Redox Reactions of Thiol Free Radicals with the Antioxidants Ascorbate and Chlorpromazine: Role in Radioprotection

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The interaction of the thiyl radical from glutathione, GS•, and its corresponding peroxyl radical adduct, GSO_2•, with the reducing agents, ascorbate and chlorpromazine, have been studied in aqueous solution at pH 5.0–5.5 using the technique of pulse radiolysis with spectrophotometric detection. The rate constants determined for interaction of GS• with ascorbate and chlorpromazine are 5.4×10^8 and 9.0×10^8 dm^3 mol^{-1} s^{-1} respectively. The reaction is thought to proceed *via* an electron transfer process. Further, the redox potential of GS•/GSH at pH 5.0 is estimated to be 0.91 V. In the presence of different concentrations of oxygen, it has been established that GSO_2•, as observed at 540 nm, interacts with ascorbate and chlorpromazine by electron transfer with rate constants of 2.1×10^8 and 5.0×10^8 dm^3 mol^{-1} s^{-1} respectively. From these kinetic observations it is inferred that GSO_2• is a weaker oxidant than GS•. From these findings the role of these glutathione radicals should be taken into account when considering the biological role of thiols in oxidative damage in biological systems.

Cellular thiols (RSH), consisting almost entirely of reduced glutathione (GSH), are involved in a number of important reductive processes. These processes contribute by exerting control over a variety of events leading to oxidative biological damage.[1]

There are several lines of evidence indicating that many physiological and pathophysiological phenomena (*i.e.* ageing, carcinogenesis, drug-toxicity, inflammation) develop through the action of electrophilic agents, predominantly involving active oxygen species.[2–4] Moreover, there are a number of significant observations on the important intracellular role of GSH in both the detoxification of highly reactive, carcinogenic electrophiles and the scavenging of a wide variety of free radicals, including reactive oxy-radicals.[5–7] In these reactions, GSH may function as a nucleophile, forming conjugates, or as a reductant.[1,8] The latter function is well known to operate in the chemical repair of radical-mediated biological damage (T•) induced by ionising radiation as well as in the scavenging of hydroxyl radicals, •OH. Both these reactions lead to the

$$T^{\bullet} + RSH/RS^{-} \longrightarrow TH/T^{-} + RS^{\bullet} \quad (1)$$

$$^{\bullet}OH + RSH/RS^{-} \longrightarrow H_2O/OH^{-} + RS^{\bullet} \quad (2)$$

formation of the thiyl free radical, RS•, which has been shown to have oxidising properties.[9]

Under aerobic conditions, RS• reacts with molecular oxygen in competition with its dimerization to RSSR and/or its reaction with the thiolate ion, RS^-.[10–12] Depending upon [O_2], [RSH] and pH, the most important reactions at low concentrations of RS• are (3) and (5). Thus, on the basis of the known rate

$$RS^{\bullet} + O_2 \rightleftharpoons RSO_2^{\bullet} \quad (3)$$

$$RS^{\bullet} + RS^{\bullet} \longrightarrow RSSR \quad (4)$$

$$RS^{\bullet} + RS^{-} \rightleftharpoons RSSR^{\bullet -} \quad (5)$$

constants for reactions (3) and (5)[8,10] and the pK_a of 9.2 for GSH,[13] GS• will be converted into predominantly the corresponding peroxyl adduct (GSO_2•) under normal physiological conditions. It has recently been confirmed,[14,15] by EPR spectroscopy, that, in frozen aqueous solutions, the thiyl peroxyl radical is formed upon interaction of oxygen with GS•.

To date, little attention has been paid to the biological importance of such reactions. However, from our recent studies on the role of thiols in modifying the effects of radiation on enzymes in the presence of oxygen, it was suggested that the RSO_2• radical may possess damaging properties.[16] In addition, from our preliminary findings on the interactions of sulphur peroxyl radicals with reductants,[17] it is inferred that they possess oxidising properties, analogous with the behaviour of peroxyl radicals in general.

The present study was undertaken to assess redox-related interactions of the RS• radical from glutathione and its corresponding peroxyl adduct, RSO_2•, with reducing agents and in particular ascorbate (AH^-) and chlorpromazine (ClPMZ). These findings may shed light on the ability of biologically available reductants to counteract oxidative damage mediated by sulphur peroxyl radicals.

