Metal specificity in DNA damage prevention by sulfur antioxidants

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ARTICLE INFO

Article history:
Received 20 February 2008
Received in revised form 4 June 2008
Accepted 14 June 2008
Available online 20 June 2008

Keywords:
Antioxidant
Sulfur
Reactive oxygen species
DNA damage

ABS T RACT

Metals such as CuI and FeII generate hydroxyl radical (·OH) by reducing endogenous hydrogen peroxide (H2O2). Because antioxidants can ameliorate metal-mediated oxidative damage, we have quantified the ability of glutathione, a primary intracellular antioxidant, and other biological sulfur-containing compounds to inhibit metal-mediated DNA damage caused hydroxyl radical. In the CuI/H2O2 system, six sulfur compounds, including both reduced and oxidized glutathione, inhibited DNA damage with IC50 values ranging from 3.4 to 12.4 µM. Glutathione and 3-carboxypropyl disulfide also demonstrated significant antioxidant activity with FeIII and H2O2. Additional gel electrophoresis and UV–vis spectroscopy studies confirm that antioxidant activity for sulfur compounds in the CuI system is attributed to metal coordination, a previously unexplored mechanism. The antioxidant mechanism for sulfur compounds in the FeIII system, however, is unlike that of CuI. Our results demonstrate that glutathione and other sulfur compounds are potent antioxidants capable of preventing metal-mediated oxidative DNA damage at well below their biological concentrations. This novel metal-binding antioxidant mechanism may play a significant role in the antioxidant behavior of these sulfur compounds and help refine understanding of glutathione function in vivo.

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1. Introduction

Reactive oxygen species (ROS), reactive nitrogen species (RNS), and UV–generated radical species have been widely studied and are responsible for cellular damage to nucleic acids, proteins, and lipids [1–2]. Reactive oxygen species, including superoxide radical (O2·−) and hydroxyl radical (·OH), cause DNA strand breaks, base modifications, and DNA cross linking and are implicated as a cause of many diseases including cancer, chronic inflammation, neurodegenerative, and cardiovascular diseases [1,3–5]. Although many ROS have been implicated in cellular damage and disease, the primary focus for this paper is on hydroxyl radical, since metal-mediated damage from ·OH is a primary source of cell death in both prokaryotes and eukaryotes, including humans [6–8].

Hydrogen peroxide (H2O2), a non-radical species derived from oxygen metabolism, is relatively non-reactive compared to other ROS. However, cellular H2O2 is reduced by metal ions to generate the highly-reactive and damaging hydroxyl radical [9]. Among the many cellular components damaged by metal-generated ·OH, DNA is particularly important because it contains the vital genetic material of a cell. DNA damage from metal-mediated ·OH formation is attributed to Fenton or Fenton-type reactions with H2O2 and FeIII or CuI [10].

FeIII/CuI + H2O2 → FeII/CuII + ·OH + OH−

Iron and copper are the two most common transition metal ions in biological systems and are required components of many essential enzymes and proteins, including transferrin, ferritin, Cu–Zn superoxide dismutase, and ceruloplasmin [11]. Both metals are required for normal physiological function, but must also be regulated due to their role in oxidative damage. In fact, the inability of cells to maintain proper iron and copper homeostasis has been associated with increased oxidative damage due to ROS generation [12,13]. Under normal conditions, the amount of non-protein-bound iron (NPBI) is low [14]. In Escherichia coli this concentration is ∼20 µM [9], but this concentration rises to between 80 and 320 µM if iron homeostasis is not maintained [15,16], causing a much greater susceptibility to oxidative DNA damage [15,17]. In humans, even normal levels of body iron are linked to oxidative DNA damage [18], and mildly elevated iron levels are attributed to increased cancer incidence, cardiovascular disease, aging, and Parkinson's disease [19–22]. These pathological conditions can also cause elevation of NPBI in cells, resulting in increased oxidative damage [9,23–26].

