Selective Sensitization of Zinc Finger Protein Oxidation by Reactive Oxygen Species through Arsenic Binding*

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Background: Cysteine oxidation of zinc finger proteins plays an important role in protein function.
Results: Arsenic binding selectively sensitizes C3H1/C4 zinc finger proteins to oxidation by ROS.
Conclusion: Selectivity in arsenic binding to zinc finger motifs determines target proteins for oxidation by ROS.
Significance: This work provides an example of how an environmental insult may alter protein oxidation profiles and redox signaling.

Cysteine oxidation induced by reactive oxygen species (ROS) on redox-sensitive targets such as zinc finger proteins plays a critical role in redox signaling and subsequent biological outcomes. We found that arsenic exposure led to oxidation of certain zinc finger proteins based on arsenic interaction with zinc finger motifs. Analysis of zinc finger proteins isolated from arsenic-exposed cells and zinc finger peptides by mass spectrometry demonstrated preferential oxidation of C3H1 and C4 zinc finger configurations. C2H2 zinc finger proteins that do not bind arsenic were not oxidized by arsenic-generated ROS in the cellular environment. The findings suggest that selectivity in arsenic binding to zinc fingers with three or more cysteines defines the target proteins for oxidation by ROS. This represents a novel mechanism of selective protein oxidation and demonstrates how an environmental factor may sensitize certain target proteins for oxidation, thus altering the oxidation profile and redox regulation.

It has become increasingly appreciated that reactive oxygen species (ROS) play a critical role in regulating various physiological signaling pathways (1, 2). The balance between ROS production systems (e.g. NADPH oxidase) and ROS-scavenging systems (e.g. superoxide dismutase) precisely maintains normal physiology (1, 3). On the other hand, oxidative stress, which refers to the imbalance of the redox system in favor of oxidation, is an underlying mechanism for developing various diseases such as cardiovascular disease, diabetes, and cancer (4–7). In such cases, the alteration of redox signaling is caused by excess accumulation of ROS. Therefore, redox-sensitive protein targets are subject to overoxidation, which is suggested as a mechanism of protein function alteration in pathophysiological processes.

In ROS target proteins, cysteine residues are redox-sensitive sites that can be covalently modified by ROS into various forms, including reversible and irreversible oxidation (8, 9), which plays a critical role in the redox-signaling system. Oxidation of reactive cysteine residues may cause active-site modification in enzymes (10) and conformational changes in proteins (9, 11), both of which can lead to changes in protein function. Therefore, cysteine oxidation largely determines the outcome of the oxidative modification of the redox-sensitive protein by ROS (12, 13).

Zinc finger proteins consisting of cysteine residues within zinc finger motifs are acknowledged as sensitive targets in redox signaling (14, 15). Functionally, zinc finger proteins play many important roles, especially in transcription and DNA repair (16, 17). In a zinc finger motif, a zinc ion is complexed through four invariant cysteine and/or histidine residues to form a stable structure and conformation, which mediates protein-DNA, protein-RNA, and protein-protein interactions (18–20). The zinc-cysteine interactions not only maintain the structural integrity of the zinc finger but also considerably reduce the sensitivity of cysteine to oxidation (21), thus regulating the threshold of oxidation potential. When oxidative modification occurs, cysteine thiols within zinc finger structures release zinc from the binding site, resulting in the loss of zinc finger protein function. This process is considered as an efficient redox-sensitive molecular switch (14). When excessive ROS are generated, such as following exposure to environmental insults, unwanted oxidative modification of these redox-sensitive proteins may occur, leading to disruption of normal physiological processes and disease development. However, despite the large number of zinc finger proteins and cysteine residues in the proteome, the underlying basis of how certain zinc finger proteins are selectively targeted and modified by ROS remains unknown.

