We have recently proposed that disulphide S-monoxides (thiosulphinates) and disulphide S-dioxides (thiosulphonates) are formed from their parent disulphides and ‘reactive oxygen species’ during oxidative stress. These ‘reactive sulphur species’ are themselves strong oxidizing agents that preferably attack the thiol functionality. We now show that under conditions where disulphides show little effect, disulphide S-oxides rapidly modify metallothionein, alcohol and glyceraldehyde 3-phosphate dehydrogenases and a zinc finger-protein fragment in vitro. The known antioxidants ascorbate, NADH, trolox and melatonin are unable to inhibit this oxidation pathway and only an excess of the cellular redox-buffer glutathione quenches the disulphide S-oxide activity. These results suggest that, under conditions of oxidative stress, despite the presence of high concentrations of antioxidants, reactive sulphur species formation may occur and inhibit the function of thiol-dependent proteins. Such a characterization of the disulphide S-oxide-oxidation pathway might also account for some previously observed anomalies in protein oxidation.

Key words: disulphide S-oxides, oxidative stress, reactive sulphur species, sulphur proteins.

INTRODUCTION

Oxidative stress is an important biochemical condition causing several human diseases. This stress is linked to the presence of unusually high concentrations of toxic ‘reactive species’, which include ‘reactive oxygen species’ (ROS), ‘reactive nitrogen species’ and unbound, adventitious metal ions [1–3]. Most of these species are highly oxidizing, readily modifying redox-sensitive proteins and enzymes, as well as attacking membranes and DNA. ROS react frequently with cellular thiols to form the corresponding disulphides, which are only mildly oxidizing under physiological conditions. It is, however, apparent that sulphur can be easily oxidized beyond the disulphide state by ROS under conditions prevalent in oxidative stress [4]. The redox activation of the disulphide bond by oxidizing species to form disulphide S-monoxides and disulphide S-dioxides has been observed both in vitro and in vivo [5–7] and it has been suggested that these species may form an important pathway by which the oxidizing stimulus is conveyed throughout the cell [8,9].

In accordance with these findings, disulphide S-oxides are formed easily in vitro by a range of possible pathways involving oxidative stressors. Oxidation of thiols and disulphides by ROS is perhaps the most obvious route. The formation of substantial proportions of disulphide S-oxides from equimolar amounts of GSH and H₂O₂ is fast at pH 6.0, exceeding the formation of both disulphides and sulphinic acid [5]. Other routes of disulphide S-oxide formation include the oxidation of GSH and GSSG by singlet oxygen [10,11] and the decomposition in aqueous solutions of S-nitrosothiolamine [6] and S-nitroglutathione (formed from peroxynitrite and GSH) [12]. It is therefore apparent that, under oxidative conditions, a significant amount of GSH is likely to be initially converted into disulphide S-monoxides and disulphide S-dioxides as well as disulphides. Examples of disulphide S-oxide formation in cell organelles have also been observed in rat-brain slices that were treated with xanthine/xanthine oxidase [6] and during the enzymic oxidation of GSH by cytochrome P450 [13]. These results were recently supported by studies, which showed that disulphides such as diallyl disulphide and dipropyl disulphide are readily oxidized by rat- and human-liver microsomes. Cytochrome P450 and, to a lesser extent, flavin-containing mono-oxygenases have been implicated in disulphide S-oxide formation [14,15].

Together with thiol radicals and sulphenic acids, disulphide S-oxides can therefore be considered as ‘reactive sulphur species’ (RSS) in analogy with ROS and reactive nitrogen species [7–9]. As yet, there are few examples in the literature of the potential targets and in vivo effects of this novel class of RSS. To identify disulphide S-oxides and to comprehend their possible in vivo role fully, it is important first to determine the chemical and in vitro properties of these agents (e.g. their ‘footprints’). The in vitro studies described here aim at a better understanding of the characteristics of these species in the presence of proteins and enzymes.

