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Presence of closely spaced protein thiols on the surface of mammalian cells

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Abstract

It has been proposed that certain cell-surface proteins undergo redox reactions, that is, transfer of hydrogens and electrons between closely spaced cysteine thiols that can lead to reduction, formation, or interchange of disulfide bonds. This concept was tested using a membrane-impermeable trivalent arsenical to identify closely spaced thiols in cell-surface proteins. We attached the trivalent arsenical, phenylarsenoxide, to the thiol of reduced glutathione to produce 4-(*N*-(*S*-glutathionylacetyl)amino)phenylarsenoxide (GSAO). GSAO bound tightly to synthetic, peptide, and protein dithiols like thioredoxin, but not to monothiols. To identify cell-surface proteins that contain closely spaced thiols, we attached a biotin moiety through a spacer arm to the primary amino group of the γ-glutamyl residue of GSAO (GSAO-B). Incorporation of GSAO-B into proteins was assessed by measuring the biotin using streptavidin-peroxidase. Up to 12 distinct proteins were labeled with GSAO-B on the surface of endothelial and fibrosarcoma cells. The pattern of labeled proteins differed between the different cell types. Protein disulfide isomerase was one of the proteins on the endothelial and fibrosarcoma cell surface that incorporated GSAO-B. These findings demonstrate that the cell-surface environment can support the existence of closely spaced protein thiols and suggest that at least some of these thiols are redox active.

Keywords: arsenical; cell surface; disulfide; protein disulfide isomerase; thiol; thioredoxin

The reducing nature of the intracellular environment facilitates interchange between the reduced and oxidized form of closely spaced dithiols (for review, see Huppa & Ploegh, 1998). In contrast, the oxidizing nature of the extracellular environment is generally considered to preclude the existence of closely spaced dithiols. Such dithiols are thought to exist as disulfide bonds or as mixed disulfides with other thiol compounds, for example, glutathione (GSH). We have suggested previously that the micro-environment of the cell surface can support redox reactions in and between certain surface proteins (Jiang et al., 1999). Transfer or shuffling of hydrogens and electrons between the cysteine thiols of these surface proteins can lead to net reduction, net formation, or net interchange of disulfide bonds.

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Abbreviations: BSA, bovine serum albumin; BRAA, 4-(*N*-(bromoacetyl)-amino)phenylarsonic acid; BRAO, 4-(*N*-(bromoacetyl)amino)phenylarsenoxide hydrate; COSY, correlation spectroscopy; DMP, 2,3-dimercaptopropanol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GSAO, 4-(*N*-(*S*-glutathionylacetyl)amino)phenylarsenoxide; GSAO-B, 4-(*N*-(*S*-(*N*-(6-(*N*-(6-(*N*-(biotinoyl)amino)hexanoyl)amino)hexanoyl)glutathionyl)-acetyl)amino)phenylarsenoxide; GSH, reduced glutathione; DTT, dithiothreitol; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum coherence; HRP, horse-radish peroxidase; PBS, phosphate buffered saline; PDI, protein disulfide isomerase; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinyldiethylene fluoride; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

This concept was tested in this study by making and using a membrane-impermeable trivalent arsenical to indentify cell-surface proteins that contain closely spaced thiols. Such thiols have the potential to interchange between the reduced dithiol and oxidized disulfide bond and are, therefore, likely to be important for the protein's function. There are several examples of intracellular proteins or complexes that contain closely spaced dithiols that are important for function, for example, protein disulfide isomerase (PDI) (Gilbert, 1997), thioredoxin (Holmgren, 1989), lecithin-cholesterol acyltransferase (Jauhiainen et al., 1988), tyrosyl phosphatases (Zhang et al., 1992), and lipoamide in the pyruvate dehydrogenase complex (Stevenson et al., 1978). PDI (Mandel et al., 1993; Täger et al., 1997; Jiang et al., 1999) and thioredoxin (Rosén et al., 1995), which are also secreted by cells, are the only known cell-surface proteins that contain closely spaced dithiols. The dithiols in these enzymes are responsible for their redox activity.

Trivalent arsenicals form high-affinity ring structures with closely spaced dithiols. For instance, the crystal structure of the adduct between a *p*-tolylarsenoxide equivalent and 2,3-dimercaptopropanol (DMP) reveals a five-membered ring in which both sulfur atoms are complexed to arsenic (Adams et al., 1990). The cyclic dithioarsinites are markedly more stable than the noncyclic products formed from trivalent arsenicals and monothiols because of entropic considerations (Stocken & Thompson, 1946). Vicinal thiols do not need to be chemically vicinal as in DMP, but can also be brought into spatial apposition by folding (Jauhiainen et al., 1988).

This has prompted the use of trivalent arsenicals for the exploration of closely spaced protein thiols (Gitler et al., 1994; Kalef & Gitler, 1994; Griffin et al., 1998). Small trivalent arsenicals, often phenylarsenoxide, have been applied to the study of proteins including thioredoxin (Brown et al., 1989), lecithin-cholesterol acyltransferase (Jauhiainen et al., 1988) and tyrosyl phosphatases (Zhang et al., 1992) and pathways including hexose transport (Frost & Lane, 1985) and ubiquitin-dependent protein degradation (Klemperer & Pickart, 1989).

We attached phenylarsenoxide to the thiol of GSH to produce a plasma membrane-impermeable trivalent arsenical, 4-(*N*-(*S*-glutathionylacetyl)amino)phenylarsenoxide (GSAO). GSH is constituitively secreted by mammalian cells (Bannai & Tsukeda, 1979) but is not taken up by these cells and was therefore an ideal pendant to limit the entry of the trivalent arsenical into the cell. GSAO bound tightly to closely spaced synthetic, peptide, and protein dithiols but not to monothiols. To identify cell-surface proteins that contain closely spaced thiols, we attached a biotin moiety through a spacer arm to the primary amino group of the γ-glutamyl residue of GSAO (GSAO-B). Incorporation of GSAO-B into proteins was assessed by measuring the biotin using streptavidin-peroxidase. The surface of endothelial and fibrosarcoma cells were found to support the existence of closely-spaced thiols in certain proteins.

Results

Synthesis and characterization of GSAO

The synthesis of GSAO is described in Figure 1. Briefly, 4-(*N*-(bromoacetyl)amino)phenylarsonic acid was synthesized from *p*-arsanilic and bromoacetyl bromide. 4-(*N*-(bromoacetyl)-amino)phenylarsonic acid (BRAA) was then reduced to 4-(*N*-(bromoacetyl)amino)phenylarsenoxide (BRAO) using sulfur dioxide with sodium iodide as a catalyst (Stevenson et al., 1978). BRAO was coupled to GSH at pH 9.6 for 8–12 h at room temperature to produce GSAO. The GSAO was resolved from unreacted BRAO and GSH by gel filtration on Bio-Gel P-2. The separation was facilitated by weak interaction of the phenyl ring of GSAO and BRAO with the Bio-Gel matrix as GSAO eluted after GSH and before BRAO.