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4 The abbreviations used are: ROS, reactive oxygen species; PARP, poly(ADP-ribose) polymerase; HEKn, normal human epidermal keratinocytes; NEM, N-ethylmaleimide; DBD, DNA-binding domain; PARP-1zf, PARP-1 zinc finger.
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In this study, we utilized the ROS-generating arsenite (As(III)) as a model environmental toxin to investigate whether and how As(III) interaction with zinc finger proteins affects protein oxidation. As we reported previously (22), trivalent As(III) selectively binds to C3H1 or C4 zinc fingers, but not the more common C2H2 zinc finger motifs. We and others have shown that As(III) exposure leads to zinc loss from the DNA repair zinc finger protein poly(ADP-ribose) polymerase 1 (PARP-1), inhibition of PARP-1 activity, and retention of DNA damage in ultraviolet radiation-exposed cells (23–28). There is also extensive evidence that As(III) generates ROS through induction of NADPH oxidase, and antioxidants partially reverse these arsenic effects (25). These findings suggest that both As(III) binding and As(III)-mediated ROS generation contribute to As(III) inhibition of PARP-1 activity, but how these two distinct mechanisms relate to one another is unknown. Herein, we demonstrate that low levels of As(III) induced oxidation of certain zinc finger proteins through selective binding to C3H1 and C4 zinc finger proteins, thereby sensitizing these targets to oxidation by As(III)-generated ROS. These findings identify a novel mechanism by which oxidation of select zinc finger proteins can be attained by an environmental metal, ultimately leading to impact on disease development and human health.

Experimental Procedures

Materials—Peptides derived from the first zinc finger motif of PARP-1 (native C3H1, C2H2, and C4 mutants, with cysteine residues indicated in boldface) were commercially synthesized by Genemed Synthesis Inc. (San Antonio, TX); PARP-1zfC2H2, GRASCKKCESIPKDKVPHWYHFSFVKV; PARP-1zfC3H1, GRASCKKCESIPKDKVPHWYHFSFVKV; and PARP-1zfC4, GRASCKKCESIPKDKVPHWYCFSFVKV. Purity confirmed by HPLC was >95%. Zinc chloride (ZnCl2, Zn(II), 99%) and sodium arsenite (NaAsO2, As(III), 99%) were obtained from Fluka Chemie (Buchs, Germany). Other chemicals were obtained from Sigma-Aldrich unless indicated otherwise.

ROS (Superoxide) Detection—Cells were cultured in 96-well cell culture plates in complete medium. ROS levels and total DNA content (see Fig. 1A) were determined as described previously (29, 30). Briefly, cells at ~50% confluent density were placed in fresh medium and treated with 0.1–3 μM As(III) for 24 h. Thirty minutes prior to cell fixation, 5 μM dihydroethidium (Sigma-Aldrich) was added as a fluorescent indicator of superoxide generated in response to the described treatment. Relative fluorescence intensity was quantified by measuring the intensity of fluorescence emission using a Wallac VICTOR2 fluorescence spectrophotometer equipped with 900-nm excitation and 410-nm emission filters. A minimum of three samples from three independent experiments were analyzed per treatment. Values were normalized to total DNA fluorescence as described previously (31). Briefly, plates previously analyzed for ROS were rinsed with Krebs-Ringer buffer (20 mM HEPES, 10 mM dextrose, 127 mM NaCl, 5.5 mM KCl, 1 mM CaCl2, and 2 mM MgSO4, pH 7.4) and then frozen overnight at −80 °C. Plates were thawed for at least 2 h at room temperature and stained overnight with Hoechst dye (10 μg/ml bisbenzimide), and fluorescence was determined using a Tecan plate reader (Tecan US, Inc., Morrisville, NC) equipped with 350-nm excitation and 460-nm emission filters. This method of fluorescence quantification was validated by comparison with data obtained using MetaMorph software (version 6.3r6) as described previously (29). Results were graphed, and statistical significance was determined by one-way analysis of variance with Tukey’s multiple-comparison tests conducted using GraphPad Prism 5.