In particular, these studies were driven by the following questions. First, how do disulphide S-oxides interact with different thiol proteins? Secondly, is the redox behaviour of disulphide S-oxides specific for the thiol–redox system or can disulphide S-oxides be ‘neutralized’ by common cellular antioxidants? Thirdly, could these interactions be relevant in vivo?

We here report in vitro ‘oxidative stressor’ qualities of cystamine S-monoxide (C1), an exemplary disulphide S-monoxide and glutathione disulphide S-dioxide (C2) in an attempt to define the scope and possible in vivo implications of disulphide S-oxide formation. These species readily attack a range of representative thiol proteins such as metallothionein (MT), alcohol dehydrogenase (ADH), glyceraldehyde 3-phosphate dehydrogenase (ADH), glyceraldehyde 3-phosphate dehydrogenase.

Abbreviations used: ADH, alcohol dehydrogenase; C1, cystamine S-monoxide; C2, glutathione disulphide S-dioxide; CDNB, 1-chloro-2,4-dinitrobenzene; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); GAP, glyceraldehyde 3-phosphate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GPx, glutathione peroxidase; GST, glutathione S-transferase; MT, metallothionein; PAR, 4-(2-pyridylazo)resorcinol; ROS, reactive oxygen species; RSS, reactive sulphur species; ZFP, zinc finger protein.

1 These authors contributed equally to this work.

2 To whom correspondence should be addressed (e-mail C.Jacob@ex.ac.uk).
(GAPDH) and a metal-binding zinc finger protein (ZFP) fragment. The oxidizing activity of the disulphide S-oxides is significantly greater than that of the parent disulphides, cystamine and GSSG. This redox interaction with thiols cannot be efficiently quenched by the cellular antioxidants ascorbate, NADH, trolox (a water-soluble vitamin E analogue) or the brain antioxidant agent melatonin and is only affected by an excess of GSH.

EXPERIMENTAL

Materials

Mass spectra were obtained using a Platform LC-MS instrument (Micromass), operating in electrospray mode (capillary voltage 3.90 kV, cone voltage 40 V). UV/VIS spectra were recorded on a CARY 50 Bio-U/VIS spectrophotometer (Varian) or Perkin-Elmer U2000. NMR spectra were recorded on a Bruker 300 MHz instrument.

GSH, GSSG, cystamine, H$_2$O$_2$, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), MT (rabbit liver Zn,Cd-MT-2), ADH (horse liver), NAD$^+$, NADH, glutathione peroxidase (GPx, bovine erythrocyte), GAPDH (muscle enzyme), glutathione S-transferase (GST, rabbit liver) and glycerolaldehyde 3-phosphate (GAP) were obtained from Sigma (Poole, Dorset, U.K.); 4-(2-pyridylazo)resorcinol (PAR) was purchased from Fluka (Gillingham, Dorset, U.K.). The metal-binding ZFP sequence of the HIV nucleocapsid protein NCp7 (VKCFNCGK-EGHTARNCRA) was obtained from Affiniti Research Products (Mamhead, Exeter, U.K.) and prepared according to the established procedure [16]. All other chemicals and reagents were purchased from Aldrich (Gillingham, Dorset, U.K.); they were of analytical grade and used without further purification. ‘Metal-free’ (i.e. ‘chelated’) nitrogen-purged buffers were used for assays involving metal release [8,17–19]. All assays were conducted at 25 °C unless otherwise specified.