One-dimensional (1D) and two-dimensional (2D) NMR spectroscopy was used to confirm the structure of GSAO. A series of ^{1}H and ^{13}C NMR spectra, ^{1}H, ^{13}C, ^{1}H–^{1}H COSY, ^{1}H–^{13}C HMQC, and ^{1}H–^{13}C HMBC, were all found to be consistent with the structure proposed in Figure 1. Considered together, all of the spectra permitted the unambiguous assignment of all carbons and nonexchangeable hydrogens. An expansion of the ^{1}H–^{13}C HMBC spectrum of GSAO, showing the aliphatic region, is shown in Figure 2. The ^{1}H–^{13}C HMBC technique correlates coupled ^{1}H and ^{13}C nuclei, but filters out directly bonded nuclei. This means that ^{1}H and ^{13}C nuclei that are separated by two, three, or (sometimes) four bonds appeared as cross peaks in the spectrum. Figure 2 shows that C11 is only strongly coupled to H7 (referring to the protons attached to C7), while C7 is strongly coupled to H11 in addition to H6. This confirms that GSH was successfully alkylated on sulfur by BRAO.

Interaction of GSAO with synthetic, peptide, and protein dithiols

GSAO bound to DMP and prevented interaction of the dithiol with DTNB while any interaction of GSAO with cysteine was displaced by DTNB (Fig. 3). This result confirmed the dithiol selectivity of GSAO.

The small synthetic dithiols, DMP, 6,8-thioctic acid, and dithiothreitol (DTT), formed high-affinity complexes with GSAO (Fig. 4A–C; Table 1). As anticipated, the affinity for GSAO decreased as the size of the ring structure with the arsenic of GSAO increased. For instance, the two thiols of DMP are on adjacent carbon atoms that form a five-membered ring with the GSAO arsenic. The affinity of GSAO for the dithiols decreased from a dissociation constant of 130 nM for a five-membered ring with DMP to 420 nM for a seven-membered ring with DTT.

GSAO also bound with high-affinity to both peptide and protein dithiols. The two peptides, TrpCysGlyProCysLys (Holmgren, 1989) and TrpCysGlyHisCysLys (Gilbert, 1997), correspond to the active-site sequences of thioredoxin and PDI, respectively. Both peptides bound GSAO with dissociation constants of $\sim$1 μM (Fig. 4D,E; Table 1). There were 15 atoms in the ring structure of the peptides with the arsenic of GSAO. Despite this large ring structure, the dissociation constant for binding of GSAO was only double that for binding of GSAO to DTT. This result implied that the secondary structure of the peptides brought the two Cys thiols into close proximity, which enabled them to complex with the trivalent arsenical. GSAO bound to the active-site dithiol of human thioredoxin with a dissociation constant of 370 $\pm$ 180 nM (Fig. 4F;

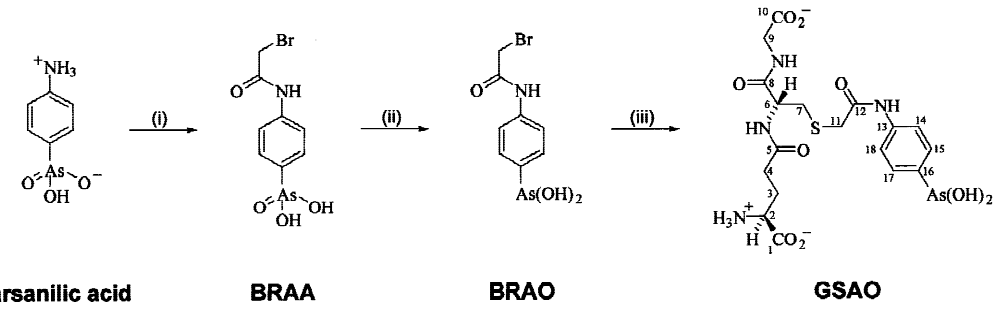


Fig. 1. Synthesis of GSAO. (i) aq. KOH, Na_2CO_3, bromoacetyl bromide in CH_2Cl_2, H_2SO_4; (ii) cat. NaI, SO_2, CH_3OH, 48% HBr, 6–8 h; (iii) DMSO, GSH, aq. $NaHCO_3$ (pH 9.6), 8–12 h. Proposed structure of GSAO showing the expected stereochemistry and the numbering scheme used in the discussion of the 2D ^{1}H–^{13}C HMBC NMR spectrum.

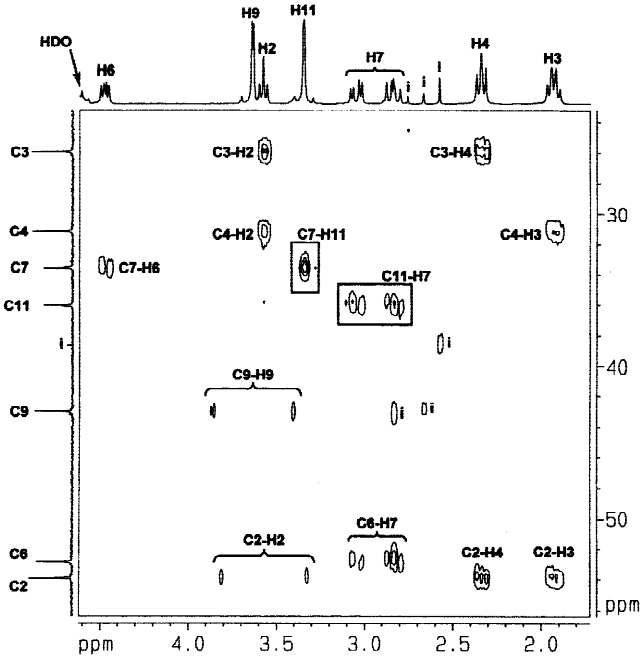


Fig. 2. Structure of GSAO. An expansion of the $^{1}H–^{13}C$ HMBC spectrum of GSAO in DCl/D_2O, showing the aliphatic region. The spectrum shows any long-range heteronuclear ($^{1}H–^{13}C$) coupling as crosspeaks, in line with the corresponding ^{1}H and ^{13}C signals along the horizontal and vertical axes. The boxed cross peaks correspond to $^{1}H–^{13}C$ coupling between the C7 and C11 methylenes, confirming that alkylation by BRAO has occurred on the glutathione sulfur atom. Peaks and cross peaks marked "i" are due to impurities; one-bond cross peaks corresponding to the C9 methylene and the C2 methine are also observable as doublets due to incomplete filtering by the HMBC pulse sequence.