Experimental

The pulse radiolysis experiments were performed using either the 12 MeV linear accelerator at Bologna or the 4.3 MeV linear accelerator at the RBU. The optical detection system with data handling for the two machines has been described previously.[17–19] All solutions were prepared with water which had been purified by a Milli-Q system. The pH values of the solutions were adjusted using either $HClO_4$ or NaOH. Solutions were saturated with N_2O (BOC, zero grade) or with varying percentages of N_2O/O_2 or air by flushing solutions with gas mixtures or by the syringe-mixing technique whereby a known volume of a saturated N_2O solution was mixed with a given volume of solution either aerated or oxygen-saturated. Solutions contained in a quartz cell of 0.2 or 0.5 dm path length were irradiated at 296 ± 2 K with an electron pulse. Optical filters were used to minimise photochemical effects.

Radiation doses were determined with the use of KSCN dosimetry at 480 nm assuming $G = 0.3$ μmol J^{-1} and $\varepsilon = 710$ m^2 mol^{-1} or by means of a charge collector placed behind the irradiation cell and calibrated with O_2-saturated 0.1 mol dm^{-3} KSCN solution.

Glutathione (Fluka), ascorbate (Merck), chlorpromazine

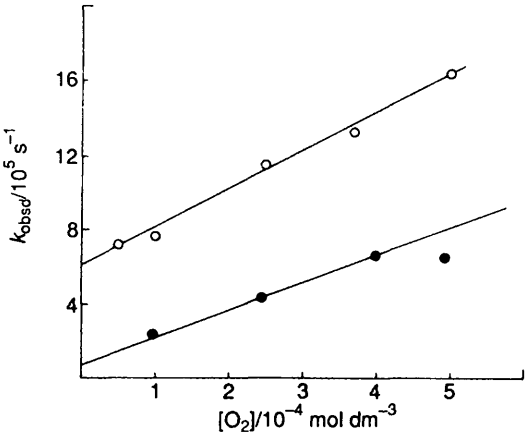

Fig. 1 The dependence on oxygen concentration of k_{obsd} for the formation of absorption at 540 nm (– – ○ – –) and for the decay at 330 nm (– – ● – –). [GSH] = 1 mmol dm^{-3} at pH 5.6; dose *ca.* 24 Gy, path length = 0.5 dm.

(Sigma) and all other chemicals of Analar grade were used as supplied.

Results and Discussion

Generation of Thiyl Radicals.—The thiyl radical from glutathione, GS$^{\bullet}$, is produced immediately upon pulse irradiation of N_2O-saturated solutions of GSH (typically 1 mmol dm^{-3}) at pH *ca.* 5.5 where the –SH group is present in its undissociated form. Under these conditions the GS$^{\bullet}$ radicals cannot be converted into GSSG$^{\bullet -}$ by reaction (5) and the observed second-order decay kinetics at 330 nm, where GS$^{\bullet}$ exhibits maximum optical absorption, are ascribed to the dimerisation reaction (4). Details of the spectra and kinetics of thiyl radicals of different origin are reported elsewhere.[8,20]

Thiyl Radicals and Oxygen.—If the N_2O-containing glutathione solutions at pH *ca.* 5.5 also contain oxygen at different concentration (ratios varying from 4:1 to 2:3), the rate of decay of GS$^{\bullet}$ is increased and becomes first order and dependent upon the oxygen concentration. A new, weak absorption forms at 540 nm at a comparable rate (Fig. 1). This absorption is attributed to the glutathione peroxyl radical, $GSO_2^{\bullet}$,[10,11] formed through the equilibrium reaction (6) and characterised by the following

$$GS^{\bullet} + O_2 \rightleftharpoons GSO_2^{\bullet} \qquad (6)$$

kinetic parameters: $k_6 = 2.0 \times 10^9$ dm^3 mol^{-1} s^{-1}, $k_{-6} = 6.2 \times 10^5$ s^{-1} and $K_6 = 3.2 \times 10^3$ dm^3 mol^{-1} from k_6/k_{-6}.