Analysis of Protein Oxidation in Cells with Modified Biotin-switch Assay—This assay was modified from previously reported biotin-switch assays (32, 33). Normal human epidermal keratinocytes (HEKn) were treated with 2 μM As(III) or 100 μM H2O2 for 12 h (see Fig. 1B) or for the indicated times in time course experiments (0–8 h) (see Fig. 3B). Cells were harvested in radioimmune precipitation assay cell lysis buffer (Thermo Scientific), sonicated, and centrifuged at 14,000 rpm for 15 min at 4 °C to remove cell debris. N-Ethylmaleimide (NEM; 10 mM; Sigma-Aldrich) was added to block all free Cys residues. Excess NEM in samples were removed three times by ice-cold acetone precipitation. Tris(2-carboxyethyl)phosphine hydrochloride (1 mM; Sigma-Aldrich) and biotin-NEM (500 μM; Thermo Scientific Pierce) were added to reduce all forms of Cys oxidation on proteins and to label the residues. Oxidized proteins were purified with streptavidin-agarose beads for further Western blot analysis using anti-PARP antibody (1:1000; Cell Signaling 9532) or anti-APTX (agraçațin) antibody (1:1000; Abcam ab66861). The membranes were developed using the SuperSignal chemiluminescence detection system (Pierce). Quantification of immunoblot results was performed using a Digital Science Image Station on a Kodak 440CF imager with ID Image Analysis software. A minimum of three independent samples were analyzed per treatment and time point. One-way analysis of variance with Tukey’s multiple-comparison tests conducted using GraphPad Prism 5 was used to determine statistical significance of data obtained from densitometry analysis.

Isolation of Zinc Finger Proteins and Zinc Content Measurement—Cells were cultured and transfected with wild-type and mutant PARP-1 expression vectors as described under “Transfection of PARP-1 DNA-binding Domain” and treated as described in the figure legends. Cells were no more than 75% confluent at the time of collection. Cells were harvested in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium vanadate, 1 μg/ml leupeptin, and 1 mM PMSF), sonicated, and centrifuged at 10,000 rpm for 5 min at 4 °C to remove cell debris. Zinc finger proteins were isolated by immunoprecipitation as follows. Protein (500 μg) was incubated with 5 μl of mouse anti-DDK monoclonal antibody (Origene TA50011) for 1–3 h at 4 °C, protein A beads (Invitrogen) were added in a 1:1 slurry, and samples were incubated for an additional 1–2 h at 4 °C. The beads were recovered by centrifugation at 10,000 rpm for 5 min at 4 °C and washed five times with 1 ml of lysis buffer. When examining the zinc content of the PARP-1 DNA-binding domain (DBD) samples (see Fig. 2B), the above protocol was amended by using biotin-coated polystyrene beads (Spherotech Inc., Lake Forest, IL) in place of antibody and agarose beads. For experiments to confirm the involvement of NADPH oxidase, transfected cells were treated for 30 min with apocynin (10 μM) prior to subsequent
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HEKn transiently expressing the PARP-1 DBD were treated to determine statistical significance of data obtained from den-
time point. One-way analysis of variance with Tukey’s multiple-
mM PMSF. Cell lysates were clarified by centrifugation at
Quantification of immunoblot results (see Figs. 1
Proteins were transferred to nitrocellulose membranes (Milli-
content was normalized to immunoprecipitated protein con-
compared with a standard curve for zinc content (34, 35). Zinc
the presence of zinc, and the 493 nm peak is recorded and
nm on a Beckman Coulter DU 800 spectrophotometer. The
resorcinol indicator absorbance shifts from 411 to 493 nm in
nm for 30 min, and added dropwise to cells cultured in 150-mm dishes.
Cells transfected with the PARP-1 DBD were incubated for 48 h
to allow protein expression before further treatment. For the
PARP WT and mutant plasmdis, isopropyl β-D-thiogalactopy-
ranoside (25 μM) was added to pTUNE vector transfections
after overnight incubation and incubated for an additional 48 h
to allow for protein expression prior to additional treatment.

