction with e_{aq}^- and $\cdot\text{CO}_2^-$ (Adams *et al.*, 1967; Willson, 1970), interacted extensively with $\text{RN} \rightarrow \text{O}$, as shown in Fig. 6a. The loss of 380 nm absorption of $\text{RN} \rightarrow \text{O}$ with increasing dose corresponds to the removal of more than 1.5 $\text{RN} \rightarrow \text{O}$ per $\text{CyaS}^\cdot$, and it is particularly interesting to note that $\text{CyaS}^\cdot$, though it is an oxidant

(Forni and Willson, 1986), generates the characteristic 565 nm absorption band of the reduced dye. Fig. 6b shows for comparison the spectra of $\text{RN} \rightarrow \text{O}$, and of RN after reduction by $(\text{CyaSe})_2/\text{NACySH}$, a system resembling the $(\text{CyaSe})_2/\text{GSH}$ system (Fig. 3b). Additional products with absorptions extending up to 700 nm are seen in Fig. 6a. Our results can be explained by assuming that $\text{RN} \rightarrow \text{O}$ reacts with $\text{CyaS}^\cdot$ by O-atom transfer to form a reactive thiyl-oxyl (sulfinyl) radical which further interacts with $\text{RN} \rightarrow \text{O}$:



It was recognized that $\text{RN} \rightarrow \text{O}$ readily interacts with a variety of radiolytically generated radicals; that the $\text{CyaSe}^\cdot$ radical is inert with $\text{RN} \rightarrow \text{O}$ appears almost as an exception. Free radicals generated by reaction of $\cdot\text{OH}$ with Gly-Gly-Gly, for instance, led to $\text{RN} \rightarrow \text{O}$ reduction at a yield of 2 RN per peptide-radical, suggesting that these radicals can accept two O-atoms from $\text{RN} \rightarrow \text{O}$ (possibly to form peroxy radicals); oxygen completely inhibited $\text{RN} \rightarrow \text{O}$ reduction by $\cdot\text{OH}$ -products of Gly-Gly-Gly. Pulse radiolysis techniques will be required to investigate in further detail the mechanisms of such interactions of resazurin with various free radicals. Also peroxides have a tendency to interact with free radicals by O-atom transfer; the reaction of $\cdot\text{OH}$ with H_2O_2 to yield $\text{HO}_2^\cdot$ and H_2O (Buxton *et al.*, 1988) is a classical example.

Discussion

SeO_3^{2-} -activated reduction of $\text{RN} \rightarrow \text{O}$ and H_2O_2 by GSH has been proposed to involve O-atom transfer from these substrates to GSSeH , reactions (7) and (9), and recycling of GSSeOH to GSSeH via reactions (8) and (3) (Prütz, 1994). The present results support this mechanism. Particularly we have shown that: (a) H_2O_2 competes with Se° -elimination from GSSeH by reaction (4) (Fig. 2), (b) inhibition of $\text{RN} \rightarrow \text{O}$ reduction by H_2O_2 (Fig. 3a) cannot be explained by reoxidation ($\text{RN} + \text{H}_2\text{O}_2 \rightarrow \text{RN} \rightarrow \text{O} + \text{H}_2\text{O}$) or by $\text{O}_2^{\cdot-}$ -scavenging ($\text{O}_2^{\cdot-} + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \text{OH}^- + \cdot\text{OH}$) and is therefore likely to be due to competition between the reactions (9) and (7), (c) the effect of preincubation of SeO_3^{2-} with GSH before addition of