At present, there is discussion in the literature concerning the assignment of the optical absorption centred around 540 nm to $GSO_2^{\bullet}$. Based upon *ab initio* calculations, an alternative suggestion was proposed[21] whereby the sulphinyl radical, RSO$^{\bullet}$, formed in reaction (7), should be considered as a possible radical which absorbs at 540 nm.

$$GSO_2^{\bullet} + GSH \longrightarrow GSO^{\bullet} + GSOH \qquad (7)$$

However, with cysteine,[14] it has been confirmed that the species assigned to the thiyl peroxyl by EPR does indeed show a visible absorption centred at 540 nm consistent with its previous assignment[10,11] to $GSO_2^{\bullet}$ but at variance with the theoretical considerations. Under the conditions of the present experiments, the species which absorbs at 540 nm decays by a first-order process with a half-life of *ca.* 86 μs[17] and independent of [GSH] and $[O_2]$.[22] Whether the first-order decay represents isomerisation of $GSO_2^{\bullet}$ into the sulphonyl radical is as yet not known. Evidence for an isomerisation is derived from EPR at low temperature[14,15] whereby $GSO_2^{\bullet}$ either photoisomerises to the sulphonyl radical or is converted to the sulphinyl radical, but only at high glutathione concentration (>3 mmol dm^{-3}). From these considerations, the assignment of the 540 nm absorption to $GSO_2^{\bullet}$ remains valid.

The extinction coefficient of $GSO_2^{\bullet}$ was determined by measuring the maximum optical densities at 540 nm in experiments performed with differing dose/pulse and oxygen concentrations and by using the following relationships.

Let $[GS^{\bullet}]_0$ be the total concentration of $[GS^{\bullet}]$ and $[GSO_2^{\bullet}]$ at equilibrium [eqn. (I)] and assuming $G(GS^{\bullet}) = G(OH) = 6.0$, by the equilibrium expression in eqn. (II) where K_6 and

$$[GS^{\bullet}]_0 = [GS^{\bullet}]_{eq} + [GSO_2^{\bullet}]_{eq} \qquad (I)$$

$$K_6 = [GSO_2]_{eq}/[GS^{\bullet}]_{eq} \times [O_2] \qquad (II)$$

$[O_2]$ are known, $[GS^{\bullet}]_{eq}$ is calculated from the dose and $G(OH)$ and therefore $[GSO_2^{\bullet}]_{eq}$ and its molar extinction coefficient are derived.

Corrections are required at the highest oxygen concentration used (*i.e.* 5 × 10^{-4} mol dm^{-3}) where a small fraction of e_{aq}^- (*ca.* 10%) can escape N_2O capture and at the lowest (< 10^{-4} mol dm^{-3}) where reaction (4) competes with reaction (6).

The experimental value of $\varepsilon_{540} = 1040 \pm 103$ dm^3 mol^{-1} cm^{-1} is considerably higher than the published value of 180 ± 35 reported for the thiyl peroxyl radical derived from 2-mercaptoethanol.[23]

In spite of the relatively low value of ε_{540}, the optical absorption at 540 nm may be used as a reference in studies on the fate of the sulphur peroxyl radical in oxidation–reduction reactions.

Reactions of Thiyl/Thiylperoxyl Radical with Antioxidants.—Due to the ease of its one-electron oxidation, $E = +0.78$ V,[24] radiolytic oxidation of ClPMZ can be achieved in aqueous solution by a variety of electrophilic free radicals, X$^{\bullet}$,[25] in agreement with the general reaction (8). The characteristic

$$ClPMZ + X^{\bullet} \longrightarrow ClPMZ^{\bullet +} + X^- \qquad (8)$$

strong visible absorption at 525 nm ($\varepsilon = 1.00 \pm 0.08 \times 10^4$ mol^{-1} dm^3 cm^{-1})[26] makes $ClPMZ^{\bullet +}$ and its parent molecule particularly useful as a reference reactant.

