Oxidation of Biological Thiols by Highly Reactive Disulfide-S-Oxides

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Abstract. Oxidative stress involves the generation of a number of reactive species, among them 'reactive oxygen species' and 'reactive nitrogen species'. Recent reports have indicated that disulfide-S-monoxides (thiosulfinates) and disulfide-S-dioxides (thiosulfonates) are formed under conditions of oxidative stress. We have now been able to demonstrate that these species are highly reactive and rapidly oxidise thiols. Glutathione and cysteine were oxidised to mixed disulfides by the action of disulfide-S-oxides. Oxidative attack on the zinc/sulfur protein metallothionein with concomitant zinc release was readily accomplished by these 'reactive sulfur species' whereas hydrogen peroxide showed minimal zinc release.

Key words: Oxidative stress — Reactive sulfur species

Abbreviations: ROS, reactive oxygen species; RNS, reactive nitrogen species; RSS, reactive sulfur species; GSH, glutathione; GSSG, glutathione disulfide; $GS(O)_2SG$, glutathione disulfide-S-dioxide; MT, metallothionein; PAR, 4-(2-pyridylazo) resorcinol; LC-MS, liquid chromatography-mass spectrometry; DTDP, 3,3'-dithiodipropionic acid.

Introduction

Oxidative stress is an important biochemical condition present in several human diseases (Munthe et al. 1986; Honkanen et al. 1991; Halliwell 1994; Sayre et al. 1999; Betteridge 2000; Bush 2000). It is most pronounced in autoimmune and inflammatory diseases such as rheumatoid arthritis (Munthe et al. 1986; Honkanen et al. 1991). In addition the involvement of oxidative stress in neurodegenerative diseases (e.g. familial amyotrophic lateral sclerosis, Alzheimer's disease, Creutzfeld-Jakob disease, Parkinson's disease and Friedrich's ataxia) and diabetes mellitus has recently been postulated (Betteridge 2000; Bush 2000; Sayre et al. 1999). Oxidative stress is linked to the presence of unusually high concentrations of toxic 'reactive species', among them 'reactive oxygen species' (ROS), 'reactive nitrogen species'

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(RNS) and unbound, adventitious metal ions (Betteridge 2000; Bush 2000; Sayre et al. 1999; Davis et al. 2001). Most of these species are highly oxidising, readily modifying redox-sensitive proteins and enzymes as well as attacking membranes and DNA.

ROS frequently react with cellular thiols. Sulfur species such as the cellular redox buffer glutathione (GSH) reduce ROS resulting in the formation of the oxidised thiol glutathione disulfide (GSSG). A number of enzyme-based cellular antioxidant defence systems utilise GSH to detoxify ROS (e.g. glutathione peroxidase, glutathione-S-transferase) resulting in an increase in the cellular concentration of GSSG. While thiols are reducing, the corresponding disulfides are only mildly oxidising under physiological conditions and are thought to have few redox implications within the cell.

It has, however, now become apparent that sulfur also occurs in a number of higher 'reactive' oxidation-states in biological systems (Figure 1) (Giles et al. 2001). Sulfenic acids have recently been identified in a number of redox-active cysteine-enzymes and the oxidation of cysteine to cysteine-sulfenic acid might be part of cellular signalling (Claiborne et al. 1999; Finkel 2000). In addition the garlic component allicin (diallyldisulfide-S-monoxide) readily oxidises thiols and might have antimicrobial properties (Rabinkov et al. 1998). While allicin exclusively occurs in plants recent reports have indicated the possible formation of disulfide-S-oxides under conditions similar to oxidative stress (Finley et al. 1981; Li et al. 2001).

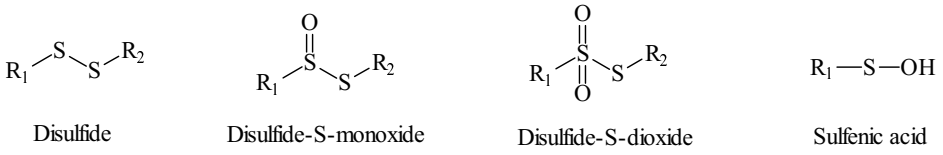

Figure 1. Chemical structures of disulfides, disulfide-S-oxides and sulfenic acids. R_1, R_2 – Chemical groups; $R_1 = R_2$ – Symmetric disulfide; $R_1 \neq R_2$ – Asymmetric (mixed) disulfide.

