Role of Thiols in Radioprotection: Radiation Chemical Aspects

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Using the technique of pulse radiolysis, it has been demonstrated that thiyl radicals ($RS^{\cdot}$) derived from glutathione (GSH), cysteine (CYSH), penicillamine (PnSH) and 2-mercaptoethanol (ME) interact with oxygen at a high rate. The resulting transient absorption, with a maximum around 540–560 nm, is characteristic of the sulphur peroxyl radical ($RSOO^{\cdot}$). The yield and the kinetic of formation of $RSOO^{\cdot}$ further support our previous suggestion that thiyl/O_2 reaction is an equilibrium. The redox properties of $RSOO^{\cdot}$ are discussed on the basis of the interaction with reductants. Studies on the radio-induced enzyme inactivation in the presence of thiols seem to suggest a damaging role for $RSOO^{\cdot}$ radicals.

Introduction

The study of chemical radioprotectors was initially stimulated from the potential military applications in protecting soldiers from nuclear weapons. The importance of sulphur compounds as modifiers of radiation response dates almost 40 years ago, when it was observed that the presence of some exogenous thiols at the time of irradiation resulted in protection of biological systems *in vitro* [1] as well as of animals *in vivo* [2]. Numerous compounds with radioprotective potential, mainly sulphur containing, have since been designed, synthesized and tested *in vitro* and *in vivo* systems.

As research developed, the interest in chemical radioprotection came also from the therapeutic potential of radioprotectors in cancer therapy [3]. The strategy is to increase by chemicals, the effectiveness of radiation therapy on the anoxic fraction of the tumor, but not on normally oxygenated tissues. This strategy is often limited by the drug toxicity at the relatively large concentration required to achieve a significant therapeutic gain, other than by the impredictable large variability of the response in both normal tissues and tumors [4, 5].

The different purposes which aim the research on chemical radioprotection help to develop considerably the scientific understanding of the action of thiols and related compounds at molecular, cellular and tissue levels. The likely involvement of free radicals in radiation-induced biological damage makes that the study of simple chemical biological model systems is invaluable in exploring the molecular basis of free-radical damage and its modification (radioprotection and radiosensitization) [6].

This paper, in addition to a brief survey of the mechanisms proposed to account for the radioprotection by thiols (RSH), gives results of experiments on the interaction of molecular oxygen with thiyl radicals ($RS^{\cdot}$). This reaction, involving the most prominent sulphur centered radical intermediate in radiobiological processes, may be of biological significance in affecting cellular radiosensitivity [7].

Materials and Methods

Glutathione (GSH), cysteine (CySH), penicillamine (PnSH) and 2-mercaptoethanol (ME) from Fluka (Buchs, Switzerland), and other chemicals of analytical reagent grade were used as received. Lysozyme was obtained from Boehringer Co. and was used without further purification.

The lysozyme stock solutions were diluted to 0.1 mg/ml with 1 mM phosphate buffer pH 7. The final concentration was estimated from the absorbance at 280 nm using an extinction factor $\varepsilon_{280} = 2.6$ for 0.1 per cent lysozyme solution [8].

Lysozyme activity was determined by monitoring the change in turbidity of a suspension of lyophylized *Micrococcus lysodeikticus* as a function of time after addition of enzyme solutions [9].

Solutions to be irradiated were saturated prior and during irradiation, with appropriate gas in a special glass vessel. To avoid foaming, the gas was passed over the surface of the solutions stirred slowly by a magnetic stirrer. Samples were taken periodically by the vessels without disturbing the gas flow.

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The steady-state irradiations were performed at room temperature in a AECL^{60} Co-cell with a dose rate of about 130 Gy per minute as determined by Fricke dosimetry.

The pulse radiolysis experiments were performed with a 12 MeV linear accelerator (Radiation Dynamics). The optical path-length of the irradiation cell was generally 5 cm. Dosimetry was carried out using the thiocyanate system. The processing of the data was made on line by a digitizer-computer system consisting of a DEC PDP 11/23 and a Tektronix Transient Digitizer AD 7912.

Results and Discussion

Chemical mechanisms of radioprotection

Various chemical mechanisms on the mode of action of thiols have been suggested and modified on the basis of the growing knowledge on the chemistry of sulphur radicals [10]. These include, as shown in Fig. 1:

i. Scavenging of damaging primary water radicals, mainly ·OH, with consequent prevention or drop of indirect damage to the biological target, TH;

$$TH + {}^{\cdot}OH \rightarrow T^{\cdot} + H_2O \quad (1)$$

$$RSH + {}^{\cdot}OH \rightarrow RS^{\cdot} + H_2O. \quad (2)$$

ii. Hydrogen atom donation from the thiol to the damaged target molecule, T·, in competition with the fixation of damage by oxygen [11].