Ascorbic acid (AH_2), plays an important antioxidant role in biological systems.[27,28] This property is mainly attributed to the stability of the ascorbyl radical ($A^{\bullet -}$), the initial product of ascorbate oxidation [reaction (9)].[29]

$$AH^- + X^{\bullet} \longrightarrow A^{\bullet -} + HX \qquad (9)$$

The ascorbyl radical, $A^{\bullet -}$, has a strong absorption at 360 nm[30,31] ($\varepsilon_{360} = 3300$ dm^3 mol cm^{-1}) and the redox potential for the couple $AH^{\bullet}/AH^-$ at pH 7 has been estimated to be +0.30 V.[32,33]

The rate constant for the interaction of glutathione thiyl radical, GS$^{\bullet}$, with either ascorbate or chlorpromazine, was determined at pH 5.5 upon pulse radiolysis of N_2O-saturated solutions containing 10^{-3} mol dm^{-3} glutathione and up to 1.25 mol dm^{-3} of antioxidants. The rate of formation of the absorption at 360 nm and at 530 nm due to the ascorbyl radical ($A^{\bullet -}$) and chlorpromazine radical ($ClPMZ^{\bullet +}$) respectively, is first-order and dependent upon the concentration of the antioxidant. These dependences are shown in Fig. 2 from which the rate constants for interaction of GS$^{\bullet}$ with the antioxidants were calculated and are shown in Table 1. The rate constant with ascorbate is consistent with the value of 6.0 × 10^8 dm^3 mol^{-1} s^{-1} previously reported.[34]

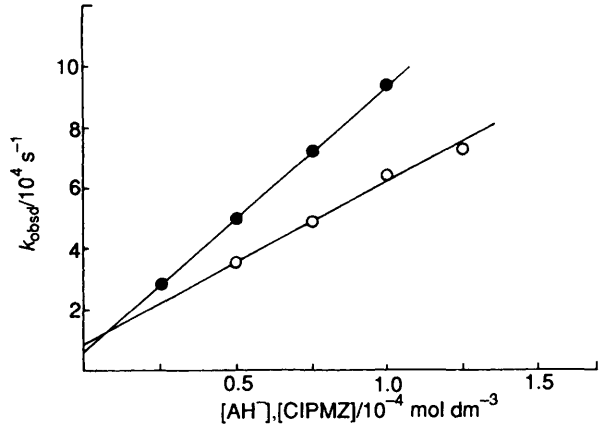

Fig. 2 Dependence of the first-order rate constant for formation of the absorption at 360 nm and 530 nm on the concentration of ascorbate (– – ○ – –) and chlorpromazine (– – ● – –). (– – ○ – –), [GSH] = 1 mmol dm^{-3} at pH *ca.* 5.6, dose = 9 Gy, path length = 0.5 dm; (– – ● – –), [GSH] = 1 mmol dm^{-3} at pH *ca.* 5.2, dose *ca.* 0.6 Gy, path length = 0.2 dm.

Table 1 Rate constants for interaction of thiyl/thiylperoxyl radicals with antioxidants at pH *ca.* 5.2

Thiol radical	Antioxidant	k/dm^3 mol^{-1} s^{-1}	k_{calc}
GS$^\bullet$	ascorbate	5.4×10^8	6.0×10^8
GS$^\bullet$	chlorpromazine	9.0×10^8	9.9×10^8
$GSO_2^\bullet$	ascorbate	2.1×10^8	2.1×10^8
$GSO_2^\bullet$	chlorpromazine	5.0×10^8	5.3×10^8

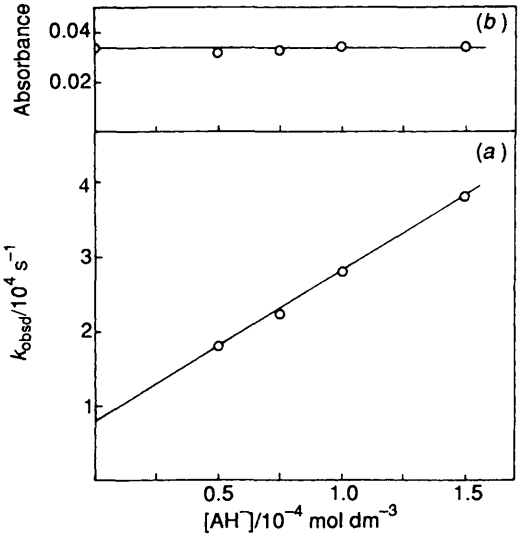

Fig. 3 (*a*) The dependence on ascorbate concentration of the first-order rate constant for formation of absorption at 360 nm in N_2O/O_2 (60:40 v/v)-saturated solutions of 1 mmol dm^{-3} GSH at pH 5.1. (*b*) Effect of ascorbate concentration on the optical absorption at 540 nm. Dose *ca.* 9 Gy, path length = 0.5 dm.