Unlike iron, the concentration of non-protein bound copper within cells is uncertain [27–29]. O'Halloran and coworkers calculated cellular copper concentrations based on the affinity of copper-binding proteins to be less than 10−18 M [28,29] in E. coli. In humans, 90% of blood copper is bound to ceruloplasmin, whereas the remaining 10% is non-specifically bound to albumin and other molecules. In addition, Fahimi and coworkers observe labile copper
localized in mouse mitochondria and Golgi [30]. This labile copper can generate ROS [5,31], and is associated with increased oxidative damage [5,32]. Increased intracellular copper levels are definitively linked to the development of cancer, atherosclerosis, and diabetes, as well as Wilson's and Alzheimer's diseases in humans [5,33–35].

Antioxidants can ameliorate oxidative damage caused by ROS and are of considerable interest for the prevention and treatment of disease [1,36]. Among the numerous antioxidants utilized by cells, sulfur compounds including cysteine, methionine, and glutathione and their derivatives have been widely studied for their antioxidant properties [37]. For example, supplementation with N-acetyl-cysteine increased immune function in HIV infection in humans [38], and consumption of sulfur-containing compounds from Allium vegetables can result in chemopreventive effects for stomach, colorectal, and prostate cancers [39–41]. Although numerous studies have demonstrated the value of antioxidant sulfur compounds in disease treatment and prevention, direct quantification and comparison of their antioxidant activity is lacking. In addition, the role of antioxidant-metal coordination as a mechanism for preventing metal-mediated oxidative damage has not been examined.

The tripeptide glutathione (GSH) has been widely studied as an antioxidant for its role in the enzymatic decomposition of H2O2 by glutathione peroxidase (GPx) and its radical scavenging ability: it also plays a critical role in cellular detoxification [42]. GSH is the most abundant non-protein thiol in cells, with concentrations of 1–11 mM in cytosol, 3–15 mM in nuclei, and 5–11 mM in mitochondrial [1,43]. Respiratory and enzymatic processes as well as drug interactions continually generate ROS and RNS, causing oxidation of intracellular components, including GSH, and increasing oxidative stress in cells [44–48]. Unsurprisingly, cells depleted of glutathione also have increased oxidative stress levels, an underlying cause of many diseases [49,50].

GSH redox state is increasingly seen as a biological indicator of oxidative stress and disease progression [48,51]. Many factors cause changes in intracellular GSH concentrations: GSH becomes oxidized as cells progress through the cell cycle due to oxidative stress, and plasma GSH oxidation is associated with aging and age-related diseases [51,52]. Jones examined the GSH/GSSG balance in one age-related study and found that glutathione in subjects over the age of 45 was increasingly oxidized [53]. GSH concentrations also decrease and GSH/GSSG balance shifts to oxidized GSSG as a result of increased cellular oxidative stress after bone marrow transplantation, high-dose chemotherapy, aging, cigarette smoking, and diabetes [51]. Although there is considerable evidence linking the redox state of GSH/GSSG to pathogenesis, less evidence linking the redox state of GSH/GSSG to pathogenesis, less antioxidant activity is examined. A greater understanding of the mechanisms responsible for antioxidant activity of these essential sulfur compounds will provide a critical link between basic biochemistry and clinical observations and lay the foundation for design and evaluation of effective antioxidant supplements to prevent and treat diseases caused by oxidative damage.

2. Materials and methods

2.1. Materials

Water was purified using a Nano Pure Diamond Ultrapure system (Barnstead International, Dubuque, IA). H2O2 solution (30%) and CuSO4 were from Fisher. NaCl (99.999% to avoid trace metal contamination) and methyl-cysteine were purchased from Sigma–Aldrich; ethanol (99.5%), 3-(N-morpholino)propanesulfonic acid (MOPS), 2,2′-dithiobisalicylic acid, 3-carboxypropyl disulfide and glutathione (oxidized and reduced) were obtained from Acros. 2-(N-Morpholino)ethanesulfonic acid (MES) was from Calbiochem; 2,2′-bipyridine (bipy) and cysteine were purchased from Alfa Aesar. EDTA (disodium salt, dihydrate, crystal) and ascorbic acid were from J.T. Baker. Methionine, cystamine, and 2-amino phenyl disulfide were purchased from TCI America, and cystine was from Lancaster.