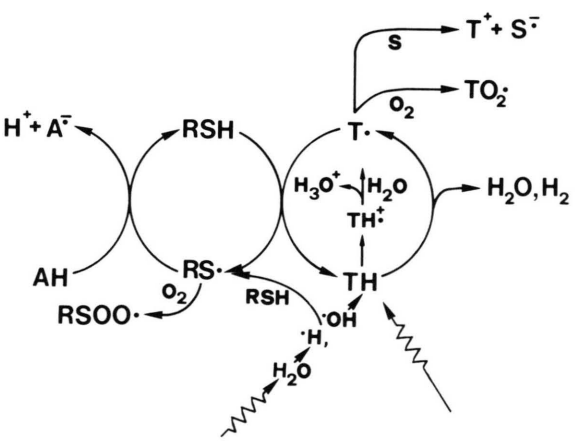

Fig. 1. Some possible mechanisms of radiation damage and protection. TH = target molecule; S = sensitizer; AH = antioxidant.

$$T^{\cdot} \xrightarrow{RSH} TH + RS^{\cdot} \quad (3)$$

$$T^{\cdot} \xrightarrow{O_2} TOO^{\cdot} \quad (4)$$

This model is kinetically feasible, considering the intracellular thiol level, mainly constituted of glutathione (0.1–10 mM), the oxygen concentration in oxic cells (0.2 mM) and the rate constant for reaction (4), about two order of magnitude faster than reaction (3), as estimated by pulse radiolysis of model systems [12, 13].

The physico-chemical properties themselves of the thiol group -SH, as shown in Table I for GSH, foretell for the effectiveness of RSH in scavenging and hydrogen-donation processes.

Both the above processes yield thiyl radicals, which were originally supposed to be quite unreactive and to decay mainly bimolecularly giving the corresponding biologically harmless disulphides.

$$RS^{\cdot} + RS^{\cdot} \rightarrow RSSR. \quad (5)$$

Recent results make the thiyl radicals much more reactive than commonly believed. In fact the radical, which has an oxidizing nature ($E°(RS^{\cdot}/RS^{-}) \approx +1.0$ V) [14, 15], has been observed to react rapidly with antioxidants, including ascorbate and NADH, to abstract carbon-bound H atoms [16] and to add rapidly molecular oxygen [17].

Interaction of thiyl radicals with oxygen

The reaction of molecular oxygen with thiyl radicals (reaction 6) has been investigated by pulse radiolysis of oxygen containing solutions of various sulphydryl compounds.

$$RS^{\cdot} + O_2 \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} RSOO^{\cdot}. \quad (6)$$

Table I. Some physico-chemical properties of the SH group of glutathione.

Parameter	
Bond length [Å]	1.33
Dissociation energy (Kcal/mol)	89
Electronegativity (Li = 1)	2.6
pK_a (–SH)	9.2
Redox potential E_o [V]	−0.205
H abstraction by H atoms [$M^{-1}s^{-1}$]	$\approx 4 \times 10^9$
H abstraction by OH [$M^{-1}s^{-1}$]	1.5×10^{10}

GSH = $O_2^-CCH(NH_3^+)CH_2CH_2CONHCH(CH_2SH)CONHCH_2CO_2^-$.