Synthesis of disulphide S-oxides

C1 and C2 were prepared from their disulphide-parent compounds by reacting with peracetic acid or H$_2$O$_2$ under acidic conditions according to procedures described in the literature [20–23]. Remaining traces of H$_2$O$_2$ were removed by the addition of MnO$_2$. C1 was purified by recrystallization and characterized by elemental analysis, $^1$H-NMR, LC-MS and IR. Experimental values were found to be in accordance with those described in the literature [21]. C2 was purified as described by Mannervik et al. [22]. C2 is chemically unstable (similar to peroxynitrite) and its rate of oxidation can be measured by the oxidation of NADH monitored for 2 min [26]. Lyophilized GAPDH and ADH were dissolved in NaPi (15 mM, pH 7.0) and Hepes (20 mM, pH 7.5), respectively, and desalted by passing through a PD10 column packed with Sephadex G25. Protein concentrations were calculated by measuring absorbance at 280 nm. GAPDH activity was assayed in a modified Krebs procedure to allow for the instability of the disulphide S-oxides to basic conditions [29]. GAPDH was incubated in NaPi (15 mM, pH 7.0) with sodium arsenate (30 mM) and GAP (450 μM) and the reduction of NAD$^+$ (250 μM) monitored spectrophotometrically ([ε$_{340}$ (NADH) = 6220 M$^{-1}$ cm$^{-1}$] for 5 min. To establish ADH activity, ADH (40 nM) was incubated in KPi (100 mM, pH 7.0) with acetaldehyde and NADH (300 μM) and the oxidation of NADH monitored for 2 min [26].

Zinc release assays for ADH and ZFP

Release of zinc from ADH (1 μM) and the ZFP fragment (2 μM) was measured spectrophotometrically in Hepes (20 mM, pH 7.5), in the presence of PAR (100 μM) as described for MT. Maximum zinc release was calculated by incubation with Ebselen (75 μM) [30]. The ZFP fragment was examined as a model for potential gene receptor and viral activity regulation by disulphide S-oxides.

Oxidation of GSH by disulphide S-oxides

The consumption of GSH by C1 and C2 was measured in two independent assays. The enzyme GST specifically conjugates GSH to CDNB, whereas the modified DTNB procedure is specific for the thiol group [31]. For both assays, selected concentrations of GSH were incubated for 30 min at 37 °C with the disulphide S-oxide (70 μM for C1 and 60 μM for C2). An aliquot of the reaction mixture (100 μl for the GST and 50 μl for the DTNB assay) was then added to the specific assay solution (900 μl for the GST and 950 μl for the DTNB assay). The GST assay solution contained GST (385 nM) and CDNB (1 mM) in KPi (100 mM, pH 7.0). The formation of the CNDB–GSH conjugate ([ε$_{340}$ = 9600 M$^{-1}$ cm$^{-1}$]) was recorded continuously until completion (2 min). The DTNB assay solution consisted of DTNB (500 μM) in KPi (100 mM, pH 7.0). The conjugate absorbance was recorded by UV/VIS spectroscopy ([ε$_{112}$ = 13600 M$^{-1}$ cm$^{-1}$]). The consumption of GSH (or thiols) was calculated from the difference in the GSH titration curves in the absence and presence of disulphide S-oxide.
RESULTS

We have recently demonstrated C1- and C2-induced zinc release from MT [7,8]. The results described in Table 1 indicate that disulphide S-oxides C1 and C2 were both able to release zinc effectively from MT, ADH and ZFP, whereas the parent compounds cystamine, GSSG and H$_2$O$_2$, at the same concentrations, had little or no observable effect. For example, C1 released approx. 10 times more zinc from MT in 60 min than the same concentration of cystamine. C1 also enhanced zinc release from ADH and freed approx. 30% zinc from the ZFP fragment in 10 min. C2 was similarly effective, releasing approx. 40% zinc from ADH in 60 min. Whereas most oxidative stressors are sensitive towards antioxidants, ascorbate, trolox, melatonin and NADH were unable to quench effectively the oxidizing effects of C1, even in 10-fold excess (Table 1). These non-thiol-based antioxidants were also unable to quench C2 activity that remained at 40% in the ADH–zinc release assay.