Our studies were therefore driven by the following questions. Firstly, how do RSS interact with thiols and zinc/sulfur proteins? Secondly, do these sulfur species exhibit an oxidising power comparable to other reactive species?

We here report 'oxidative stressor' qualities of disulfide-S-monoxides and dioxides. These species readily attack thiols and oxidise the zinc/sulfur protein metallothionein (MT) considerably faster and more effectively than the ROS hydrogen peroxide.

Materials and Methods

GSH, GSSG, cysteine, cystamine, hydrogen peroxide and metallothionein (rabbit liver Zn, Cd-MT-2), were obtained from Sigma (Poole, Dorset, U.K.). 4-(2-pyridylazo) resorcinol (PAR) and L-cystine were purchased from Fluka (Gillingham, Dorset, U.K.) and 3,3'-dithiodipropionic acid (DTDP) from Aldrich (Gillingham, Dorset, U.K.). All chemicals were analytical grade and used without further purification. 'Metal free' nitrogen-purged buffers were used for the MT 'oxidation assays' (Jacob et al. 1998a,b, 2000).

Synthesis of disulfide-S-oxides

L-cystine-S-monoxide, cystamine-S-monoxide, DTDP-S-monoxide and glutathione disulfide-S-dioxide were prepared by reaction with H_2O_2 or peracetic acid under acidic conditions according to literature procedures (Steinman and Richards 1970; Wälti and Hope 1971; Mannervik and Larson 1981; Rajca et al. 1990). The monoxides were analysed by elemental analysis, ^{1}H-NMR, LC-MS and IR. Experimental values were found to be in accordance with literature values (Steinman and Richards 1970). The optical rotation of L-cystine-S-monoxide was in line with literature values (Wälti and Hope 1971). Remaining traces of hydrogen peroxide were removed by the addition of MnO_2.

LC-MS experiments

Mass spectra were obtained using a Platform LC-MS instrument (Micromass) operating in electrospray mode (capillary voltage 3.90 kV, cone voltage 40 V). Disulfide-S-monoxides and dioxides (10 mmol/l) were incubated with different thiols (10 mmol/l) in deionised H_2O at 25 ℃. The reaction mixtures were analysed by LC-MS after 30 min.

In vitro oxidation assays

Zn_7MT-2 was prepared from the Zn, Cd-form according to an established procedure (Vašák 1991). MT is a small protein (approximately 6 kDa) that tightly binds zinc in a Zn_4Cys_{11} and a Zn_3Cys_9 cluster. Its thiol ligands are redox-sensitive but their oxidation is generally considerably slower than the oxidation of 'free thiols'. MT in combination with the chromophoric dye PAR can be used in 'oxidation assays' to study the oxidising power (i.e. extent and overall rate of oxidation) of strong oxidants and oxidation catalysis (Jacob et al. 1998a,b, 1999, 2000). The release of zinc from MT was measured spectrophotometrically using a CARY 50 Bio UV/VIS spectrophotometer (Varian). The formation of the $Zn(PAR)_2$ complex (ε_{500} = 65,000 $mol^{-1} \cdot cm^{-1}$ (Shaw et al. 1990) at a PAR concentration of 100 μmol/l in 20 mmol/l HEPES-Na^+, pH 7.5, 25 ℃ was monitored at 500 nm. The use of HEPES buffer is essential in these assays since it does not interact with MT or zinc ions (unlike phosphate buffer) (Maret et al. 1999). Nitrogen gas purged buffers were used throughout.

Results

Redox-transformations of disulfide-S-oxides in the presence of thiols

All disulfide-S-oxides studied reacted with thiols leading to the formation of mixed disulfides. The latter were detected by LC-MS. Cysteine was oxidised by glutathione disulfide-S-dioxide to form the glutathione-cysteine mixed disulfide ($(M + 1)^+$; $m/z = 427$). Mixed disulfides were also obtained from the oxidation of GSH by cystamine-S-monoxide ($(M-1)^-$; $m/z = 381$ for glutathione-cysteamine disulfide) and by DTDP-S-monoxide ($(M-1)^-$; $m/z = 410$ for glutathione-thiopropionic acid disulfide).