2.2. Purification of plasmid DNA

Plasmid DNA (pBBSK) was purified from E. coli DH1 using a Qia prep Spin Miniprep kit (Qiagen, Chatsworth, CA). Plasmid DNA was eluted in Tris–EDTA (TE) buffer and dialyzed for 24 h at 4 °C against 130 mM NaCl to remove metal ions. UV–vis measurements were acquired from a Shimadzu UV-3101 PC spectrophotometer. Absorbance ratios of A260/A280 ≤ 0.95 and A260/A230 ≥ 1.8 were determined for all DNA used.

2.3. DNA damage experiments

CuSO4 (6 μM), the indicated concentrations of sulfur compound, ethanol (10 mM), dH2O2, MOPS (10 mM), NaCl (130 mM), and ascorbic acid (7.5 μM) at pH 7 were allowed to stand for 5 min at room temperature. Plasmid (pBBSK, 0.1 pmol in 130 mM NaCl) was added to the reaction mixture and allowed to stand for an additional 5 min. The reaction was then initiated by the addition of H2O2 (50 μM). After 30 min, EDTA (50 μM) quenched the reaction. For Cu(bipy)2+ gel electrophoresis studies, Cu(bipy)2+ (50 μM) was used in place of CuSO4, and the concentration of ascorbic acid was increased to 62.5 μM. For experiments with iron, FeSO4 (2 mM) and MES (10 mM, pH 6) was substituted for the CuSO4, ascorbic acid, and MOPS; the pH was decreased due to the insolubility of iron at pH 7. All Fe8 solutions were freshly prepared and used immediately as previously reported [58]. All concentrations indicated are final concentrations in a 10 μL volume. For Fe8 experiments with GSSG and GSH, the final MES buffer concentration was increased to 100 mM (pH 6) for 10,000 μM GSSG/GSH.

Damaged and undamaged forms of plasmid DNA were separated using 1% agarose gel electrophoresis in TAE buffer (140 V
for 30 min). The gels were then stained with ethidium bromide and imaged under UV light, and the amount of damaged (nicked) DNA versus undamaged (circular) DNA was determined using UVi-ProMW [Jencons Scientific Inc., Bridgeville, PA, 2003]. Ethidium bromide stains circular DNA less efficiently than nicked DNA; therefore, circular DNA band intensities were multiplied by 1.24 prior to comparison [59,60]. The intensities of the DNA bands were normalized for each lane so that addition of circular and nicked DNA equals 100%.

2.4. Calculation of DNA damage inhibition

DNA damage inhibition was calculated using the formula 1 – [%N/[%B] × 100, where %N is the percentage of nicked DNA in the sulfur-containing lanes (lanes 5–9) and %B is the percentage of nicked DNA in the CuII/H2O2 or FeII/H2O2 lane (lane 4). Both %N and %B were normalized for residual nicking (lane 2) prior to calculation, and results are the average of at least three trials with standard deviations indicated by error bars. Statistical significance was determined by calculating p values at 95% confidence (p < 0.05 indicates significance) as described by Perkowski et al. [61]; these values can be found in the supplementary material.

2.5. IC50 determination

The plots of percent inhibition of DNA damage versus log concentration of sulfur compound (μM) were fit to a variable-slope sigmoidal dose-response curve using SigmaPlot (v. 9.01, Systat Software, Inc., San Jose, CA). The error given for each IC50 value in Table 1 represents the standard deviation of the values obtained from the fits of three separate experiments.

2.6. Quantification of thiol

Quantification of thiol for both cysteine and reduced glutathione was completed using Thiol and Sulfide Quantitation Kit (T-6060, Molecular Probes, Eugene, OR). For cysteine and GSH, 92% and 75% were in the reduced form, respectively. Since corrections for GSH oxidation would be very small, reported values for GSH antioxidant activity are uncorrected.