Zinc release from ADH by C1 corresponded to enzyme inhibition (IC$_{50}$: 70 μM for 40 nM ADH). Under the same conditions, C2 inhibited ADH with an IC$_{50}$ value of 60 μM. Enzyme inhibition was also not quenched significantly by the presence of non-thiol-based antioxidants (Table 2). The in-

### Table 1: Effect of antioxidants on zinc release from metalloproteins by the action of C1

<table>
<thead>
<tr>
<th>Inhibitor*</th>
<th>Antioxidant†</th>
<th>Zinc release (%) (±5%‡)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>From MT</td>
</tr>
<tr>
<td>--</td>
<td>--</td>
<td>0</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>--</td>
<td>9.5</td>
</tr>
<tr>
<td>Cystamine</td>
<td>--</td>
<td>0</td>
</tr>
<tr>
<td>GSSG</td>
<td>--</td>
<td>0.5</td>
</tr>
<tr>
<td>C1</td>
<td>--</td>
<td>76</td>
</tr>
<tr>
<td>C1</td>
<td>Trolox</td>
<td>59</td>
</tr>
<tr>
<td>C1</td>
<td>Ascorbate</td>
<td>76</td>
</tr>
<tr>
<td>C1</td>
<td>NADH</td>
<td>92</td>
</tr>
<tr>
<td>C1</td>
<td>Melatonin</td>
<td>72</td>
</tr>
<tr>
<td>C1</td>
<td>GSH</td>
<td>3</td>
</tr>
</tbody>
</table>

* C1, H$_2$O$_2$, cystamine and GSSG were used at concentrations of 20 μM for determining zinc release from MT (0.5 μM) and ZFP (2 μM) and at concentrations of 70 μM for ADH (1 μM).
† Antioxidants were used at 200 and 700 μM, respectively (10-fold excess). Readings were taken after 60 min (MT and ADH).
‡ Maximum zinc release was established by incubation with 75 μM Ebselen [30]. Assays were complete after 60 min for MT and ADH and 10 min for ZFP.

### Table 2: Effect of antioxidant quenching on enzyme inhibition by disulphide S-oxides

<table>
<thead>
<tr>
<th>Inhibitor*</th>
<th>Antioxidant†</th>
<th>ADH $V_{max}$ (%) (±5%)</th>
<th>GAPDH $V_{max}$ (%) (±5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>--</td>
<td>--</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>--</td>
<td>95</td>
<td>100</td>
</tr>
<tr>
<td>Cystamine</td>
<td>--</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>GSSG</td>
<td>--</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>C1</td>
<td>--</td>
<td>51</td>
<td>68</td>
</tr>
<tr>
<td>C1</td>
<td>Trolox</td>
<td>44</td>
<td>31</td>
</tr>
<tr>
<td>C1</td>
<td>Ascorbate</td>
<td>50</td>
<td>44</td>
</tr>
<tr>
<td>C1</td>
<td>NADH</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>C1</td>
<td>Melatonin</td>
<td>46</td>
<td>38</td>
</tr>
<tr>
<td>C1</td>
<td>GSH</td>
<td>86</td>
<td>88</td>
</tr>
<tr>
<td>C2</td>
<td>--</td>
<td>47</td>
<td>58</td>
</tr>
<tr>
<td>C2</td>
<td>Trolox</td>
<td>36</td>
<td>41</td>
</tr>
<tr>
<td>C2</td>
<td>Ascorbate</td>
<td>6</td>
<td>51</td>
</tr>
<tr>
<td>C2</td>
<td>NADH</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>C2</td>
<td>Melatonin</td>
<td>37</td>
<td>47</td>
</tr>
<tr>
<td>C2</td>
<td>GSH</td>
<td>100</td>
<td>82</td>
</tr>
</tbody>
</table>