Destruction of MT by disulfide-S-oxides in 'oxidation assays'

The reaction of the disulfide-S-oxides with the zinc/sulfur protein MT was analysed by monitoring zinc release (Table 1). The disulfide-S-oxides were incubated with MT and zinc release recorded as a percentage of maximum zinc release (ebselen standard (Jacob et al. 1998b)). Glutathione disulfide-S-dioxide showed superior activity to the monoxides with almost total zinc release within the time period (Figure 2). Cystine-S-monoxide and cystamine-S-monoxide showed comparable activity although at a lower level than the dioxide, whereas DTDP-S-monoxide was the least active of the disulfide-S-oxides. The initial rates of zinc release indicated that all of the disulfide-S-oxides were highly reactive compared to their biological disulfide precursors and the ROS hydrogen peroxide (Table 1). Under the same condi-

Table 1. Reaction of disulfide-S-oxides with MT. MT (0.5 μmol/l) was incubated with different RSS (15 μmol/l, 1.5 fold excess based on MT thiols) in the presence of PAR (100 μmol/l) in HEPES-Na^+ buffer (20 mmol/l) at 25 °C. The reaction was monitored by UV/VIS at 500 nm and readings were taken after 30 min. The maximum zinc release was calculated by incubation of MT (0.5 μmol/l) with ebselen (20 μmol/l) under the same experimental conditions. Initial rates were calculated for 0 to 10 min. Relative experimental error <10 %.

Compound	% of maximum zinc release	Initial rate of zinc release ($\times 10^{-10}$ mol·l^{-1}·s^{-1})
$GS(O)_2SG$	89.9	42.7
Cystine-S-monoxide	59.8	12.3
Cystamine-S-monoxide	44.4	9.2
DTDP-S-monoxide	19.5	16.4
GSSG	0.5	2.1
Cystine	1.5	0.5
Cystamine	0.0	n.a.
DTDP	0.0	n.a.
Hydrogen peroxide	11.3	5.9

n.a., not applicable (no significant zinc release).

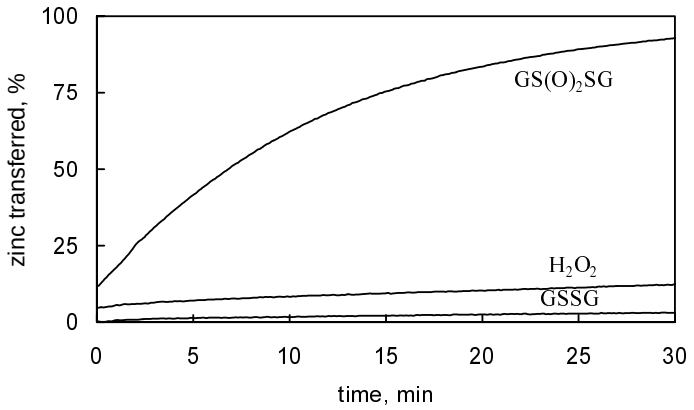

Figure 2. Zinc release from MT in the presence of glutathione disulfide-S-dioxide ($GS(O)_2SG$). MT (0.5 μmol/l) was incubated with glutathione disulfide-S-dioxide (15 μmol/l) in the presence of PAR (100 μmol/l) in HEPES-Na^+ buffer (20 mmol/l) at 25 ℃. The reaction was monitored by UV/VIS at 500 nm. GSSG (15 μmol/l) and H_2O_2 (15 μmol/l) were used as controls.

tions the latter only released a small percentage of the total zinc. L-cysteinesulfinic acid, L-cysteine-S-sulfate and glutathione sulfonic acid were inactive in these assays.

Discussion

Formation of disulfide-S-oxides in vitro

During oxidative stress a number of ROS such as superoxide and peroxides are generated enzymatically. Other reactive species are then formed chemically by the interaction of ROS with other cellular components. For example, peroxynitrite is generated from superoxide and nitrogen monoxide. Peroxynitrite itself is an aggressive stressor that oxidises, nitrates and hydroxylates small molecules, proteins and enzymes (Beckman et al. 1990). The reaction of thiols and disulfides with ROS (i.e. hydrogen peroxide) generates 'reactive sulfur species' (RSS). Hydrogen peroxide is known to oxidise disulfides to disulfide-S-monoxides and dioxides at physiological pH and relatively low peroxide concentrations at a biologically relevant rate ($t_{1/2}$ = 2 min) (Finley et al. 1981). *In vitro* glutathione disulfide-S-oxide formation during the spontaneous degradation of S-nitrosoglutathione (SNG) has also been reported (Li et al. 2001). Interestingly, the enzymatic formation of disulfide-S-monoxides has also been observed in rabbit liver microsomes due to the presence of cytochrome P-450 (Fukushima et al. 1978). These results indicate that disulfide-S-oxides can be generated within animal cells under conditions of oxidative stress.