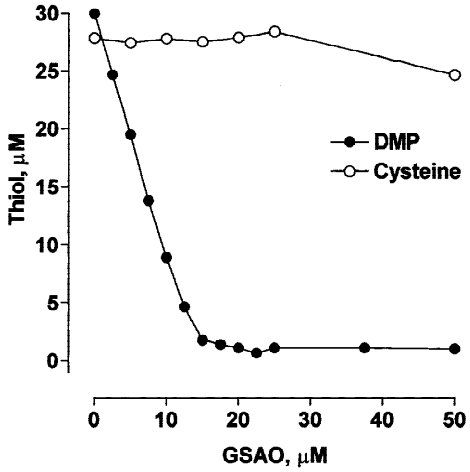


Fig. 3. Titration of DMP or cysteine with GSAO. DMP (15 μM corresponding to 30 μM thiols) or cysteine (28 μM) was incubated with GSAO (0–50 μM) for 10 min. DTNB (950 μM) was then added and the reactions incubated for a further 10 min. The concentration of thiol in the reactions was determined from the absorbance of the TNB dianion at 412 nm. GSAO bound to DMP and prevented interaction of the dithiol with DTNB while any interaction of GSAO with cysteine was displaced by DTNB.

Table 1), which was approximately fourfold higher affinity than that of GSAO binding to the thioredoxin active-site hexapeptide, 1,420 ± 450 nM. This result implied that the distance between the active-site thiols in thioredoxin was closer than their distance in the hexapeptide.

Considered together, these results indicated that GSAO would selectively bind proteins containing closely spaced thiols. To identify these proteins on the cell surface, we attached a biotin moiety through a spacer arm to the primary amino group of the γ-glutamyl residue of GSAO. Incorporation of GSAO-B into proteins could be assessed by measuring the biotin using streptavidin-peroxidase.

Interaction of GSAO-B with PDI and thioredoxin

Human recombinant PDI and thioredoxin bound GSAO-B (Fig. 5). In the experiment, purified PDI, thioredoxin, or albumin as negative control were incubated with a twofold molar excess of DTT for 60 min to ensure that the active-site disulfides of PDI and thioredoxin were in the reduced dithiol state. The proteins were then incubated with GSAO-B or GSAO-B and a fourfold molar excess of DMP for 30 min. Equivalent moles of the labeled proteins were resolved on SDS-PAGE, transferred to PVDF membrane, and blotted with streptavidin-peroxidase to detect the GSAO-B label. Both PDI and thioredoxin incorporated GSAO-B but albumin did not. The higher M_r band in lane 1 of Figure 5B was a small amount of aggregated PDI in the preparation (Jiang et al., 1999).

It is noteworthy that the density of labeling of PDI was approximately twice that of thioredoxin, which is consistent with the two active-site dithiols of PDI vs. the one of thioredoxin.

Identification of endothelial and fibrosarcoma cell-surface proteins that contain closely spaced dithiol(s)

GSAO-B was used to identify proteins on the endothelial and fibrosarcoma cell surfaces that contained closely spaced thiols. BAE cell lysate or the surface of intact endothelial and fibrosarcoma cells was labeled with GSAO-B for 30 min at room temperature in the absence or presence of DMP. The intact cells were lysed, and the lysate from both incubations was resolved on SDS-PAGE, transferred to PVDF membrane, and blotted with streptavidin-peroxidase to detect the GSAO-B label.

The pattern of labeling of the endothelial cell lysate (Fig. 6A, lanes 1 and 2) with GSAO-B was very different from the pattern of labeling of the cell surface proteins (Fig. 6A, lanes 3 and 4). This result supports the membrane-impermeable nature of GSAO-B. The pattern of labeling of the cell lysate was not dissimilar to the pattern of labeling of 3T3 fibroblasts proteins with a tritiated phenylarsenoxide (Frost & Schwalbe, 1990).

There were approximately 10 proteins on the endothelial cell surface (Fig. 6A) and 12 proteins on the fibrosarcoma cell-surface (Fig. 6C) that incorporated GSAO-B. The molecular masses of these proteins varied from 13 to 153 kDa for endothelial cells (Fig. 6B) and 19 to 104 kDa for fibrosarcoma cells (Fig. 6D). The intensity of labeling of the proteins varied considerably, which probably reflected their abundance on the cell surface, although it may also have reflected differences in the affinity of GSAO for the protein dithiols.

There was some labeling of two of the proteins on either cell surface in the presence of DMP, the 86 and 153 kDa endothelial surface proteins, and the 66 and 104 kDa fibrosarcoma surface proteins. The binding of GSAO-B to these proteins was further