* C1 and C2 were used at concentrations of 70 and 60 μM, respectively for determining $V_{max}$ from ADH (40 nM). C1 and C2 were used at a concentration of 8 μM, each for determining $V_{max}$ from GAPDH (15 nM).
† Antioxidants were used at 700 μM (in the case of C1) and 600 μM (in the case of C2) for ADH and 80 μM for GAPDH (10-fold excess over inhibitor).
hitory effects of C1 and C2 were even more pronounced when GAPDH (with a non-ligating cysteine residue positioned at the active site of the enzyme) was used instead of ADH (Figure 1): an IC \textsubscript{50} value of just 8 \mu M was obtained by incubating C1 with GAPDH (15 nM). This value was essentially unaffected by including a 10-fold excess of non-thiol-based antioxidant (i.e. ascorbate, trolox, melatonin and NADH) in the incubation (Table 2). Some of these antioxidants even seemed to enhance the inhibitory effects of C1 and C2, although the precise mechanism for this enhancement remains to be studied.

Lineweaver–Burk analysis for both GAPDH and ADH activity was performed to decide if C1 acted as a non-competitive enzyme inhibitor (Figure 2). Under the experimental conditions employed, GAPDH possessed a \( V \text{\textsubscript{max}} \) of 0.006 mM \text{-} min\textsuperscript{-1} and a \( K \text{\textsubscript{m}} \) of 0.04 mM. The value for the \( K \text{\textsubscript{m}} \) was unchanged upon incubation with C1, whereas \( V \text{\textsubscript{max}} \) was reduced to 0.004 mM \text{-} min\textsuperscript{-1} (Figure 2a). Analysis of ADH revealed a similar relationship between inhibitor concentration and \( V \text{\textsubscript{max}} \), with a significant decrease of \( V \text{\textsubscript{max}} \) values (uninhibited 0.067 mM \text{-} min\textsuperscript{-1}) in the presence of 70 (0.036 mM \text{-} min\textsuperscript{-1}) and 140 \mu M C1 (0.028 mM \text{-} min\textsuperscript{-1}). Again, the \( K \text{\textsubscript{m}} \) value of 0.3 mM was unchanged upon addition of the inhibitor (Figure 2b).

The Lineweaver–Burk plots are consistent with the proposed enzyme model of non-competitive inhibition. Oxidation of the enzymes’ cysteine residues, with concomitant release of zinc for ADH and inactivation of the essential catalytic cysteine residue in the case of GAPDH, requires C1 to function in a non-competitive manner. The disulphide S-oxides can therefore be seen to be partaking in thiol-specific interactions \textit{in vitro}.

The only antioxidant to interact effectively with the disulphide S-oxides in all assays was GSH (Tables 1 and 2). In the zinc-release experiments, a 10-fold excess of GSH was sufficient to prevent zinc release caused by C1 and C2 (Table 1), and a similar trend was observed in the kinetic studies (Table 2). This quenching reaction was further investigated by incubating increasing concentrations of GSH with C1 and studying the effect on ADH activity (Figure 3). In this case, some quenching activity was observed with 1 and 2 mol of thiol compared with C1, but a > 10-fold excess of thiol over C1 was required to quench fully the oxidizing activity of the disulphide S-monoxyde. The activity of C1 (70 \mu M) was still observable at millimolar concentrations of GSH, indicating that in the presence of ADH and GSH, C1 did not preferably react with GSH.

To gain a better understanding of this interaction, the redox reaction between GSH and the disulphide S-oxides was investigated further by monitoring the \textit{in vitro} consumption of GSH in the presence of C1 and C2 in two independent assays (Figure 4). GST specifically conjugates GSH to CDNB and can therefore be used to probe the GSH concentration, whereas a modified DTNB procedure allows the total number of reduced thiol groups to be quantified. As shown in Figure 4a, approx. 2–3 molar equivalents of GSH were required in the GST assay to quench fully the oxidizing power of C1. This stoichiometry indicated that a multi-step reaction mechanism was in operation, potentially involving the generation of a cascade of redox-active products such as mixed disulphides and sulphenic acids (Scheme 1) [7–9]. C2 oxidized approx. 1–2 molar equivalents of GSH, again in agreement with the proposed mechanism in Scheme 1.