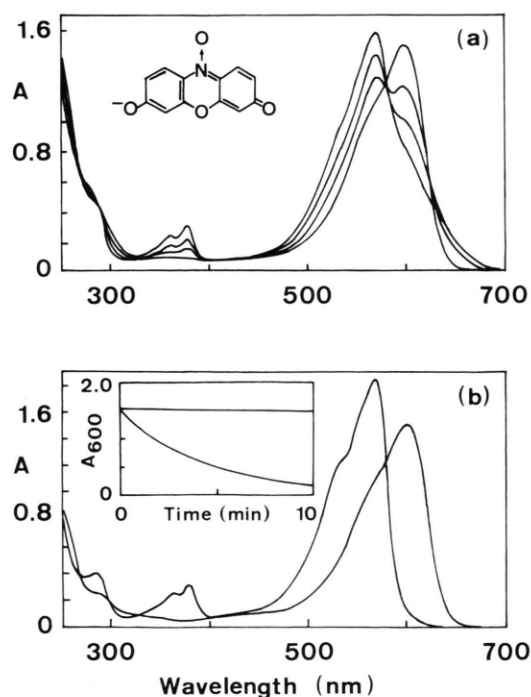


Fig. 6 Absorption spectra of $\text{RN} \rightarrow \text{O}$, and of products formed by interaction (a) with $\text{CyaS}^\cdot$, and (b) with $(\text{CyaSe})_2/\text{NACySH}$. (a) A N_2 -saturated solution containing 2 mM $(\text{CyaS})_2$, 40 mM HCOONa , 50 μM $\text{RN} \rightarrow \text{O}$ and 10 mM phosphate (pH 6.8) was irradiated with 0, 20, 40 and 60 Gy; in this order the absorption of $\text{RN} \rightarrow \text{O}$ (380 and 600 nm peaks) is bleached, due to reaction of $\text{CyaS}^\cdot$, with formation of RN (565 nm peak) and other products absorbing above 650 nm. The structure of $\text{RN} \rightarrow \text{O}$ is shown. (b) 50 μM $\text{RN} \rightarrow \text{O}$ was incubated with 50 μM $(\text{CyaSe})_2$ and 10 mM NACySH in 50 mM phosphate (pH 6.8) to give complete reduction to RN after 15 min, shown by the 565 nm absorption which is identical to authentic RN . Insert: time profiles at 600 nm in presence and absence (upper curve) of $(\text{CyaSe})_2$.

$\text{RN} \rightarrow \text{O}$ or H_2O_2 is also consistent with a loss of the reducing entity GSSeH by reactions (4) and (16), (d) H_2O_2 is in fact decomposed catalytically in the SeO_3^{2-} /GSH system (Fig. 5a, Table II). The possibility of electron transfer, as opposed to O-atom transfer, is discussed below.

The $(\text{CyaSe})_2$ /GSH system bears analogies to the SeO_3^{2-} /GSH system (Figs. 3 and 5). It is long known that H_2O_2 -induced oxidation of GSH can be accelerated by selenocystine. However, this was tentatively explained (Caldwell and Tappel, 1965) by a mechanism which is not catalytic, and we have already pointed out that the proposed O-atom transfer from H_2O_2 to diselenide is not detectable in the case of $(\text{CyaSe})_2$. Interaction of seleno-cystine with thiols was later found to yield selenol (Dickson and Tappel, 1969), and we adopt this reaction scheme for the $(\text{CyaSe})_2$ system:



In the present paper it is shown that CyaSeH reacts fairly fast with H_2O_2 (Fig. 4b, Table I). The results in Fig. 3b can again be explained by an O-atom transfer mechanism with competition between $\text{RN} \rightarrow \text{O}$ and H_2O_2 , i.e. reactions (21) and (22),



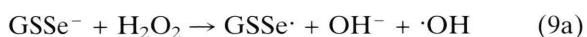
followed by the reactions (23) and (20),



The reaction cycle (22)-(23)-(20), involving formation of a selenenic acid transient, CyaSeOH , actually corresponds to the reaction mechanism proposed for the seleno-enzyme GPx (Wendel, 1980; Flohé, 1982). In adopting the selenenic acid pathway of GPx in the above reaction cycle we do not exclude seleninic acid pathways (Wendel, 1980; Kice, 1981; Flohé, 1982), i.e. H_2O_2 or $\text{RN} \rightarrow \text{O}$ reacting by O-atom transfer with CyaSeOH to form $\text{CyaSe}(\text{O})\text{OH}$ which then interacts with two GSH to regenerate CyaSeOH (+ H_2O and GSSG). Selenite-activated reduction, proposed to involve the reaction cycle (9)-(8)-(3), might alternatively also be explained by O-atom transfer from H_2O_2 (or $\text{RN} \rightarrow \text{O}$) to GSSeOH , and recycling of $\text{GSSe}(\text{O})\text{OH}$ by GSH.

The rate constant $k_{22} = 9.7 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$ (Table I) means that CyaSeH removal at 2 mM H_2O_2 (used in Fig. 5) would proceed with a half-life of about 0.35 s. The rate-determining step in the relatively slow decomposition of H_2O_2 ($t_{1/2} \sim 7$ min, see Fig. 5) is therefore certainly not the reaction (22), but rather the recycling reaction sequence (23)-(20). The high turnover rate of GPx, as compared to the selenium compounds tested in this study, is obviously due to a much faster recycling of the active selenol group.