Cysteine Oxidation Immunoblot Analyses—Control and
treated cells were washed with ice-cold PBS and harvested in 20
mm Tris, pH 7.5, 150 mm NaCl, 1 mm EDTA, 1 mm EGTA, 1%
Triton X-100, 2.5 mm sodium pyrophosphate, 1 mm β-glycero-
phosphate, 1 mm sodium vanadate, 1 μg/ml leupeptin, and 1
mm PMSF. Cell lysates were clarified by centrifugation at
10,000 rpm for 10 min at 4 °C, and 30 μg of total cell lysate was
resolved by electrophoresis on 10% SDS-polyacrylamide gel.
Proteins were transferred to nitrocellulose membranes (Milli-
pore, Bedford, MA) and probed with anti-cysteine sulfenic acid
antibody (Millipore 07-2139). The membranes were developed
using the SuperSignal chemiluminescence detection system.
Quantification of immunoblot results (see Figs. 1C and 3C) was
performed using a Digital Science Image Station on a Kodak
440CF imager with ID Image Analysis software. A minimum of
three independent samples were analyzed per treatment and
time point. One-way analysis of variance with Tukey’s multiple-
comparison tests conducted using GraphPad Prism 5 was used
to determine statistical significance of data obtained from den-
sitometry analysis.

Inductively Coupled Plasma Mass Spectrometry Analyses—
HEKn transiently expressing the PARP-1 DBD were treated with 2 μM As(III) for 0–8 h, and total protein collected in lysis
buffer and protein content were determined using the BCA
assay (Pierce). The PARP-1 DBD was immunoprecipitated by
incubating 1 mg of protein with biotin-coated polystyrene
beads for 1 h at room temperature, and the PARP-1 DBD was
isolated by centrifugation at 10,000 rpm for 10 min at 4 °C.
Beads bound to protein were washed three times with cold lysis
buffer. Pellets were resuspended in 100 μl of lysis buffer, and a
10-μl aliquot was removed for protein content analysis by gel
electrophoresis. Briefly, samples were mixed with 3× SDS load-
ing buffer (187.5 mM Tris-HCl, pH 6.8, 6% (w/v) SDS, 150 mM
DTT, and 0.03% (w/v) bromphenol blue) and separated by 10%
SDS-PAGE in parallel with known concentrations of purified
BSA (Sigma-Aldrich). Gels were fixed and stained with Coom-
assie Brilliant Blue R (Sigma-Aldrich), and bands were ana-
lized with a Carestream imager and Carestream molecular
imaging software (version 5.3.4). The bands from the known
concentrations of BSA were used to generate a standard con-
centration curve, and sample concentrations were determined.
Pellets were air-dried and sent to Huffman Laboratories Inc.
(Golden, CO) for microwave digestion and dilution in high-
purity nitric acid, followed by arsenic analysis by inductively
coupled plasma mass spectrometry. Spiked samples, untreated
beads, and blanks were included with experimental samples as
additional quality control for preparation and analyses. Results
were normalized to immunoprecipitated protein content, the
results of at least three independent experiments were averaged
and graphed, and statistically significant differences were
determined by one-way analysis of variance with Tukey’s mul-
tiple-comparison tests using GraphPad Prism 5 (see Fig. 3A).