From the yield of $A^{\bullet -}$ determined at 360 nm, the efficiency of the electron transfer process shown in reaction (10), is

$$GS^\bullet + AH^- \longrightarrow GS^- + A^{\bullet -} + H^+ \qquad (10)$$

determined to be 96% (taking as 100% the yield of $A^{\bullet -}$ using bromine free radical).

The rate constant determined for interaction of GS$^\bullet$ with chlorpromazine is greater than the value of 1.4×10^8 dm^3 mol^{-1} s^{-1} determined previously at pH 3.0.[34] This apparent discrepancy can be explained on the basis of the state of protonation of GSH at these different pH values. Since the net charge (Z) of GSH changes from −1 to 0,[35] and that of chlorpromazine (Z = 1) remains constant,[36] a marked decrease in the rate constant is expected upon lowering the pH.

Assuming that the optical absorption at 530 nm is due to the chlorpromazine radical-cation ($ClPMZ^{\bullet +}$),[26] the efficiency of electron transfer from GS$^\bullet$ to ClPMZ was determined to be 65% at pH 5.1. However, formation of an adduct cannot be ruled out since the transient optical absorption spectrum is different at $\lambda > 560$ nm from that assigned to $ClPMZ^{\bullet +}$. This low yield of $ClPMZ^{\bullet +}$ cannot be due to the interaction with GS$^\bullet$ involving an equilibrium, such as reaction (11). Assuming that this

$$GS^\bullet + ClPMZ + H^+ \rightleftharpoons GSH + ClPMZ^{\bullet +} \qquad (11)$$

equilibrium is established, from the dependence of the formation of $ClPMZ^{\bullet +}$ at pH 5.0 on the concentration of ClPMZ and GSH, an equilibrium constant (K_{11}) of *ca.* 83 is determined from the kinetic information (data not shown). This value of K_{11} corresponds to a redox potential for the couple GS$^\bullet$/GSH of 0.91 V at pH 5.0. Therefore, under the concentration conditions used for GSH and ClPMZ, equilibrium (11) is such that the formation of $ClPMZ^{\bullet +}$ is preferred (>90%). Taking this equilibrium into account, therefore, does not explain the interaction of GS$^\bullet$ with ClPMZ yielding its one-electron oxidised product with less than unit efficiency. Other reaction pathways in competition with one-electron oxidation processes are probably involved in the interaction between GS$^\bullet$ and chlorpromazine.

If aqueous solutions containing both GSH and ascorbate at pH 5.1 are irradiated in the presence of a fixed oxygen concentration (*i.e.* 5×10^{-4} mol dm^{-3}), the kinetics of formation of the ascorbyl radical at 360 nm remain first-order and dependent upon the ascorbate concentration as shown in Fig. 3(*a*). The growth of the absorption at 360 nm is attributed to the interaction of ascorbate with the sulphur peroxyl radical, $GSO_2^\bullet$, formed under these experimental conditions through the equilibrium reaction (6) [reaction (12)]. The rate constant

$$GSO_2^\bullet + AH^- \longrightarrow GSO_2^- + A^{\bullet -} + H^+ \qquad (12)$$

determined is presented in Table 1. We have assessed the possibility that under our experimental conditions the thiyl radical GS$^\bullet$ could compete with $GSO_2^\bullet$ towards ascorbate, thus introducing an error into the derived rate constant value. The observed invariance of the optical density at 540 nm, attributed to $GSO_2^\bullet$ radical, with ascorbate concentration at fixed oxygen concentration [Fig. 3(*b*)] is, however, inconsistent with this competition. In fact, if such a mechanism occurs, a decrease in the signal at 540 nm would be expected owing to the shift of the equilibrium (6) towards the left on increasing AH^- concentration.