2.7. UV–vis measurements

CuSO4 (58 μM) and ascorbic acid (72.5 μM) were combined in MOPS buffer (10 mM) at pH 7 and allowed to stand for 3 min at room temperature. The sulfur compound (116 μM) was then added to the reaction mixture and the solution was allowed to stand for an additional 3 min. For spectra with only copper/ascorbic acid or sulfur compound, an equivalent volume of MOPS buffer was added. A similar procedure was used for Cu(bipy)2+ studies except Cu(bipy)2+ (29 μM), ascorbic acid (36.25 μM), and the sulfur compound (58 μM) were used. For experiments with FeII, FeSO4 (300 μM) and the sulfur compound (600 μM) were combined in MES buffer (10 mM; pH 6) and allowed to stand for 3 min at room temperature prior to obtaining UV–vis spectra. All concentrations indicated are final concentrations in a 3 mL reaction volume. Exact values for kmax was determined by subtracting the CuII/ascorbic acid and sulfur compound spectra from the combined CuII/sulfur compound spectrum.

3. Results and discussion

3.1. Determining antioxidant activity of sulfur compounds with Cu

Ten sulfur compounds (the compounds shown in Fig. 1 and oxidized glutathione) were examined using gel electrophoresis to determine their ability to prevent DNA damage by CuII/H2O2. Fig. 2 shows the effects of oxidized glutathione (GSSG) in inhibiting copper-mediated oxidative DNA damage. H2O2 alone does not damage DNA; however, combining CuII, ascorbate, and H2O2 results in 97% DNA damage (Fig. 2, lane 4). Addition of increasing concentrations of GSSG causes a substantial decrease in oxidative DNA damage as compared to lane 4, with 10 μM GSSG inhibiting 99% (p < 0.001) of DNA damage (Fig. 2, lane 9). Fig. 3 illustrates the best-fit dose–response curve for GSSG, demonstrating that the concentration of GSSG required to inhibit 50% of copper-mediated oxidative DNA damage (IC50) is 6.6 ± 1.0 μM (Table 1). Similarly, the IC50 for reduced glutathione is 12.4 ± 1.0 μM, and GSH inhibited 97% of DNA damage at 30 μM (p < 0.001). Notably, these IC50 values are a thousand times less than the measured intracellular concentrations of glutathione [1–15 mM] [1,43].

Because cysteine (Cys) and cystine (Cys2) are also important intracellular sulfur antioxidants, we quantified the ability of these compounds to inhibit copper-mediated DNA damage using the same method. Cys2 inhibits 100% of copper-mediated DNA damage at 10 μM and has an IC50 value of 3.4 ± 1.1 μM, demonstrating that Cys2 is an excellent antioxidant with activity almost twice that of the major cellular disulfide, GSSG. Cysteine is also a potent antioxidant, with an IC50 value of 7.6 ± 1.2 μM (Table 1). As was observed for the thiol GSH, higher concentrations of reduced Cys are required to inhibit DNA damage compared to the disulfide Cys2.

The sulfur compounds methionine, methylv-cysteine, 3-carboxypropyl disulfide, cystamine, 2-amino-phenyl disulfide, and 2,2’dithiolalcalic acid (Fig. 1) were also examined for their ability to

Table 1

IC50 values and kmax for sulfur compounds with copper and maximum DNA damage inhibition with iron