MALDI-TOF-MS Analysis and Data Processing—Peptide
samples were diluted 100 times in 2 mg/ml α-cyano-4-hydroxy-
cinnamic acid (Sigma-Aldrich) in a 1:1 (v/v) water/acetonitrile
solution, and 0.5 μl of each sample was deposited in tripli
cate on the MALDI plate and allowed to dry at room temperature.
MALDI-TOF-MS analyses were performed on an Applied Bio-
systems 4700 proteomics analyzer (TOF/TOF) operating in MS
reflector-positive ion mode. The total acceleration voltage was
20 kV. Desorption was performed using a neodymium/ytrrium-
aluminum-garnet laser (355 nm, 3-ns pulse width, 200-Hz repe-
tition rate). Mass spectra were acquired with laser pulses over
a mass range of m/z 1000–5000 Da using a focus mass of 3500
Da. Final mass spectra were the summation of 10 subspectra,
each acquired with 200 laser pulses. Peaks were addressed
according to monoisotopic molecular mass. For disulfide for-
mation analysis, full mass spectra were obtained. Specifically,
the molecular mass of the apo-PARP-1 zinc finger (PARP-1zf)
is 3454 Da. Disulfide formation released two hydrogens from
two Cys residues, and one molecule of iodoacetamide label on
the third Cys residue introduced a +57 m/z shift. PARP-1zf
with a disulfide bond was observed at m/z 3509 (3454 + 2 + 57).
Quantitative results (see Fig. 4B) were obtained by summariz-
ing the intensities of the isotope peak family utilizing mMass
5.5.0 (36). Based on selective oxidation analysis, full mass spec-
tra are shown in Fig. 5 (A–E). Reduced forms of zinc fingers
were first addressed in spectra: PARP-1zfC2H2 + 2 NEM, m/z
3739; PARP-1zfC3H1 + 3 NEM, m/z 3829; and PARP-1zfC4 + 4
NEM, m/z 3921. Peaks that appeared at lower m/z in the
absence of a certain number of NEM molecules (125 m/z for
each NEM molecule) represent oxidized forms of the corre-
sponding configuration of zinc fingers with a certain number of
Cys residues being oxidized. NEM-labeled Cys residues were
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![Graph](https://via.placeholder.com/150)

**FIGURE 1. As(III)-generated ROS oxidizes PARP-1 and XPA (but not APTX) in cells.** A, superoxide generation by As(III). HEKn were treated with increasing concentrations of As(III) (0–3 μM) for 24 h in 96-well plates. The superoxide indicator dihydroethidium (DHE; 5 μM) was added, and fluorescence levels were analyzed after an additional 30 min of incubation. Plates were subsequently analyzed for total DNA content, and ROS fluorescence was normalized to total DNA. Graph represents mean ± S.D. (n = 4). B, Western blot analysis of oxidized PARP-1, XPA, and APTX. HEKn were treated with H₂O₂ (100 μM) or As(III) (2 μM) or left untreated (Ctrl) for 12 h. Oxidized proteins were analyzed using the modified biotin-switch method. Blots shown are representative of three independent experiments. IP, immunoprecipitation; IB, immunoblot. C, densitometry analysis of protein oxidation Western blots. Graphs represent mean ± S.D. (n = 3), *p < 0.05.

counted as reduced. Cys residues without NEM labeling were counted as oxidized. Quantitative results were also obtained by summarizing the intensities of the isotope peak family. Zn(II)- or As(III)-binding signals were not counted as oxidized or reduced in the calculation. Cys oxidation percentage in each type of zinc finger is thus summarized in bar charts in Fig. 5 (F−I).

**Results**

**Selective Oxidation of C3H1 versus C2H2 Zinc Finger Protein in Cells—**Oxidation by ROS functionally inhibits zinc finger protein activity, and cysteine residues within zinc finger motifs are targets of oxidation. Exposure of human keratinocytes to As(III) rapidly increases intracellular ROS (30, 37). In HEKn, elevation of superoxide following As(III) exposure is evident within 10 min and is sustained for at least 24 h (38). Although 5 μM arsenite did not alter glutathione levels in HEKn (data not shown), As(III) concentrations as low as 0.1 μM were sufficient to significantly increase superoxide 24 h after exposure (Fig. 1A). To investigate whether As(III)-stimulated ROS generation displays any selectivity for oxidative modification of zinc finger proteins, we exposed HEKn to H₂O₂ (10 or 100 μM) or As(III) (2 μM) for 12 h and analyzed the cysteine oxidation of three representative DNA repair proteins with different number of cysteines in the zinc finger motif: APTX (C2H2 zinc finger), PARP-1 (C3H1 zinc finger), and XPA (C4 zinc finger). Protein oxidation was measured by the modified biotin-switch assay. Exposure to 10 μM H₂O₂ did not cause measurable change in oxidation of any of the three proteins, whereas 100 μM H₂O₂ was able to significantly oxidize each protein (Fig. 1, B and C). Surprisingly, treatment of cells with 2 μM As(III) resulted in the oxidation of PARP-1 and XPA, but not APTX (Fig. 1, B and C). These findings suggest that although proteins with different zinc finger configurations can be oxidized by high concentration of H₂O₂, oxidation of these proteins occurs with exposure to very low concentrations of As(III) and also demonstrates selectivity.