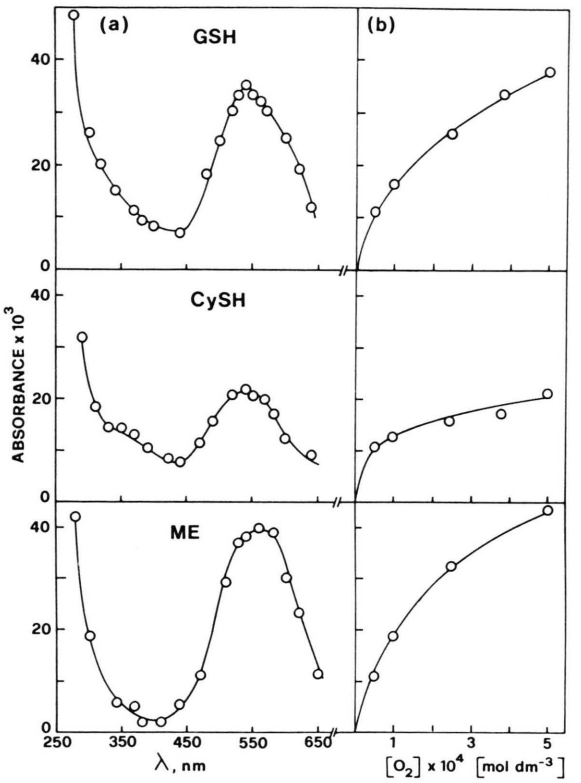

Fig. 2. Panel (a): transient spectra of 1 mM glutathione (GSH), cysteine (CySH) and 2 mercaptoethanol (ME) in N_2O/O_2 (60:40 v/v) saturated solutions of pH 5.5, ≈4 μs after the pulse. Panel (b): maximum optical absorption at 540 nm as a function of oxygen concentration, ≈2–4 μs after the pulse. Dose ≅ 25 Gy, pathlenght = 5 cm.

Our previous observations for GSH [18] interpreted the thiyl/oxygen reaction 6 as an equilibrium. The resulting weak absorption with a maximum around 540 nm was attributed to the sulphur peroxyl radical GSOO·.

The present results further support the above findings. In fact, on the irradiation of CySH, PnSH and ME under the same experimental conditions, the resulting transient spectra are all characterized by a weak absorption with a maximum around 540–560 nm (Fig. 2a). Again, the yield of the 540 nm absorption increases with oxygen concentration even at levels where RS· radicals would be expected to be completely converted into peroxyl radicals (Fig. 2b). These data, together with the kinetic observations showing as RSOO· formation is related to oxygen concentration according to

$$k_{obs} = k_6 [O_2] + k_{-6} \quad (I)$$

are clearly consistent with reaction 6 being reversible.

The rate constants (k_6), as calculated from pulse radiolysis techniques for the different RSH compounds by using direct or indirect methods, are listed in Table II. Values taken from the literature are also reported in the table for comparison. The results obtained by pulse radiolysis are self consistent and further support the earliest observations indicating the remarkably fast addition of oxygen to thiyl radicals derived from CySH and GSH [19, 20].

An apparent discrepancy exists for penicillamine (PnSH), for which the value derived from the decay

Table II. Rate constants (k_6) for the reaction of RS· radicals with oxygen as determinated with different methods. k values are in unit of 10^8 $dm^3mol^{-1}s^{-1}$.

Compound	Indirect (A)	Indirect (B)	Direct (C)	Direct (D)
Glutathione (GSH)	16[1]	0.3[2]	14[1]	20[1]
Cysteine (CySH)	80[3] 81[4]	0.6[2]	–	32[5]
Penicillamine (PnSH)	14[5]	0.25[2]	0.27[5] 0.40[4]	17[5]
Ethanethiol	3.4[4]	–	–	–
2-Mercaptoethanol (ME)	2.7[4]	–	–	–
t-Butylmercaptane	7.8[4]	–	–	–

(A) Method based on the competition between RS^- and O_2 for thiyl radicals according to equations
$RS^{\cdot} + RS^- \rightleftharpoons RSSR^-$
$RS^{\cdot} + O_2 \rightleftharpoons RSOO^{\cdot}$.

(B) Method based on RS· formation by reduction of the corresponding disulphide RSSR and competition according to
$RS^{\cdot} + O_2 \rightarrow RSOO^{\cdot}$
$RS^{\cdot} + D \rightarrow RS^- + D^{+\cdot}$
where D is on electron donor such ascorbate. RS^- is colorimetrically determined by Ellman's reagent.

(C) From the exponential decay of RS· at 320–330 nm at various oxygen concentrations (pH 5.5).

(D) From the exponential build-up of the absorption of RSOO· at 540 nm at various oxygen concentrations (pH 5.5).

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of PnS$^{\cdot}$ at 330 nm is considerable lower than that derived from the build-up at 540 nm. The discrepancy might be attributed to the overlapping of different radical species in the 330 nm region, as supported by the different time scale for the formation (≈ 3 μs) and decay (≈ 200 μs) of the signal at 540 and 330 nm, respectively.