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (μM)a</th>
<th>kmax (nm)b</th>
<th>Maximum DNA damage inhibition (% with FeII)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSSG</td>
<td>6.6 ± 1.0</td>
<td>241</td>
<td>50.2 ± 4.5 at 10,000 μM</td>
</tr>
<tr>
<td>GSH</td>
<td>12.4 ± 1.0</td>
<td>242</td>
<td>23.5 ± 8.4 at 10,000 μM</td>
</tr>
<tr>
<td>Cysteine</td>
<td>3.4 ± 1.1</td>
<td>239</td>
<td>–</td>
</tr>
<tr>
<td>Methyl-cysteine</td>
<td>0.7 ± 1.2</td>
<td>239</td>
<td>–</td>
</tr>
<tr>
<td>Methionine</td>
<td>9.6 ± 1.0</td>
<td>239</td>
<td>–</td>
</tr>
<tr>
<td>Cystamine</td>
<td>11.8 ± 1.3</td>
<td>233</td>
<td>–</td>
</tr>
<tr>
<td>3-Carboxypropyl disulfide</td>
<td>–</td>
<td>–</td>
<td>21.8 ± 6.2 at 1000 μM</td>
</tr>
<tr>
<td>2-Amino-phenyl disulfide</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2,2’dithiolalcalic acid</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

a IC50 is defined as the concentration at which the compound inhibits 50% of DNA damage.
b kmax was calculated based on the difference in absorption for each spectrum.

Fig. 1. Structures of sulfur compounds tested for DNA damage inhibition.
inhibit copper-mediated DNA damage (see supplementary material for gel images and inhibition data). The amino acids methionine and methyl-cysteine have IC_{50} values higher than the disulfides cystine or oxidized glutathione (11.8 ± 1.3 and 9.6 ± 1.0 μM, respectively, Table 1), and inhibit > 95% of DNA damage at 100 μM. 2-Aminophenyl disulfide, cystamine, 3-carboxypropyl disulfide, and 2,2'-dithiosalicylic acid do not inhibit copper-mediated DNA damage.

3.2. Structural effects of sulfur compounds on antioxidant activity with Cu^{2+}

Directly quantifying and comparing the DNA damage inhibition of a range of sulfur compounds enables evaluation of the effects of structure on antioxidant activity. Of the sulfur compounds tested, GSSG, GSH, cystine, methionine, cysteine, and methyl-cysteine prevent DNA damage with Cu^{2+}. The disulfide compounds GSSG and cystine have the lowest IC_{50} values, likely due to the presence of two sulfur atoms in these molecules. In addition, the thiol compounds GSH and cysteine inhibit approximately half the copper-mediated DNA damage of their corresponding disulfides. Cystine inhibits 50% DNA damage at approximately a 0.5:1 mole ratio of cystine to copper, but this is unlikely to be a direct stoichiometry effect, since IC_{50} values for most other sulfur compounds do not correlate to stoichiometric equivalents of copper.

Interestingly, the six sulfur compounds that prevent DNA damage with Cu^{2+} and H_{2}O_{2} have both amino and carboxylate substituents, whereas cystamine and 3-carboxypropyl disulfide with only amine or carboxylic acid substituents, respectively, show no activity. Additionally, disulfide compounds with only amine or carboxylic acid aromatic substituents, 2-aminophenyl disulfide and 2,2'-dithiosalicylic acid, showed no antioxidant activity in the copper system, indicating that the presence of both amine and carboxylic acid substituents, in addition to the two sulfur atoms, also significantly affects antioxidant activity.

3.3. Inhibition of iron-mediated oxidative DNA damage with sulfur compounds

Antioxidant activity for the ten sulfur compounds was also determined for iron-mediated DNA damage using similar gel electrophoresis methods with Fe^{3+} and H_{2}O_{2}. The gel in Fig. 4 shows that GSSG inhibits iron-mediated DNA damage (lanes 9 and 10), with a maximum inhibition of 50% of oxidative DNA damage (p = 0.003) at 10,000 μM. In addition to GSSG, GSH and 3-carboxypropyl disulfide also inhibit iron-mediated DNA damage: GSH inhibits 23% of oxidative damage at 10,000 μM (p = 0.04), and 3-carboxypropyl disulfide inhibits 22% of iron-mediated DNA damage at 1000 μM (p = 0.03). The significant inhibition of iron-mediated DNA damage by both reduced and oxidized glutathione is surprising, since the other amino acid compounds have no effect on this type of damage. These results suggest an additional reason for the presence of large concentrations of glutathione in vivo, since this antioxidant would prevent both copper- and iron-mediated oxidative damage at high concentrations. The observed antioxidant activity of 3-carboxypropyl disulfide with Fe^{3+}/H_{2}O_{2} was unexpected, since this compound did not prevent oxidative DNA damage with Cu^{2+}/H_{2}O_{2}.