The nature of the thiol-specific interaction between GSH and C1 was therefore studied further by the DTNB assay that measures the total thiol content (Figure 4b). The results obtained also indicated that approx. 2–3 molar equivalents of GSH were...

---

**Figure 3** Effect of GSH on ADH inhibition by C1

ADH (40 nM) was incubated with C1 (70 \mu M) at 37 \(^\circ\)C in KPi (100 mM, pH 7.0) and selected concentrations of GSH for 30 min and the initial rate assayed spectrophotometrically by the addition of acetaldehyde (2 mM) and NADH (300 \mu M) [26].

**Figure 4** Oxidation of GSH by C1

Selected concentrations of GSH were incubated for 30 min at 37 \(^\circ\)C with C1 (70 \mu M). (a) An aliquot was added to the GST assay mixture (GST, 385 nM; CDNB, 1 mM; KPi, 100 mM, pH 7.0) and the absorbance at 340 nm recorded for 2 min. (b) An aliquot was added to the DTNB assay mixture (DTNB, 500 \mu M; KPi, 100 mM, pH 7.0; C1, 7 \mu M) and the absorbance at 412 nm recorded for 1 min.
required to quench C1 completely. They also suggested that few or no other thiol-containing species (e.g. cystamine) were generated as a product of the redox reaction between C1 and GSH as these species would also be detected by DTNB. Similar results were obtained with the analogous reaction of C2 and GSH, where C2 consumed approx. 1–2 molar equivalents of GSH. These findings were in agreement with the generally accepted reaction–oxidation pathways of disulphide S-oxides (Scheme 1). Assuming that the thiol/disulphide exchange reactions between mixed disulphides and GSH played a minor role (although they might be responsible for the approximate values), C1 was expected to oxidize 2 molar equivalents of GSH and C2 to oxidize 1 molar equivalent of GSH. 

In vitro, the antioxidant activity of GSH is enhanced greatly by enzymes such as GPx. The potential for GPx to catalyse the antioxidant effect of GSH upon the disulphide S-oxides was therefore examined by incubating GPx with GSH and C1, and monitoring the release of zinc from ADH for 60 min. GPx was not able to affect the extent of the reaction up to the maximum concentration studied (70 µM C1, 140 µM GSH, 1 µM ADH and 100 nM GPx). This agrees with the substrate specificity of GPx for ROS rather than RSS. Similarly, when C2 was used instead of C1, GPx had no significant effect on zinc release from ADH.

DISCUSSION

The redox behaviour of disulphide S-oxides

During oxidative stress, a number of ROS such as superoxide and peroxides are generated enzymically. Other reactive species such as peroxynitrite are then formed chemically by the interaction of ROS with other cellular components. High cellular concentrations of thiols and disulphides make sulphur a prime target for oxidation under conditions of oxidative stress. Under these conditions, the oxidation of thiols can lead to sulphur species of different oxidation states and various redox behaviours. Whereas disulphides are generally only mild oxidizing species (although they contribute towards ‘disulphide stress’), the reaction of disulphides with ROS (i.e. H₂O₂) also generates RSS in vitro. As discussed in the Introduction section, the formation of various RSS has now been postulated firmly, based on in vitro and cell-fragment studies and, for sulphur-centred radicals at least, has directly been observed in vivo [9]. The precise redox behaviour of such oxidizing sulphur species is therefore of increasing importance in oxidative stress-related research.