As well as ascorbate, the rate constant determined for the interaction of $GSO_2^\bullet$ with ClPMZ [reaction (13)] at fixed oxygen concentration is given in Table 1.

$$GSO_2^\bullet + ClPMZ \longrightarrow GSO_2^- + ClPMZ^{\bullet +} \qquad (13)$$

In order to gain further kinetic indications about the possibility of the competition between GS$^\bullet$ and $GSO_2^\bullet$ in the presence of oxygen, an additional set of experiments at different oxygen concentrations was carried out. Fig. 4 shows the dependence upon the concentration of oxygen of the first-order rate constant for formation of $A^{\bullet -}$ radical at fixed concentrations of GSH and AH^-. The first-order rate constant increases with decreasing concentration of oxygen and reaches a plateau at $[O_2] \leq 10^{-4}$ mol dm^{-3}.

In solutions containing GSH and different concentrations of

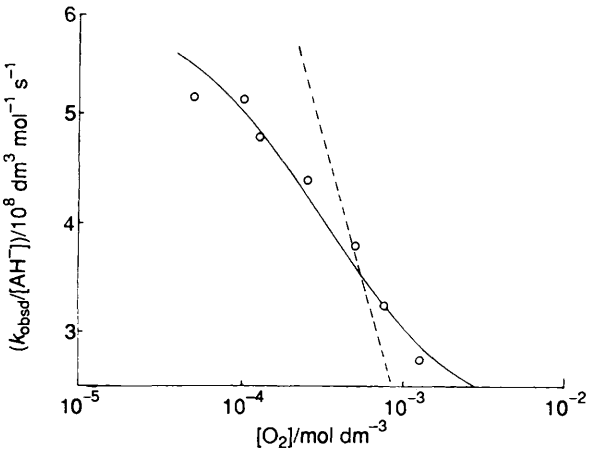

Fig. 4 The dependence on oxygen concentration of the first-order rate constant for formation of $A^{\bullet -}$ radical at 360 nm at fixed [GSH] (1 mmol dm^{-3}) and [AH^-] (0.1 mmol dm^{-3}). ○, experimental points; solid line, the calculated curve obtained using eqn. (III) of text; dotted line, the calculated curve assuming $k_{12} = 0$. pH *ca.* 5.6, dose *ca.* 9 Gy, path length = 0.5 dm.

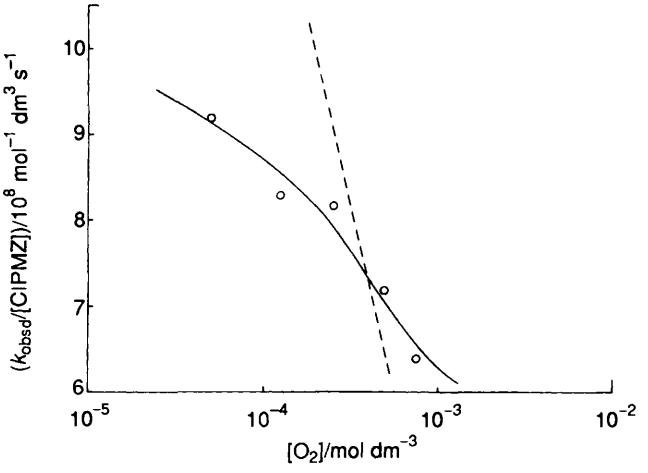

Fig. 5 The dependence on oxygen concentration of the first-order rate constant for formation of $ClPMZ^{\bullet +}$ radical at 530 nm at fixed [GSH] (1 mmol dm^{-3}) and [ClPMZ] (50 μmol dm^{-3}). ○, experimental points; solid line, the calculated curve obtained using the corresponding eqn. (III) of text; dotted line, the calculated curve assuming $k_{13} = 0$. pH *ca.* 5.1, dose *ca.* 0.6 Gy, path length = 0.2 dm.

oxygen, similar observations were obtained for interaction of the glutathione radicals with chlorpromazine (Fig. 5).