Selective Oxidation of PARP-1 in Cells Correlates with As(III) Binding and Zinc Release—Oxidation of C3H1 and C4 zinc finger proteins PARP-1 and XPA (but not C2H2 APTX) shows a similar pattern to our earlier finding that As(III) binds with zinc fingers containing three or more cysteine residues (22). To investigate whether the observed oxidation of C3H1 and C4 (but not C2H2) zinc finger proteins is linked to selective As(III) binding to certain zinc finger motifs, we transfected HEKn with cDNA representing native PARP-1 or PARP-1 harboring mutations within the PARP-1 DBD that convert the zinc fingers to C2H2 motifs that do not bind As(III) (22). Transfected cells were treated with As(III), and PARP-1 protein was isolated by affinity tag immunoprecipitation, followed by immunoblotting to detect oxidized cysteine residues. Significant cysteine oxidation was evident with the native PARP-1 protein, but not with the PARP-1 protein expressing the mutant C2H2 zinc fingers (Fig. 2A). Cysteine oxidation corresponded to zinc loss from native PARP-1, but not the mutant C2H2 PARP-1 protein (Fig. 2B). Our previous work identified NADPH oxidase as a major contributor to arsenic-induced ROS (30), which may reflect an underlying mechanism for As(III)-induced PARP-1 oxidation. NADPH oxidase inhibition by apocynin (10 μM) significantly decreased As(III)-dependent native PARP-1 cysteine oxidation and increased zinc retention in PARP-1 protein, but had no impact on the mutant C2H2 PARP-1 proteins (Fig. 2B). These findings were also confirmed with a different C2H2 zinc finger mutation (HCHC versus CCHH zinc finger) (data not shown). These results obtained with site-directed mutations to the PARP-1zf confirm that As(III) binding facilitates selective oxidation on C3H1 zinc fingers and further suggest that As(III)-dependent activation of NADPH oxidase, in conjunction with selective As(III) binding, is necessary for zinc finger oxidation by As(III) within the cellular environment.

To further investigate the observed correlation between selective oxidation and As(III) binding to zinc finger motifs, we performed time course analysis of As(III) binding, zinc loss, and
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FIGURE 2. As(III) binding and stimulation of NADPH oxidase activity are necessary for PARP-1 oxidation by As(III) in cells. HEK293 expressing DDK-tagged native PARP-1 (WT) or PARP-1 with DBD zinc fingers mutated to CCHH (M1) were left untreated (control (Ctrl); black bars), treated with As(III) alone (As; 1 μM) for 6 h (red bars), or pretreated with apocynin (apo; 10 μM) for 30 min to inhibit NADPH oxidase before treatment with As(III) for 6 h (green bars). Cell lysates were collected, and the native and C2H2 mutant expressed PARP proteins were immunoprecipitated with anti-DDK antibody. H2O2 (100 μM, 6 h; gray bars) was used as an oxidation positive control. A, oxidized cysteine residues were visualized by immunoblotting with anti-cysteine sulfenic acid, and densitometry was used to quantify oxidized cysteine residues. B, the zinc content in immunoprecipitated PARP-1 (WT) or C2H2 mutant (M1) protein was determined using the zinc release assay as described (22). The M2 mutant (HCHC zinc fingers) was also analyzed in these experiments with results similar to M1. Graphs represent mean ± S.D. (*, p < 0.01 versus Ctrl; **, p < 0.01 versus As(III) group.