Redox-behaviour of reactive sulfur species (RSS)

Disulfide-S-monoxides and dioxides readily react with thiols to form mixed disulfides, the presence of which has been demonstrated by LC-MS. As a consequence, the formation of disulfide-S-oxides from disulfides and ROS under oxidative stress has two important implications. Firstly, disulfides can behave as antioxidants that reduce hydrogen peroxide and possibly other ROS (such as superoxide and hydroxyl radicals) under oxidative stress conditions (Finley et al. 1981). Secondly, the reaction of disulfides with ROS leads to the (non-enzymatic) formation of RSS (similar to the formation of peroxynitrite). The high oxidising power of disulfide-S-oxides is apparent when their interactions with the zinc/sulfur protein MT are considered. The thiols of MT are generally less reactive than the ones of GSH or of uncoordinated surface cysteines in proteins and enzymes. Oxidation of MT can therefore be seen as 'litmus test' for strong oxidising agents. The rapid and efficient zinc release from MT caused by disulfide-S-monoxides and dioxides (Figure 2) shows that disulfide-S-oxides are among the more aggressive oxidising agents interacting with MT.

A comparison of the available standard redox potentials (Table 2) shows that while the ROS such as hydrogen peroxide and the hydroxyl radical are strongly oxidising ($E^{0\prime}$ of +1.35 and +2.33 V at pH 7.0 respectively (Loach 1976)) thiols are relatively redox-stable under physiological conditions ($E^{0\prime}$ of approximately −0.23 V). Although reliable electrochemical data for RSS such as the disulfide-S-oxides and the sulfenic acids is not yet available, the results of the MT zinc assay indicate that these species will prove to be highly oxidising. These RSS release zinc up to 20 times faster than GSSG and up to seven times faster than hydrogen peroxide (Table 1). The particularly high reactivity of disulfide-S-oxides towards more stable protein and enzyme thiolates has not yet been fully realised. While the interaction of these species with thiols has previously been reported, their high activity at low concentrations and rapid rate of reaction in our 'oxidation assays' indicates that disulfide-S-oxides are considerably more damaging towards proteins than previously thought. This finding is particularly important since disulfide-S-oxides can clearly be formed under relatively mild oxidising conditions *in vivo*. As a consequence disulfide-S-oxides are likely to be among the physiologically most important 'reactive sulfur species'.

Table 2. Standard oxidation-reduction potentials of ROS and sulfur compounds

Redox-Couple	$E^{0\prime}$/V
GSSG/GSH	−0.23
Cystine/Cysteine	−0.34
H_2O_2/H_2O	+1.35
O_2/H_2O	+0.82
O_2/H_2O_2	+0.30
$O_2/O_2^{\bullet -}$	−0.33
$O_2^{\bullet -}/H_2O_2$	+0.94
$HO^{\bullet}/H_2O$	+2.33

$E^{0\prime}$ indicates the mid-point potential for a particular couple at pH 7.0 (Loach 1976).

Biochemical implications

The oxidising power of disulfide-S-oxides has important biochemical implications. Although glutathiolation itself is not necessarily damaging to cells, the MT experiments have shown that redox-sensitive proteins can be modified (i.e. inhibited) by disulfide-S-monoxides and dioxides. As a consequence RSS have to be considered as oxidative stressors with their own particular cellular targets and redox-transformation pathways. Oxidation of cellular thiols not only inhibits a number of redox-sensitive proteins and enzymes, it also consumes GSH and hence tilts the cellular redox-balance towards disulfide stress. These studies have shown that the important zinc/sulfur protein MT is a surprisingly easy target for RSS. Other zinc/sulfur proteins and enzymes with similar reactivity include zinc-finger transcription factors (e.g. TFIIIA), a number of important cellular receptors (e.g. estrogen receptor) and alcohol dehydrogenase (Jacob et al. 1998b). The wider biochemical implications of RSS formation are therefore comparable to the effects of peroxynitrite. Unlike peroxynitrite, however, disulfide-S-monoxides and dioxides seem to specifically interact with thiols and might therefore prove to have specific cellular targets. Other sulfur compounds in higher oxidation states include thiyl radicals (Abedinzadeh 2001), trisulfides (Agarwal 1996) and sulfenic acids (Claiborne et al. 1999). It remains to be shown if these species also exhibit RSS characteristics. Nevertheless, the biochemical basis for RSS as oxidative stressors is rapidly developing. The *in vitro* and *in vivo* behaviour of RSS therefore merits further investigations.

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