Whereas disulphides and thyl radicals contain sulphur in the oxidation state −1, disulphide S-monoxides and dioxides have sulphur in the oxidation states of +1 and +3, respectively [7–9]. These species readily react with thiols to form mixed disulphides and sulphenic or sulphonic acids (oxidation states −1, 0 and +1, respectively). This kind of oxidation (i.e. thiolation) of cysteine residues modifies and subsequently inhibits a number of important proteins and enzymes. Although thiolation itself is not necessarily damaging to proteins, the experiments with ADH and GAPDH have shown that these redox-sensitive proteins are readily modified (i.e. inhibited) by disulphide S-oxides. Interestingly, although zinc–sulphur complexes are generally more resistant towards oxidation, similar effects were observed with MT and ZFP. Passing this ‘litmus test’ for strong oxidants further highlights the aggressive, oxidising behaviour of disulphide S-oxides that is not limited to particular proteins but seems to have a more general effect on sulphur and zinc–sulphur proteins and enzymes. The oxidizing power of disulphide S-oxides is further highlighted by the stoichiometric ratio of thiol oxidation, where one C1 molecule consumes approx. 2–3 GSH molecules and C2 consumes approx. 1–2 GSH molecules.

As a consequence, disulphide S-monoxides and dioxides have to be considered as oxidative stressors with their own particular cellular targets and redox-transformation pathways (Scheme 1). Disulphide S-oxide-induced oxidation of thiols not only inhibits a number of redox-sensitive proteins and enzymes in vitro, but also consumes GSH and hence could tilt the cellular redox balance towards disulphide stress. Furthermore, disulphide S-oxides easily form sulphenic and sulphonic acids whose own toxicity remains to be studied in detail.

Influence of common cellular antioxidants

The living cell contains a number of important antioxidants and antioxidant catalysts. Their presence counteracts oxidative stress and also ‘neutralizes’ a range of oxidizing species. Ascorbate, NADH, melatonin, trolox and GSH are common antioxidants that frequently occur in the cell (apart from trolox which here represents vitamin E). NADH is generally considered to be an indirectly operating antioxidant as it serves as a cofactor for glutathione reductase in converting GSSG into GSH. However, Kirsch and De Groot [32] have recently proposed that this molecule, in addition to its classically recognized role, may also act as a direct antioxidant for scavenging free radicals. The antioxidant species studied here can be considered to represent different chemotypes with a wide-ranging antioxidant redox behaviour.

Interestingly, most of these antioxidants (i.e. ascorbate, NADH, melatonin and trolox) are not effective against disulphide S-oxides and might even slightly enhance the oxidizing power of these species in vitro. From the chemical point of view, this is hardly surprising. The atom-transfer mechanism at the heart of thiol-disulphide exchange reactions cannot be coupled easily to the kind of electron-transfer reactions these antioxidants undergo [9]. As a consequence, disulphide S-oxides cannot be neutralized effectively by such common antioxidants. Like other RSS (e.g. sulphenic acids), disulphide S-oxides have well-defined biological targets (i.e. thiol proteins) that could make them very effective and very harmful.

At the same time, however, the thiol-peptide GSH readily interacts with disulphide S-oxides. This result is hardly surprising
since thiols are the prime targets of these RSS. Interestingly, an excess of GSH is required to abolish the reactivity of disulphide S-oxides and fully ‘neutralizing’ these species consumes up to 3 molar equivalents of GSH. This finding is in good agreement with the proposed reaction pathway of disulphide S-oxides, assuming that mixed disulphides and sulphenic acids do not readily oxidize GSH [9]. Importantly, there is no indication that C1 or C2 preferentially reacts with GSH, allowing other thiol-containing species (e.g. MT, ADH, GAPDH) to undergo redox interactions with C1, even in the presence of a larger excess of GSH (Figure 3). One of the reasons for this activity might be that the redox potential of cysteine residues in peptides varies widely and the cysteine residues in enzymes such as GAPDH might be even more susceptible towards disulphide S-oxides than the one in GSH. In addition, initial reduction of C1 by GSH forms other transient-reactive species such as a sulphenic acid (Scheme 1) that might be even more aggressive than C1. Interestingly, this reaction pathway of disulphide S-monoxides implies the reductive activation of an oxidizing species.