For GSH, the decay of the absorption at 540 nm and attributed to GSOO$^{\cdot}$, occurs with first-order kinetics (8×10^3 s^{-1} at pH 5.5). Deviations from linearity were observed only at the highest doses used (≥ 25 Gy). Moreover, GSOO$^{\cdot}$ results to be quite unreactive towards hydrogen abstraction reaction from GSH itself ($\leq 10^6$ dm^{3}mol^{-1}s^{-1}).

$$GSOO^{\cdot} + GSH \rightarrow GSOOH + GS^{\cdot}. \quad (7)$$

Thus, these results suggest that the peroxyl radical is likely to undergo other paths in competition with reaction (7). Possible mechanisms might include an intramolecular H-abstraction of carbon bound H atoms, with conversion of the sulphur peroxyl radical, GSOO$^{\cdot}$, to carbon-centered radical, $^{\cdot}$G(-H)SOOH, (reaction (8)).

$$GSOO^{\cdot} \rightarrow {}^{\cdot}G(-H)SOOH. \quad (8)$$

This reaction cannot easily be envisaged, considering our previous observations concerning reaction (7). Unfortunately, at the present, the knowledge on the preferred steric arrangement of RSOO$^{\cdot}$ in solution is still scarce and inadequate to provide support to the above suggestion. However, in spite of the structural differences between thiyl and sulphur peroxyl radicals, we did an estimate for the reduction potential of the couple $GSO_2^{\cdot}/GSO_2^-$ close to that reported for $RS^{\cdot}/RS^-$ [14, 15].

This may account for a mechanism of reduction of GSOO$^{\cdot}$ as suggested in reaction (8) and as observed recently for GS$^{\cdot}$ radical by ESR investigations [21]. The authors also found that the sulphynyl radical, RSO$^{\cdot}$, is a ubiquitos intermediate in the irradiation of frozen solution of thiols in presence of oxygen. It is formed as suggested in reaction (9).

$$RS^{\cdot} + O_2 \rightarrow [RSOO^{\cdot}] \rightarrow RSO^{\cdot} + \text{“O”}. \quad (9)$$

The thiyl peroxyl radical, at first considered to be a highly unstable intermediate, has been more recently detected by the same authors [22].

Despite of the significant differences existing between the chemistry in solution at room temperature and that in frozen state, we cannot exclude the possibility that GSO$^{\cdot}$ may be formed and may finally lead to the formation of products with sulphur in a higher oxidation state and/or to the formation of disulphide, GSSG [23, 24].

This mechanism requires the formation of the sulphenic acid, GSOH, as reactive intermediate.

$$GSOH + H_2O_2 \rightarrow GSO_2H + H_2O. \quad (10)$$

$$GSOH + GSH \rightarrow GSSG + H_2O. \quad (11)$$

Unfortunately, it is very difficult to establish unequivocally if the relatively high value for G(−GSH) [24] may justify the formation of GSOH according to reaction (12).

$$GSO^{\cdot} + GSH \rightarrow GSOH + GS^{\cdot}. \quad (12)$$

The sulphur peroxyl radical, GSOO$^{\cdot}$, efficiently accept electrons from several natural and man-made electron donors (D), including ascorbic acid, NADH, and ABTS (2,2′-azinobis-(3-ethyl-benzthiazoline-6-sulphonate) at a rate comparable with that of GS$^{\cdot}$ and in the order of 10^8 dm^{3}mol^{-1}s^{-1}.

$$GSOO^{\cdot} + D \rightarrow GSOO^- + D^{+\cdot}. \quad (13)$$

$$GS^{\cdot} + D \rightarrow GS^- + D^{+\cdot}. \quad (14)$$

The rate constants for reactions (13) and (14) were determined directly by following the build-up of the donor radical product ($D^{+\cdot}$). These radicals present absorptions in useful spectral regions, *i.e.*, at 360 nm and 415 for ascorbyl radical $A^{-\cdot}$ and ABTS radical-cation, $ABTS^{+\cdot}$, respectively. In all cases, the build-up of $D^{+\cdot}$ was exponential and first-order in [D]. Taking into account the equilibrium 6 linking GS$^{\cdot}$ and GSOO$^{\cdot}$ one another, it is possible for reaction (14) to compete and interfere with the “observed“ rate of build-up of $D^{+\cdot}$ in reaction (13). This aspect has been carefully evaluated, at least for ascorbate. From the kinetics of reaction (6) [18], one can derive half-lives for GSOO$^{\cdot}$ formation of about 1 μs and 0.4 μs at oxygen concentration of 50 and 500 μmol dm^{-3}, respectively. Our estimate of k_{14} is in fairly agreement with the value of 6.0×10^8 dm^{3}mol^{-1}s^{-1} previously reported [25]. Even if the ascorbate concentration were as high as 150 μmol dm^{-3}, the half-life of GS$^{\cdot}$ with respect to reaction 14 (which would then about 4 μs) would be significantly longer than half-life for GS$^{\cdot}$ conversion to GSOO$^{\cdot}$ by reaction (6). Consequently, reaction (14) is not likely to compete favourably towards reaction (13) except at rather low concentration of oxygen and relatively high concentration of donor. Work is in progress in order to define a general kinetic equation which could pro-

vide corrections for the possible overestimate of k_{13} in particular experimental conditions.