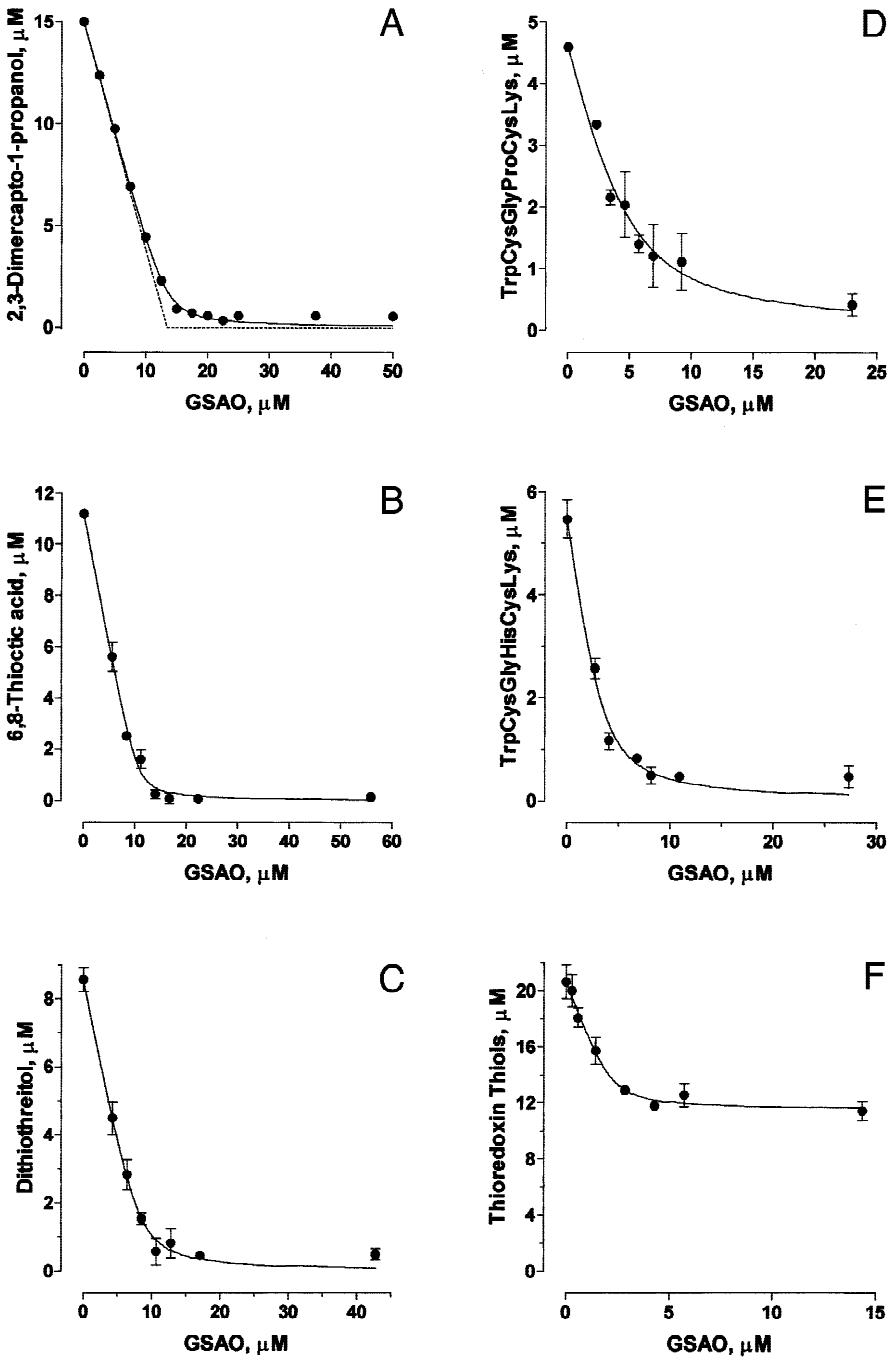


Fig. 4. Interaction of GSAO with synthetic, peptide, and protein dithiols. (**A**) DMP (15 μM), (**B**) 6,8-thioctic acid (11 μM), (**C**) DTT (8.5 μM), (**D**) TrpCysGlyProCysLys (4.6 μM), (**E**) TrpCysGlyHisCysLys (5.5 μM), or thioredoxin (4.1 μM corresponding to 20.6 μM thiols) was incubated with GSAO (0–56 μM) for 10 min DTNB (950 μM) was then added and the reactions incubated for a further 10 min. The concentration of thiol in the reactions was determined from the absorbance of the TNB dianion at 412 nm. The solid lines represent the best nonlinear least-squares fit of the data to (**A–E**) Equation 1 or (**F**) Equation 2, and have been drawn using the parameter estimates in Table 1. The dotted line in **A** represents a simulated titration assuming an infinite affinity of GSAO for the dithiol. GSAO bound to both synthetic, peptide, and protein dithiols with dissociation constants in the range 130 nM to 1.4 μM.

reduced when the concentration of DMP in the labeling reaction was increased from 400 to 800 μM (not shown). This result supports the differential affinity of GSAO for different protein dithiols; that is, these four proteins bound GSAO-B more avidly than the other proteins.

PDI was one of the GSAO-B labeled proteins on the endothelial and fibrosarcoma cell surfaces

BAE cells were labeled with GSAO-B in the absence or presence of DMP, lysed, and incubated with streptavidin-agarose beads to

Table 1. *Dissociation constants for binding of GSAO to synthetic and protein dithiol*

Dithiol	Ring size[a]	Dissociation constant (nM)
2,3-Dimercapto-1-propanol	5	130 ± 40[b]
6,8-Thioctic acid	6	200 ± 50
DTT	7	420 ± 80
TrpCysGlyProCysLys	15	1,420 ± 450
TrpCysGlyHisCysLys	15	870 ± 270
Thioredoxin	15	370 ± 180

[a]Number of atoms in the ring structure with the arsenic of GSAO.
[b]Errors are 1 SD.

collect the biotin-labeled proteins. The labeled proteins were eluted from the beads, resolved on SDS-PAGE, transferred to PVDF membrane, and blotted with anti-PDI polyclonal antibodies.

The results shown in Figure 7 indicate that PDI was one of the proteins on the endothelial cell surface that incorporated GSAO-B. There was no labeling of PDI in the presence of DMP, which is supported by the results of Figures 5 and 6. The higher M_r band in lane 1 of Figure 7 was some aggregated PDI (see Fig. 5B). PDI was also one of the labeled proteins on the fibrosarcoma surface (not shown).

Discussion

GSAO bound tightly to closely spaced dithiols but not to monothiols, which is in accordance with the chemical reactivity of trivalent arsenicals. Small synthetic dithiols, peptide dithiols, and protein dithiols all formed high-affinity complexes with GSAO. The affinity of GSAO for synthetic dithiols decreased as the size of the ring structure with the arsenic of GSAO increased. That is, the stability of the cyclic complex with the trivalent arsenical decreased as the distance between the thiols increased. This observation is in accordance with those of Hannestad et al. (1982), who reported that elution of 1,2- or 1,3-dihiols from *p*-aminophenylarsine oxide linked to Sepharose 6B required more alkaline conditions than elution of monothiols or 1,4-dithiols. It is noteworthy that GSAO bound with relatively high affinity to two hexapeptide dithiols and a protein dithiol that formed 15-membered ring structures with the arsenic of GSAO. Despite this large ring structure, the dissociation constant for binding of GSAO to the active-site dithiol of human thioredoxin, for example, was the same as that for binding of GSAO to DTT. This result is in accordance with the crystal structure of thioredoxin showing that the active-site thiols are brought into close proximity by the secondary structure of the polypeptide backbone (Weichsel et al., 1996). These findings indicated that GSAO bound specifically to dithiols and that the affinity of the interaction was influenced by the spacing of the thiols.

To identify cell-surface proteins that contain closely spaced thiols, we attached a biotin moiety through a spacer arm to the primary amino group of the γ-glutamyl residue of GSAO. Incorporation of GSAO-B into proteins was assessed by measuring the biotin using streptavidin-peroxidase. GSAO-B specifically bound to the active-site dithiols of human recombinant PDI and thioredoxin. In contrast, GSAO-B did not bind to the single free thiol of albumin.