Nevertheless, the in vitro activity of disulphide S-oxides must be closely related to the cellular ratio of GSH and GSSG and therefore depends critically on the microenvironment of the cell. Reports in the literature have suggested that the GSH/GSSG ratio normally lies between 30:1 and 100:1 in most cell phenotypes [33]. However, there is substantial evidence to propose that in certain instances this ratio is drastically disturbed, with GSSG levels more than 25% of GSH levels [12,33]. Previous work has indicated that, under these conditions, physiologically relevant concentrations of disulphide S-oxides are formed in vitro, primarily dependent on the ROS concentration [5,7,8]. From the results presented in this paper, it is evident that if these levels of disulphide S-oxide formation did indeed occur under oxidative stress in vivo, they would be sufficient to lead to a widespread, rapid and non-selective oxidation of biological thiol species (obviously also including GSH).

Although an excess of GSH can prevent some of the damage to proteins and enzymes, the high susceptibility of some thiol proteins towards disulphide S-oxide-induced oxidation compared with GSH, the reductive activation of sulphenic acids and the formation of disulphide stress highlights the oxidizing damage that these RSS, together with thiyl radicals and sulphenic acids, can cause in the presence of GSH. The activity of disulphide S-oxides not only decreases the GSH but also increases the GSSG concentrations (hence increasing the chance of further RSS formation from GSSG). Disulphide S-oxides can also easily inhibit metabolic enzymes such as GAPDH, consequently impairing the cellular mechanisms involved in GSSG reduction.

Although highly speculative at this stage, it is possible that during oxidative stress the formation of disulphide S-oxides, ‘after’ the GSH redox–buffering system has broken down, actually initiates cell death by oxidation of ZFPs, MT and other essential thiol-containing enzymes.

Biochemical implications

The in vitro properties of disulphide S-oxides discussed here have several important biochemical implications. The formation of these species under relatively mild conditions is equally surprising and important supporting the idea that oxidation of thiols is not limited to the cysteine/cysteine-redox pair commonly investigated. In a biochemical context, this notion is significant because of the high and rather specific reactivity of these species. Disulphide S-oxides increase the reactivity of some of their parent compounds, especially H2O2 and disulphides. There are various reports in the literature that have shown rather unusual reactivities of disulphides [34,35]. Some of these results have already been discussed in the light of disulphide S-oxide formation, whereas other findings may be rationalized in this way [36].

The high reactivity of these species is coupled with a high selectivity. Unlike many reactive species (including thiyl radicals), disulphide S-oxides have a rather specific mode of action, undergoing atom-transfer redox reactions and almost exclusively interacting with other sulphur-containing systems. These interactions can lead to the formation of other characteristic sulphur species such as disulphides, thiolated proteins, sulphenic and sulphenic acids. Interestingly, the disulphide S-oxides studied did not seem to discriminate between specific thiol peptides and proteins. The proteins investigated were selected to represent various cysteine-containing species, specifically the catalytic and structural zinc–sulphur complexes and enzymes containing active-site cysteines. All of those proteins were modified by C1 and C2. The representative use of these four proteins is therefore only the starting point. Future studies will focus on the specificity of this exclusive kind of sulphur-based redox chemistry in vitro and subsequently in vivo.

This work was financially supported by the Engineering and Physical Sciences Research Council (grant no. GR/N22007), an Alzheimer’s Society Innovation Grant, the Royal Society, the Wellcome Trust, the Leverhulme Trust and the University of Exeter.

REFERENCES

1 Betteridge, D. J. (2000) What is oxidative stress? Metabolism 49, 3–8
15 Teyssier, C. and Siess, M. H. (2000) Metabolism of dipropyl disulphide by rat liver phase I and phase II enzymes and by isolated perfused rat liver. Drug Metab. Dispos. 28, 648–654

© 2002 Biochemical Society
Reactive sulphur species


Received 21 December 2001/15 February 2002; accepted 2 March 2002