3.4. The role of metal coordination in antioxidant activity

Previous work determined that selenium compounds inhibit copper-mediated oxidative DNA damage through Cu^{2+} coordination.
Similarly, sulfur compounds might also exert their antioxidant effects through copper binding. This antioxidant mechanism would likely have biological relevance, since glutathione–copper binding is observed in human cells, and glutathione reduces cell death in copper-treated cultures [54,55]. To test whether metal binding is responsible for the antioxidant activity of the tested sulfur compounds, we conducted gel electrophoresis experiments with Cu(bipy)$_2$ (bipy = 2,2′-bipyridine) instead of CuI as the copper source. The bipyridine ligands fully coordinate the copper ion, leaving no vacant coordination site for a sulfur compound to bind, but this copper complex can reduce H$_2$O$_2$ to form DNA-damaging hydroxyl radical (Fig. 5, lane 4). If metal coordination is required for the antioxidant activity of the sulfur compound, no inhibition of DNA damage should be observed.

The gel in Fig. 5 shows the effect of increasing GSSG concentration on DNA damage for the Cu(bipy)$_2$/H$_2$O$_2$ system. In contrast to experiments with CuI as the copper source (Fig. 2), GSSG shows no DNA damage inhibition even at high concentrations (lanes 5–9), strongly suggesting that copper coordination is required for antioxidant activity. Similarly, GSH, methionine, methyl-cysteine, cysteine, and cysteine demonstrate significant antioxidant activity with CuI, but showed no inhibition of DNA damage with Cu(bipy)$_2$ (supplementary material). Thus, for all of the sulfur compounds that prevent copper-mediated DNA damage, interaction with the copper is required.

Using UV–vis spectroscopy we examined this potential sulfur-metal coordination by adding a sulfur compound to solutions of CuI/ascorbic acid. Upon addition of GSSG to CuI/ascorbate, a new absorption band ($\lambda_{\text{max}}$ = 240 nm, Fig. 6) that may correspond to a Cu–S charge transfer absorbance [53,62] is observed. For all sulfur compounds that prevented copper-mediated DNA damage, similar absorption bands are seen in the UV–vis spectra (Table 1 and supplementary material), but these absorption bands are not observed for sulfur compounds with no antioxidant activity.

In addition, for sulfur compounds showing antioxidant activity, these UV–vis absorbances are not observed when the sulfur compounds are combined with Cu(bipy)$_2$/ascorbic acid, suggesting that sulfur–copper coordination is responsible for their observed antioxidant activity in the gel studies (supplementary material). Our results also indicate that these UV–vis studies provide a simple diagnostic tool for preliminary assessment of the antioxidant efficacy of sulfur compounds with Cu/H$_2$O$_2$.

It is clear that metal coordination is required for the observed antioxidant activity of the tested sulfur compounds with copper. Although an IC$_{50}$ value of 3.4 ± 1.1 μM for cystine suggests a 0.5:1 ratio of antioxidant to metal, IC$_{50}$ values for other sulfur compounds do not follow similar stoichiometric trends, suggesting that a simple copper-to-sulfur antioxidant ratio does not determine the efficacy of a given sulfur compound. Interestingly, the IC$_{50}$ values for disulfides are approximately half those of the analogous thiol; however, this relationship is likely coincidental since reduction potentials for Fe$^{III}$/II (770 mV), Fe[EDTA]$^{2-}$ (120 mV), Cu$^{II}$/II (153 mV), and ascorbic acid (60 mV) are much higher than those for cysteine (−250 mV) and GSSG (−264 mV) [9,63–66]. Thus, reduction of these disulfide compounds to their corresponding thiolates by the metal ions is not favorable. Work is ongoing to elucidate the coordination environment of these sulfur antioxidant complexes with Cu$^1$.