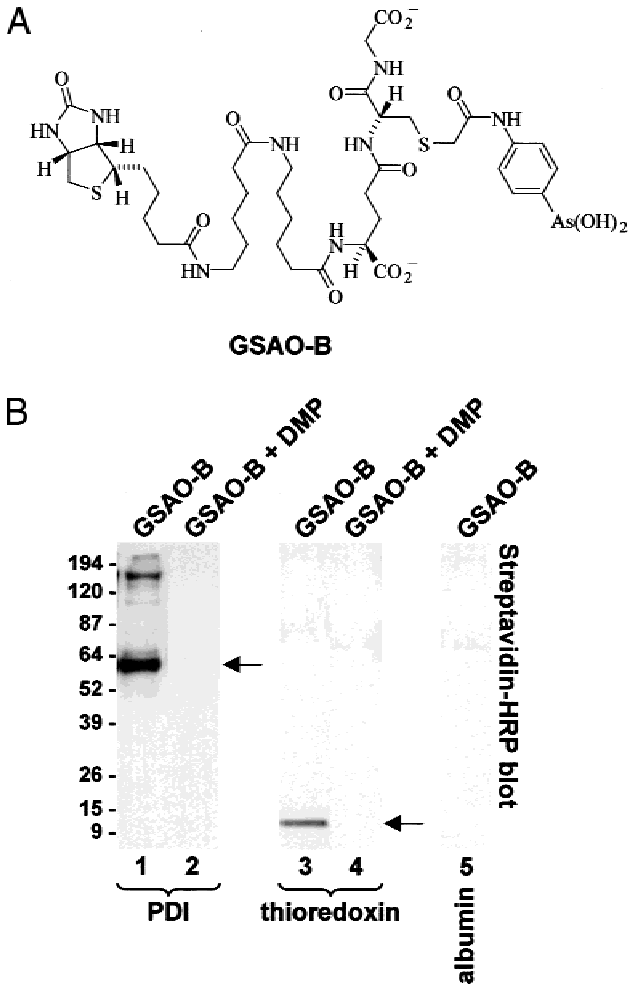


Fig. 5. Interaction of GSAO-B with PDI and thioredoxin. **A:** Structure of GSAO-B. **B:** Purified human recombinant PDI (5 μM), human recombinant thioredoxin (5 μM), or bovine serum albumin (5 μM) was incubated with DTT (10 μM) for 60 min at room temperature to ensure that the active-site disulfide(s) of PDI and thioredoxin were in the reduced dithiol form. GSAO-B (100 μM) or GSAO-B and DMP (400 μM) was then added and the reactions incubated for 30 min at room temperature. The labeled PDI (lanes 1 and 2), thioredoxin (lanes 3 and 4), and albumin (lane 5) (75 pmol) was resolved on 4–16% SDS-PAGE, transferred to PVDF membrane, and blotted with streptavidin-peroxidase to detect the GSAO-B label. The positions of Mr markers are shown at left.

These results demonstrated that biotinylation of GSAO did not affect its binding to closely-spaced protein thiols.

There were 10 distinct proteins on the endothelial cell surface and 12 on the fibrosarcoma cell surface that incorporated GSAO-B. The pattern of labeling of proteins on the two cell surfaces was very different. Proteins with molecular masses of between 13 and 153 kDa were labeled on the endothelial cell surface, while proteins with masses of between 19 to 104 kDa were labeled on the fibrosarcoma cell surface. The cell-type differences were probably a consequence of the different makeup of the cell-surface proteins, which reflect the different functions of the cells. It is likely that some cell-surface proteins containing closely spaced thiols were labeled with GSAO-B, but were not detected because of their paucity. It is also possible that some closely spaced dithiols were