Using ascorbate as the example, Scheme 1 was assumed to

$$GS^{\bullet} + AH^- \xrightarrow{k_{10}} GS^- + A^{\bullet -} + H^+$$

$$\begin{matrix} k_6 \uparrow \downarrow k_{-6} \\ + \\ O_2 \quad O_2 \end{matrix}$$

$$GSO_2^{\bullet} + AH^- \xrightarrow{k_{12}} GSO_2^- + A^{\bullet -} + H^+$$

Scheme 1

describe the formation of the ascorbate radical, $A^{\bullet -}$, from the interaction of $GS^{\bullet}$ and $GSO_2^{\bullet}$ with ascorbate. For oxygen concentrations $\geq 5 \times 10^{-5}$ mol dm^{-3} it is assumed that equilibrium (6) involving interaction of oxygen with $GS^{\bullet}$ is established prior to significant occurrence of reactions (10) and (12) so that the equilibrium is not rate determining.

From Scheme 1 eqn. (III) is obtained for the dependence on

$$k_{obsd}/[AH^-] = (k_{10} + k_{12} \times K_6[O_2])/(1 + K_6 \times [O_2]) \quad \text{(III)}$$

O_2 concentration of the rate constant observed for formation of $A^{\bullet -}$ upon reaction of $GS^{\bullet}/GSO_2^{\bullet}$ with ascorbate where $K_6 = k_6/k_{-6}$. Assuming $K_6 = 3.2 \times 10^3$ mol^{-1} dm^3,[10] values for the rate constants k_{10} and k_{12} were calculated. The best fit line determined using eqn. (III) is shown in Fig. 4 and Fig. 5 for ascorbate and chlorpromazine, respectively. The calculated values of k_{10} and k_{12} as reported in Table 1 are in good agreement with those determined experimentally under the appropriate conditions. The values of k_{12} were determined at high concentration of oxygen whereby $k_{12} \times [AH^-] > k_{-6}$ and $k_6 \times [O_2] > k_{10} \times [AH^-]$.

Conclusions

From the values of these rate constants, it is assumed that reactions of $GS^{\bullet}$ and $GSO_2^{\bullet}$ with antioxidants proceed *via* electron transfer and not H-atom abstraction. This fact is also supported by our observations that the spectral characteristics of the radical products from ascorbate and chlorpromazine are very similar to those obtained using $Br_2^{\bullet -}$ or other electrophilic radicals as oxidising agent.[25,31] Moreover, H-atom abstraction by both antioxidants is considered to be an inefficient process as indicated by their low reactivity with a variety of C-centred radicals.[37,38]

The rate constants for interaction of glutathione radicals with ascorbate and chlorpromazine reflect, to some extent, the redox potential for the couples $GS^{\bullet}/GS^-$ and $GSO_2^{\bullet}/GSO_2^-$. Since $GS^{\bullet}$ oxidises both ascorbate and chlorpromazine more rapidly than $GSO_2^{\bullet}$, it is inferred that $GSO_2^{\bullet}$ is a weaker oxidant than $GS^{\bullet}$. If we consider that the behaviour of $GS^{\bullet}$ will be quite similar to that of other thiols of simple structure, for which $E(RS^{\bullet},H^+/RSH)$ is estimated to be *ca.* +1.0 V at slightly acid pH,[39] and that $E(ClPMZ/ClPMZ^{\bullet +})$ is +0.78 V, independent of pH in the range 0–7,[24] we may conclude that $E(GSO_2^{\bullet}/GSO_2^-)$ is $\leq +1.0$ V. A similar qualitative trend in the redox properties of glutathione radicals has been recently derived from their interaction with phenothiazines.[40]

The formation of potentially harmful oxidising radicals like $GS^{\bullet}$ and $GSO_2^{\bullet}$ when GSH performs its radioprotective and antioxidant function adds further to the complications in understanding the biological role of thiols. The finding that both radicals can be removed efficiently by reducing agents may be indicative of cellular defence mechanisms played by low molecular weight antioxidants in controlling the cellular level of reduced glutathione and in limiting peroxidation reactions. It is evident that further studies, both at the molecular and biological levels, are necessary in order to evaluate the role of secondary thiol radicals in radioprotection and, more generally, in the repair of radical-mediated biological damage.

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