As shown by reaction (5), GSSe^- has been assumed to react with cytochrome *c* by one-electron transfer to generate free radicals ($\text{GSSe}^\cdot$). Also other Se-derivatives have been suggested to interact with electron acceptors to produce free radicals (Levander *et al.*, 1973; Rhead and Schrauzer, 1974; Seko *et al.*, 1989). If the reactions (9) and (22) were to proceed by one-electron transfer steps, one might even expect that the primary step involves generation of $\cdot\text{OH}$ radicals, as in the Fenton reaction (see Introduction):



We have no evidence, however, for the occurrence of reactions (9a) and (22a). As shown in Table II (no. 6), the addition of NADH to the GSH/selenite system has no effect on H_2O_2 decomposition. Particularly, there was no indication of NADH oxidation when SeO_3^{2-} or $(\text{CyaSe})_2$ systems were incubated as shown in Scheme 1 in presence of H_2O_2 (1 mM) and NADH (100 to 500 μM , data not shown). If $\cdot\text{OH}$ had been generated in reactions (9a) and (22a) it would either have oxidized NADH directly, or it would have interacted with GSH (present in excess) to form the $\text{GS}^\cdot$ radical which is known to oxidize NADH (Forni and Willson, 1986). Little is known about reactions of Se-containing free radicals, with exception of the selenite radical $\text{SeO}_3^{\cdot-}$ which is a powerful oxidant (Tamba and Badiello, 1985; Neta *et al.*, 1988) and reactions of some organic selenyl radicals of more chemical interest (Deryagina *et al.*, 1993). By analogy with sulfur-centered radicals (Chatgililoglu and Asmus, 1990), one might expect that $\text{RSe}^\cdot$ and $\text{RSSe}^\cdot$ species are fairly strong oxidants, thus reaction (5) would rather proceed to the left (Prütz, 1993). We are unable,

though, to offer an alternative to the proposed electron transfer between selenium centres and cytochrome *c*.

$\text{RN} \rightarrow \text{O}$, as mentioned above, is not reduced by $\text{O}_2^{\cdot-}$. The inference is that the one-electron reduction potential of the $\text{RN} \rightarrow \text{O}/(\text{RN} \rightarrow \text{O})^{\cdot-}$ couple is likely to be more negative than that of oxygen, $E^\circ(\text{O}_2/\text{O}_2^{\cdot-}) = -155 \text{ mV}$. The additional experience that reduction of $\text{RN} \rightarrow \text{O}$ by GSSeH is not impeded by O_2 (Prütz, 1994) seems to imply that GSSeH is not removed by reducing O_2 to $\text{O}_2^{\cdot-}$. Since one-electron reduction of $\text{RN} \rightarrow \text{O}$ is even less feasible than of O_2 , we conclude that also reduction of $\text{RN} \rightarrow \text{O}$ by GSSeH is not a two-step electron transfer process, with formation of free radical intermediates, but rather an O-atom transfer process as depicted by reaction (7). $\text{RN} \rightarrow \text{O}$ seems on the other hand capable of interacting with certain free radicals to form oxyl radicals, e.g. reaction (17). Heterocyclic N-oxides like benzotriazine-di-N-oxides are hypoxic cell toxins (Laderoute *et al.*, 1988; Butler and Hoey, 1993), and nitro-quinoline-N-oxide, as another example, is a potent mutagen and carcinogen which might be activated by ascorbate (Bielski, 1982). The deleterious effects of these N-oxides have been proposed to be mediated by the generation of one-electron reduction products of the parent

compounds; the biological effects of N-oxides might, on the other hand, be related also to their ability to act as O-atom donors. Further investigations are required to test this concept.

The catalytic reduction of H_2O_2 by simple selenium compounds in combination with thiols is an interesting example of their antioxidative capability. In biological systems the instability of these compounds, and formation of inactive metabolites such as Se° and trimethylselenonium ions (Martin, 1973; Vadhanavikit *et al.*, 1993; Groeger and Ganther, 1994) may limit their activity. Proteins appear to provide specific stabilizing interactions for the intermediates of selenium-catalysed reactions, not only in the case of GPx, but also with GPx-mimic proteins such as subtilisin modified by incorporation of a selenol group (Wu and Hilvert, 1990). The question arises whether the SSeH group, which appears to be more active than the SeH group, at least with H_2O_2 (Table II), can be stabilized in proteins or by certain carrier molecules.

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