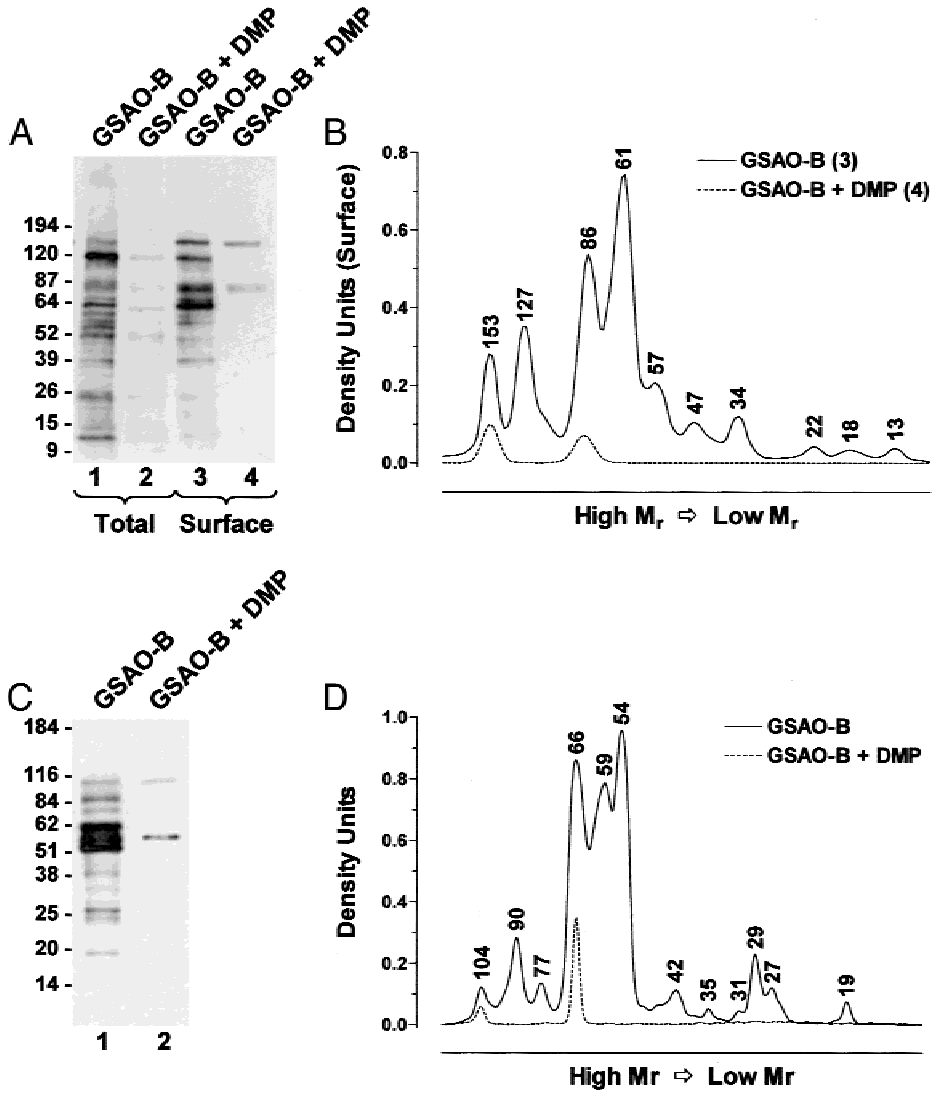


Fig. 6. Identification of endothelial and fibrosarcoma cell-surface proteins that contain closely spaced dithiol(s). **A:** BAE cell lysate (0.5×10^6 cells in 0.2 mL) or the surface of intact endothelial cells (2×10^6 cells in 0.4 mL) was labeled with GSAO-B (100 μM) for 30 min at room temperature in the absence (lanes 1 and 3) or presence of DMP (400 μM) (lanes 2 and 4). The intact endothelial cells were lysed and the lysate from both incubations was resolved on 4–15% SDS-PAGE, transferred to PVDF membrane, and blotted with streptavidin peroxidase to detect the GSAO-B label. The results represent labeling of 6×103 lysed endothelial cells (lanes 1 and 2) or 3×10^4 intact endothelial cells (lanes 3 and 4). The positions of Mr markers are shown at the left. **B:** Densitometry profile of the surface labeled proteins (lanes 3 and 4). The apparent Mrs of the individual proteins are indicated. **C:** HT1080 cells (3×10^6 cells in 0.5 mL) were labeled with GSAO-B (100 μM) for 30 min at room temperature in the absence (lane 1) or presence of DMP (400 μM) (lane 2). The cells were lysed and the lysate was resolved on 4–15% SDS-PAGE, transferred to PVDF membrane, and blotted with streptavidin peroxidase to detect the GSAO-B label. The results represent labeling of 2×10^5 HT1080 cells. The positions of Mr markers are shown at left. **D:** Densitometry profile of the GSAO-B labeled proteins on the fibrosarcoma surface. The apparent Mrs of the individual proteins are indicated.

refractive to labeling by GSAO-B due to steric considerations. These findings imply that closely spaced dithiols exist in the extracellular environment.

PDI was one of the proteins on the endothelial and fibrosarcoma cell surface that incorporated GSAO-B. PDI has been implicated in redox control of protein thiols/disulfides on the surface of fibroblasts (Jiang et al., 1999), lymphocytes (Lawrence et al., 1996; Täger et al., 1997), and platelets (Burgess et al., 2000). The thiol content of 11 proteins on the fibrosarcoma cell surface was increased with overexpression of PDI, while the thiol content of 3 of the 11 proteins was decreased with underexpression of PDI (Jiang et al., 1999). PDI contains two pairs of closely spaced thiols that cycle between the reduced dithiol and oxidized disulfide bond in coordination with a dithiol or disulfide of a protein substrate. Considering that PDI's active-site dithiols are known to participate in interchanges with disulfide bonds of other proteins, it is conceivable that some of the other proteins resolved on the surface of endothelial and fibrosarcoma cells may serve similar functions. Such oxidoreduction may regulate protein function and may be involved in the recently observed dimerization of the extracellular

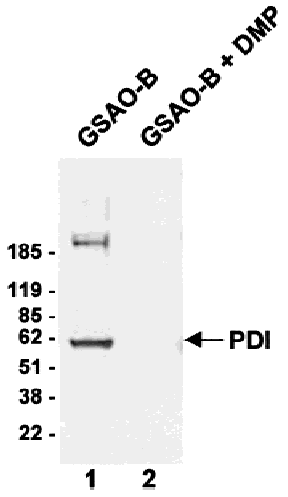


Fig. 7. PDI was one of the GSAO-B labeled proteins on the endothelial cell surface. Intact BAE cells (5×10^6 cells in 0.75 mL) were labeled with GSAO-B (100 μM) for 30 min at 37 °C in the absence (lanes 1) or presence of DMP (400 μM) (lanes 2). The cells were lysed and incubated with streptavidin-agarose beads to collect the biotin-labeled proteins. The labeled proteins were resolved on 4–15% SDS-PAGE, transferred to PVDF membrane, and blotted with anti-PDI monoclonal antibodies. The results represent labeling of 5×10^6 endothelial cells. The positions of Mr markers are shown at left.

domains of the interleukin 3 receptor (Le et al., 2000) and CD4 (Lynch et al., 1999).

Our observations raise two important questions. First, the identity of the other resolved dithiol-containing proteins, and second, the identification of the mechanisms capable of maintaining these dithiols in reduced state on a surface that is commonly believed to face a predominantly oxidizing environment.

Materials and methods

Chemicals and proteins

The following chemicals were purchased and used without further purification: bromoacetyl bromide, sulfur dioxide, d_6-dimethylsulfoxide, deuterium oxide (Aldrich, Castle Hill, NSW, Australia); methanol, 98% sulfuric acid, 48% hydrobromic acid, 37% hydrochloric acid (Ajax, Auburn, NSW, Australia); dichloromethane, potassium hydroxide, sodium hydrogen carbonate, sodium hydroxide (BDH, Kilsyth, VIC, Australia); P-2 Gel extra fine 1,800 MW cut-off (Bio-Rad, Hercules, California); 2,3-dimercaptopropanol (DMP) (Fluka, Castle Hill, NSW, Australia); thionyl chloride (Merck, Darmstadt, Germany); 6,8-thioctic acid, DTT, dimethylsulfoxide, 5,5′-dithiobis(2-nitrobenzoic acid), ethylenediaminetetraacetic acid (EDTA), *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid), glutathione, sodium carbonate, sodium chloride, sodium iodide, streptavidin-agarose (Sigma, Castle Hill, NSW, Australia); *p*-arsanilic acid (Tokyo Kasei Kogyo, Tokyo, Japan); glycine (ICN, Aurora, Ohio). Biotin-XX, SE [6-((6-((biotinoyl)amino)hexanoyl)amino)hexanoic acid, succinimidyl ester] was obtained from Molecular Probes (Eugene, Oregon). Recombinant human thioredoxin produced in *Escherichia coli* was from American Diagnostica (Greenwich, Connecticut). The hexapeptides, TrpCysGlyProCysLys and TrpCysGlyHisCysLys, were from Auspep (Parkville, Australia). Recombinant human protein disulfide isomerase (PDI) was produced in *E. coli* and purified according to Jiang et al. (1999). All other reagents were of analytical grade.

Instrumentation

1D and 2D spectra were obtained using a Bruker DPX300 NMR spectrometer, with ^{1}H and ^{13}C detected at 300.17 and 75.48 MHz, respectively.

Preparation of acidified deuterium oxide

Fresh thionyl chloride was cautiously added to an excess of deuterium oxide. After evolution of SO_2 had ceased, the resulting solution (0.6 mL) was added to GSAO (ca. 50 mg) in a 5 mm NMR tube. This sample was used to obtain the NMR spectra.