From the rates of reaction of GSOO· with different donors, the radical is argued to behave as a mild oxidant, with a redox potential comparable with that of the corresponding thiyl radical.

Further studies with other reductants as reference are being undertaken in the attempt to established more firmly the redox properties of the sulphur peroxyl radicals. These findings may be indicative for understanding the possible harmful role of peroxyl radicals in cells and in evaluating the chances of biologically available antioxidants to counteract their deleterious consequences.

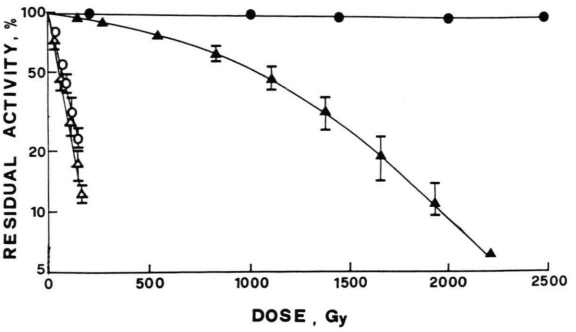

Fig. 3. Radiation induced inactivation of lysozyme (0.1 mg/ml) in 1 mM phosphate buffer pH 6.5: in presence of 5 mM GSH, under N_2 (—●—), under O_2 (—▲—) without GSH, under N_2 (—○—), under O_2 (—△—).

In vitro biological activity of RSOO· radicals

The oxygen enhancement (OE) of the radiation effects in cellular and multicellular systems is widely believed to be due to the radiation-induced formation of peroxyl radicals within the cells, very likely at the DNA level [26]. According to the "radical repair model" proposed by Howard-Flanders [27], DNA-peroxyl radical, DNA-$O_2^{\cdot}$, cannot be repaired chemically as, on the contrary, it happens for its radical precursor DNA·. Recent experimental findings obtained from simple chemical and biochemical systems appear to suggest that the model as outlined above, may be too restrictive to explain the oxygen effect in irradiated living systems. These findings include:

– the presence in the target molecule, presumably DNA, of both reparable and nonreparable forms of damage;

– harmful interactions with DNA of peroxyl radicals of several low molecular weight biomolecules, as well as, of some proteins [28, 29].

With regard to the irradiation of purified macromolecules in solution the loss of activity is observed to occur practically at the same rate both in oxic and anoxic conditions. Instead, a large oxygen effect can be observed if appropriate concentrations of thiols are present during irradiation.

These results are represented in Fig. 3 for the enzyme lysozyme and the thiol GSH. Similar findings are found for other enzymes and thiols, including trypsin and yeast-alcohol dehydrogenase and CySH and PnSH.

These data support the interpretation that RSOO· radicals, formed by reaction (6), are damaging and responsible in part for the greater inactivation in the presence of oxygen [30]. In cells, considering the relatively high GSH concentration, GSOO· has good chances to be produced at a concentration suitable to contribute to the enhancement by oxygen of the radiation response. However, mammalian cells depleted of GSH show a parallel increase in radiosensivity both in absence and in presence of oxygen. These findings are not consistent with the possible contribution of GSOO· radicals to oxygen enhancement and with the original "radical repair/fixation model". The modified version of the "radical repair model", as proposed by Biaglow [31], postulates the participation of GSH and, finally, of GSH peroxidase, in detoxifying potential harmful peroxyl radicals into harmless hydroxylated products. This model could be well integrated postulating that the fast addition of oxygen to RS· radicals (reaction (6)) might effectively reduce the intracellularly concentration of oxygen available for damage fixation. Such an integration possibly improves the compatibility of the Biaglow model with the time-scale of the OE in living cells.

In conclusion, in spite of the growing interest on this subject, it is evident that the mechanism(s) whereby thiols exert radioprotections is only in part understood. Additional information are necessary in order to: assess the application of the "repair/fixation model" to RSH radioprotection; sort out the role of secondary peroxyl radicals, including thiol-peroxyl, in alterating the cellular radiation response; modulate selectively in therapeutic useful way the cellular GSH levels.

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