Because GSSG, GSH, and 3-carboxypropyl disulfide also demonstrated antioxidant activity in the Fe$^{II}$/H$_2$O$_2$ system, we conducted similar gel electrophoresis experiments with [Fe(EDTA)]$^{2-}$ to determine whether the observed antioxidant activity was the result of iron coordination. Prior coordination of Fe$^{II}$ with EDTA will prevent antioxidant-iron interactions, although [Fe(EDTA)]$^{2-}$ still reduces hydrogen peroxide and generates hydroxyl radical. Under these conditions, no DNA damage inhibition for 3-carboxypropyl disulfide was observed, indicating that antioxidant-iron coordination may be required for antioxidant activity.

In contrast, similar experiments with GSSG show 68% DNA damage inhibition at 10,000 μM ($p = 0.001$) with [Fe(EDTA)]$^{2-}$, greater than the 50% inhibition observed with Fe$^{II}$ alone. GSH (10,000 μM) also shows an increase in DNA damage inhibition with [Fe(EDTA)]$^{2-}$: 76% compared to 23% inhibition observed with Fe$^{II}$ ($p = 0.001$). Glutathione is unlikely to effectively compete with EDTA for iron binding sites due to the high Fe$^{II}$–EDTA formation constant (2 × 10$^{14}$) [67]. The reasons for this increase in DNA damage inhibition with reduced and oxidized glutathione when [Fe(EDTA)]$^{2-}$ is used as the iron source is unclear, but it is apparent that for glutathione, the mechanisms for copper-mediated and iron-mediated inhibition DNA damage are substantially different. It is highly unlikely that this antioxidant activity can be attributed to radical scavenging alone, since both Cu$^1$ and Fe$^{II}$ generate hydroxyl radical, but the antioxidant activity of glutathione with these two metal ions is significantly different.

UV–vis experiments with Fe$^{II}$ and GSSG or GSH do not show an absorption band corresponding to Cu–S coordination ($\lambda_{\text{max}}$ ≈ 320 nm) [68–70]; however, at the maximum concentrations of GSSG (300 μM) and GSH (600 μM) that could be used in these experiments, GSSG and GSH show very little antioxidant activity in the Fe$^{II}$/H$_2$O$_2$ system by electrophoresis. 3-Carboxypropyl disulfide does inhibit DNA damage at 600 μM; however, no Fe–S
absorption band is observed for this compound in UV–vis experiments at this concentration. It is possible that 3-carboxypropyl disulfide coordinates to the iron through the carboxylate oxygen atoms rather than the sulfur atoms: in infrared spectroscopy cysteine carboxylate O–ZnII bond stretches are observed at 1634 and 1418 cm⁻¹ [71], methyl-cysteine O–ZnII and O–NiII bond stretches are between 1593–1622 cm⁻¹ and 1408–1411 cm⁻¹ [71,72], and methionine M–O bond stretches are observed between 1610 and 1625 cm⁻¹ and 1392–1415 cm⁻¹ for FeII, NiII, CuI, and ZnII [73]. It is also possible that the FeIII–sulfur interactions are weak and not detectable using this method due to concentration limitations of the experiments. That 3-carboxypropyl disulfide inhibits iron but not copper-mediated DNA damage is an unexpected result, since copper readily binds methionine and cysteine in vivo [74,75]. Thus, experimental evidence that antioxidant-iron coordination is required for DNA damage inhibition is ambiguous, and another mechanism may be partially responsible for the observed activity.