Synthesis of 4-(N-(bromoacetyl)amino)phenylarsonic acid (BRAA)

Into a stirred solution of potassium hydroxide (10.31 g, 184 mmol) in water (100 mL) was added *p*-arsanilic acid (20.39 g, 94 mmol), the flask swirled until the acid completely dissolved. Sodium carbonate (30.56 g, 288 mmol) and water (100 mL) were added, with stirring continued until all solids had dissolved to give a warm solution. Ice chips were added to the solution until it was cold enough not to melt the ice immediately. It was then poured into a 500 mL separating funnel, and half of a solution of bromoacetyl bromide (12 mL, 27.8 g, 138 mmol) in dichloromethane (50 mL) was cautiously added. The funnel was stoppered and shaken until production of gas had ceased before the remaining bromoacetyl bromide was added. This time, the mixture was shaken vigorously for about 5 min, by which time no more gas was evolved. The organic layer was drained off, and the aqueous layer (found to be pH 10) was transferred to a 500 mL conical flask. Careful acidification of the aqueous layer with 98% sulfuric acid to approximately pH 1 resulted in the formation of the product as a fine white precipitate, which was collected and air dried overnight using a Büchner funnel, giving 24.55 g (73 mmol, 78% yield). ^{1}H-NMR (d_6-DMSO): δ 4.09 (s, 2H), 7.73 (d, J = 9 Hz, 2H), 7.83 (d, J = 9 Hz, 2H), 10.87 (s, 1H). ^{13}C-NMR (d_6-DMSO): δ 30.53, 119.97, 127.34, 131.56, 143.08, 166.00 ppm.

Synthesis of 4-(N-(bromoacetyl)amino)phenylarsenoxide hydrate (BRAO·xH_2O)

Into a three-necked 500 mL round-bottomed flask was placed BRAA (12.15 g, 36 mmol). The solid was dissolved with swirling by the addition of methanol (75 mL) and 48% hydrobromic acid (75 mL), giving a transparent yellow solution. Any undissolved solid left at this point was removed by filtration. Sodium iodide (0.20 g, 1.3 mmol) was added as a catalyst, causing the color of the solution to darken to an orange-brown, and sulfur dioxide gas was slowly (ca. 2 bubbles per second) passed through the stirred solution for 6 to 8 h. The white precipitated product was collected using a Büchner funnel, giving 17.43 g of damp white solid. The activity of a solution made by dissolving 40.7 mg of solid in 800 μL of deoxygenated DMSO was determined to be 56 mM (see below). Hence, in this case the molecular weight of BRAO·xH$_2$O was found to be 908.5, that is 35% w/w BRAO and 65% w/w H_2O

(however, BRAO·xH$_2$O was found to lose water over time). Therefore, the "anhydrous" weight for BRAO was calculated to be 35% of 17.43 g, that is 6.10 g (19 mmol, 53% yield). ^{1}H-NMR (d_6-DMSO): δ 4.85 (s, 2H), 7.78 (d, J = 9 Hz, 2H), 7.86 (d, J = 9 Hz, 2H), 11.36 (s, 1H). ^{13}C-NMR (d_6-DMSO): δ 30.55, 119.22, 130.52, 140.04, 145.04, 165.52 ppm.

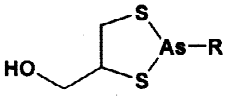
Synthesis of 4-(N-(S-glutathionylacetyl)amino)-phenylarsenoxide (GSAO)

DMSO (10 mL) was deoxygenated by passing a stream of nitrogen gas through it for a few minutes and used to dissolve BRAO·xH$_2$O (1.00 g, 2.48 mmol active arsenoxide). Glutathione (1.15 g, 3.74 mmol, 1.5 eq) was dissolved in 0.5 M bicarbonate buffer, pH 9.6 (35 mL), and added to the solution of BRAO·xH$_2$O in DMSO. The total volume was made up to 50 mL with 0.5 M bicarbonate buffer, and the solution gently agitated at room temperature overnight. Lyophilization gave a white powdery product that was freely soluble in water with no solid residue. The active arsenoxide concentration of the solution was 49.6 mM (see below).

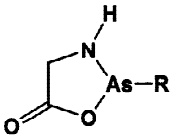
The product was purified by gel filtration on a 570 mL column (2.5 × 117 cm) of Bio-Gel P-2 extra fine (BioRad) using deoxygenated deionized water as the eluant at a flow rate of 0.1 mL per minute. Four peaks with UV absorbance were resolved, A through D. Peaks B and C were GSAO and unreacted BRAO, respectively (see below). Peak A was oxidized GSAO and peak D was probably oxidised BRAO (BRAA, see below). Peak B was pooled and deoxygenated with nitrogen gas. Approximately 12 mL of 15 mM GSAO was obtained. Lyophilization again gave a white powdery product that was freely soluble in water with no solid residue. ^{1}H-NMR (D$_2$O): δ1.93 (q, J = 7 Hz, 2H), 2.35 (t, J = 8 Hz, 2H), 2.84 (dd, J = 14 Hz, J = 9 Hz, 1H), 3.05 (dd, J = 14 Hz, J = 5 Hz, 1H), 3.35 (s, 2H), 3.58 (t, J = 6 Hz, 1H), 3.64 (d, J = 2 Hz, 2H), 4.48 (dd, J = 9 Hz, J = 5 Hz, 1H), 7.44 (d, J = 8 Hz, 2H), 7.58 (d, J = 8 Hz, 2H). ^{13}C-NMR (D$_2$O): δ 25.93, 31.16, 33.53, 36.01, 42.97, 52.83, 53.89, 121.29, 129.97, 138.77, 144.09, 170.90, 171.73, 173.75, 174.68, 175.76 ppm.

Stability of the trivalent arsenicals toward oxidation

Arsenoxides (RAs=O) have been shown not to possess an arsenic-oxygen double bond as is usually written, but are likely to exist either as cyclic polymers (containing As-O-As linkages) or, more likely, as the hydrate RAs(OH)$_2$, an organoarsonous acid, in aqueous solution (Doak & Freedman, 1970; Knoch et al., 1995). Solutions of organoarsenicals, such as GSAO and BRAO, which presumably exist as organoarsonous acids, are deactivated by dissolved oxygen over time. Although the product of the deactivation has yet to be characterized, our results suggest that it is due to an oxidation of arsenic from the trivalent to the pentavalent state, that is oxidation of GSAO to GSAA. This oxidation can be slowed in three ways: removal of dissolved O$_2$ from the solutions containing the arsenoxides, lowering the pH of these solutions, or addition of glycine to the solutions. We have routinely chosen to use glycine to prevent oxidation of the stock solutions of the trivalent organoarsenicals. Similar to the reaction between 2,3-dimercaptopropanol and RAs(OH)$_2$ in which a five-membered dithioarsonite (I) is formed, reaction of glycine with RAs(OH)$_2$ is thought to give a five-membered cyclic 1,3,2-oxazarsolidin-5-one (II). The NH group is not as acidic as OH and is not deprotonated at neutral pH. Solutions of GSAO in which an excess of glycine has been added have been found to be resistant to oxidation by dissolved oxygen for days to weeks. The glycine adduct is readily displaced from the trivalent arsenical by closely spaced dithiols (see below).



I



II

Synthesis of 4-(N-(S-(N-(6-(N-(6-(N-(biotinoyl)amino)-hexanoyl)amino)hexanoyl)glutathionyl)acetyl)amino)-phenylarsenoxide (GSAO-B)

GSAO (0.13 g) was dissolved in 0.5 M sodium bicarbonate buffer (5 mL, pH 8.5) and was found to contain 39 mM of active arsenical. The buffered arsenical solution (4.2 mL, containing 165 μmol active arsenical) was added to a solution of biotin-XX, SE (100 mg, 176 μmol) in DMSO (1 mL), the mixture inverted a few times and then incubated at 4 °C for 4 h. Glycine (17.5 mg, 233 μmol) was added and the mixture kept at 4 °C overnight. The concentration of trivalent arsenical was determined to be 31 mM, and the solution was used without further modification.

Method of assay of BRAO, GSAO, and GSAO-B

A stock solution of DMP (5 μL, 50 μmol) was dissolved in DMSO (995 μL), giving a concentration of 50 mM DMP. A second dilution of the 50 mM DMP stock solution (10 μL) in pH 7.0 buffer (0.1 M HEPES, 0.3 M NaCl, 1 mM EDTA) (990 μL) gave a working solution of 500 μM DMP. The activity of the arsenical could then be determined by the titration of varying amounts of arsenical against the DMP working solution (10 μL) in a 96-well microtitre plate, with the total volume made up to 195 μL by addition of buffer. After a 10 min incubation at room temperature, during which time the solutions were agitated on a plate shaker, 5 μL of a 37.9 mM stock solution of DTNB (15 mg) in DMSO (1 mL) was added, and the plate incubated with shaking for another 10 min. The absorbance at 412 nm due to the formation of the TNB dianion was measured using a Molecular Devices Thermomax Plus (Palo Alto, California) microplate reader. The extinction coefficient for the TNB dianion at pH 7.0 is 14,150 M^{-1} cm^{-1} at 412 nm (Riddles et al., 1983).

Calculation of the dissociation constant for binding of GSAO to dithiols

Binding of GSAO to dithiols was measured from loss of thiols using the DTNB assay described above. The dissociation constant K_d for GSAO binding to dithiols was determined by incubating increasing concentrations of GSAO, I, with a fixed dithiol concentration $[S]_T$, and measuring the remaining dithiol using DTNB. Note that the concentration of dithiol equals half the TNB concentration. The concentration of dithiol·GSAO complex, SI, as a function of the total GSAO concentration, $[I]T$, is described by Equation 1 (Hogg & Jackson, 1990):

$$[SI] = 0.5 \cdot \{([S]_T + x \cdot [I]_T + K_d) - (([S]_T + x \cdot [I]_T + K_d)2 - 4 \cdot [S]_T \cdot x \cdot [I]_T)0.5\} \quad (1)$$

where x is a factor that, when multiplied together with $[I]_T$, will yield the active concentration of GSAO. Data were fit to Equation 1 by nonlinear least-squares regression with K_d and x the unknown parameters (Scientist software, Micromath, Salt Lake City, Utah). x was 1 ± 0.2 for all the dithiols tested.

Thioredoxin contains five accessible thiols that react with DTNB (Holmgren, 1989), two of which form a cyclic complex with trivalent arsenicals (Brown et al., 1989). Titration of thioredoxin with GSAO resulted in a decrease from five to three thiols upon complex formation. The dissociation constant K_d for GSAO binding to thioredoxin was determined by incubating increasing concentrations of GSAO, I, with a fixed thioredoxin thiol concentration, $[S]_T$, and measuring the remaining thiol groups using DTNB. Note that the concentration of thiol groups equals the TNB concentration. The concentration of thioredoxin thiol·GSAO complex, SI, as a function of the total GSAO concentration, $[I]_T$, is described by Equation 2,

$$[S]_T = 2 \cdot [SI] + 2 \cdot [S]_D + [S]_M \quad (2A)$$

$$[SI] = 0.5 \cdot \{([S]_D + x \cdot [I]_D + K_d) - (([S]_D + x \cdot [I]_T + K_d)^2 - 4 \cdot [S]_D \cdot x \cdot [I]_T)^{0.5}\} \quad (2B)$$

where $[S]_D$ is the concentration of thioredoxin dithiol that complexes with GSAO and $[S]_M$ is the concentration of the remaining thiol groups. Data were fit to Equation 2 by nonlinear least-squares regression with K_d and x the unknown parameters (Scientist software, Micromath, Salt Lake City, Utah). x was 1.5 ± 0.2 for thioredoxin.

Electrophoresis and blotting

Samples were resolved on 4–15% SDS-PAGE under nonreducing conditions (Laemmli, 1970) and transferred to PVDF membrane. Proteins were detected by Western blot using an anti-PDI murine monoclonal antibody (Jiang et al., 1999) (used at 2 μg per mL). Rabbit anti-mouse horseradish peroxidase conjugated antibodies (Dako Corporation, Carpinteria, California) were used at a 1:2,000 dilution. GSAO-B-labeled proteins were blotted with streptavidin peroxidase (Amersham, Sydney, NSW, Australia) used at 1:1,000 dilution. Proteins were visualized using chemiluminescence (DuPont NEN, Boston, Massachusetts) according to the manufacturer's instructions. Chemiluminescence films were analyzed using a GS-700 Imaging Densitometer and Multi-Analyst software (Bio-Rad).

Labeling of cell surfaces with GSAO-B

Bovine aortic endothelial (BAE) (Hotchkiss et al., 1996) and HT1080 (Jiang et al., 1999) cells were harvested and cultured as indicated. BAE and HT1080 cells (5×10^6) were detached from culture flasks using 5 mM EDTA in PBS at 37 °C, washed three times with PBS, resuspended in PBS containing 100 μM of GSAO-B in the absence or presence of 400 μM DMP, and incubated for 30 min at room temperature. The cells were washed three times with 1 mL of PBS, resuspended in 0.2 mL of ice-cold 50 mM Tris/HCl, pH 8 buffer containing 0.5 M NaCl, 1% Triton X-100, 10 μM leupeptin, 2 mM PMSF (Sigma Chemical Company, St. Louis, Missouri), 5 mM EDTA, and 10 μM aprotinin (Bayer Australia Ltd., Sydney, NSW, Australia), and sonicated on ice. On one occasion, the endothelial cell lysate was incubated with streptavidin-agarose beads (25 μL of packed beads in a total volume of 1 mL) for 60 min at 4 °C with rotary mixing. Bound proteins were washed five times with 50 mM Tris/HCl, pH 8 buffer containing 0.15 M NaCl and 0.05% Triton X-100, resolved on SDS-PAGE, transferred to PVDF membrane, and the GSAO-B-labeled PDI detected by Western blot.

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