Overall, the greater antioxidant efficacy of sulfur compounds to prevent copper-mediated DNA damage is consistent with metal coordination behavior as predicted by hard-soft acid-base theory. CuI is a soft metal ion that is larger and more polarizable than the harder FeII. Since sulfur is a soft ligand, sulfur compounds should more strongly interact with CuI, promoting greater antioxidant activity. Harder ligands like the oxygen-containing carboxylate substituents should coordinate more favorably to the harder FeII than CuI [76]. This may partially account for the observation that 3-carboxypropyl disulfide prevents DNA damage with FeII since it has two hard oxygen donors in the carboxylate groups that would promote iron coordination. Our experimental evidence confirms sulfur antioxidant coordination to copper as a mechanism of activity, and experiments to establish the precise nature of this coordination (e.g. through sulfur, oxygen, and nitrogen atoms of the amino acids) and stoichiometry of the antioxidant-copper complexes using EXAFS and mass spectrometry are ongoing. Understanding this copper-antioxidant coordination will help determine why compounds such as cystine have potent antioxidant behavior, whereas similar compounds such as cysteamine do not. Further work is also necessary to establish the role of iron coordination in sulfur antioxidant activity.

3.5. Implications of antioxidant activity with CuI and FeII

Both reduced and oxidized forms of glutathione are capable of preventing copper- and iron-mediated DNA damage at biologically-relevant concentrations (1–15 mM) [1,43]. Cystine and cysteine are effective at preventing copper-mediated damage (IC50 = 3.4 ± 1.1 and 7.6 ± 1.2 μM, respectively) well below biologically-relevant concentrations (100–200 μM in E. coli) [9], but not effective at preventing iron-mediated DNA damage. Because FeII is a significant source of cellular oxidative stress, the ability to prevent oxidative DNA damage from both copper and iron is a significant survival advantage and may be one reason that glutathione, and not cysteine, is the primary sulfur-containing antioxidant in vivo. In cells, the redox balance for both GSH/GSSG and Cys/Cys2 is increasingly oxidized in response to continuous generation of ROS resulting in oxidative stress [48,51]. Although considered a deleterious indicator, higher concentrations of the oxidized form of these compounds may provide more protection against oxidative damage, since our studies show that the oxidized disulfide compounds are more effective antioxidants than their reduced forms. In addition, our results suggest that targeted antioxidant supplementation specifically to prevent either copper- or iron-mediated oxidative damage should be considered, and that the chemical form of the sulfur compound could potentially have a dramatic effect on its antioxidant efficacy. Because mis-regulation of copper or iron homeostasis occurs in cancer and neurodegenerative diseases [5,18,19,35,77,78], specifically preventing oxidative DNA damage from one or both of these metal ions may provide a powerful tool for treatment and prevention of these diseases.

4. Conclusions

We have directly quantified and compared antioxidant activities for a variety of sulfur compounds with CuI and FeII using a biologically-relevant DNA damage assay. Over half of the sulfur compounds tested showed antioxidant activity with CuI/H2O2 and had IC50 values from 3.4 to 12.4 μM. In addition, we have confirmed metal coordination as a novel mechanism for sulfur antioxidant activity with CuI and established that UV–vis spectroscopy as a qualitative diagnostic screening tool for determining the antioxidant efficacy of sulfur compounds with CuI. Oxidized and reduced forms of glutathione inhibited DNA damage from both CuI and FeII, revealing a unique role for this critical antioxidant. Our findings suggest that in addition to the vast array of defense mechanisms utilized by cells to reduce oxidative damage, metal complexation by sulfur antioxidants may also afford significant protection against cellular damage, and we are pursuing coordination chemistry and cellular studies to further confirm this hypothesis. In addition, sulfur compounds can selectively prevent oxidative DNA damage from either CuI or FeII. This work provides a critical link between clinical observations of sulfur antioxidant activity and basic biochemistry, and will enable optimization and design of antioxidants to prevent and treat disease.

5. Abbreviation

ROS reactive oxygen species  
GSSG oxidized glutathione  
GSH reduced glutathione  
Cys cysteine  
CyS2 cystine  
H2O2 hydrogen peroxide  
Bipy bipyridine  
MOPS 3-(N-morpholino)propanesulfonic acid  
MES 2-(N-morpholino)ethanesulfonic acid

Acknowledgments

We thank the American Heart Association (0665344 U) and the Clemson University Research Grant Committee.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jinorgbio.2008.06.010.

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