Cysteine oxidation. Cells expressing the native PARP-1 DBD were treated with 2 μM As(III) for 0–24 h, and the PARP-1 DBD was isolated by immunoprecipitation and then analyzed for arsenic content by inductively coupled plasma mass spectrometry. Arsenic content in the PARP-1 DBD was maximal at 1 h post-treatment and rapidly declined to undetectable levels by 4–6 h (Fig. 3A). The zinc content of the PARP-1 DBD was measured in parallel, and zinc loss was evident at 1 h and continued to decline over time, with no recovery of zinc binding evident as arsenic binding decreased (Fig. 3A). After 24 h of treatment of cells with As(III), little arsenic or zinc was associated with the PARP-1 DBD, suggesting disruption of the zinc finger metal-binding properties. Meanwhile, oxidation of PARP-1 after cell exposure to As(III) was significantly increased within 30 min of As(III) treatment (Fig. 3, B and C). Furthermore, PARP-1 oxidation was found to occur on the zinc finger DBDs based on analysis of the expressed PARP-1 DBD after As(III) treatment. In contrast, there was no increase in APTX oxidation over the same time course (Fig. 3, B and C). The protein levels of PARP-1 and APTX were not altered as a consequence of As(III) treatment (data not shown). These results confirm the selective oxidation of a C3H1 versus C2H2 zinc finger protein after As(III) exposure and demonstrate a temporal sequence of events of As(III)-stimulated ROS production, As(III) binding to the PARP-1 DBD, zinc release from PARP-1, and PARP-1 oxidation.

Zinc (but Not Arsenite) Protects PARP-1zf Peptide from Oxidation—The above findings raised the important question of why zinc finger protein oxidation is correlated with As(III) binding and Zn(II) release. We addressed this question by comparing the oxidation potential between Zn(II)-bound and As(III)-bound zinc fingers using a peptide representing a PARP-1zf motif. Zn(II)- or As(III)-bound PARP-1zf peptides were exposed to H2O2, and the free cysteine content of the peptides was analyzed by Ellman’s assay (39). Exposure of apo-PARP-1zf1 to H2O2 rapidly decreased the amount of free thiols, whereas the Zn(II)-bound peptide largely retained the free thiol content (Fig. 4A). However, thiols in the As(III)-bound peptide were mostly oxidized within 30 min, reaching similar levels as in apo-PARP-1zf1. More intriguingly, the rate of oxidation was greater for the As(III)-bound peptide than apo-PARP-1zf1 during the early time points (Fig. 4A). We next analyzed a product of the cysteine oxidation by H2O2, disulfide bond formation of PARP-1zf1, using reverse labeling and measuring the m/z shifts in the MALDI mass spectra. Quantification of peptide oxidation confirmed the protective effect of Zn(II), but not As(III) (Fig. 4B). These findings demonstrate that although Zn(II) binding protects the PARP-1zf1 peptide from oxidation, As(III) binding does not, suggesting that As(III) replacement of Zn(II) exposes the cysteine in the zinc finger motif to oxidation by ROS.

Selective Arsenite Binding Leads to Selective Oxidation on C3H1 and C4 Zinc Fingers—The above results imply a new mechanism whereby As(III) binding sensitizes certain zinc fingers to oxidation. To demonstrate the selectivity in a complex and competing environment, we analyzed the oxidation profile in detail with all three configurations of zinc fingers in a single experimental system. We performed MALDI-MS analysis on a mixture of three peptides: PARP-1zf1 (representing the native motif, C3H1) and peptides with site-specific substitutions of cysteine or histidine to yield the corresponding C2H2 and C4 PARP-1zf1 mutant peptides, respectively. Due to the complexity of oxidation forms and instability of oxidation modifications in MALDI-MS, we utilized a reverse-labeling method by labeling free cysteine thiols with NEM. Full mass spectra are shown in Fig. 5 (A–L). Peaks of the reduced forms of each zinc finger in spectra were first assigned: PARP-1zfC2H2 + 2 NEM, m/z 3739; PARP-1zfC3H1 + 3 NEM, m/z 3829; and PARP-
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1zfC4 + 4 NEM, m/z 3921. The loss of a certain number of NEM labels (125 m/z) from each of the three peaks was used to assign the oxidized forms for the corresponding zinc finger configurations. NEM-labeled Cys residues were counted as reduced. Cys residues without NEM labeling were counted as oxidized. Zn(II)- or As(III)-binding signals were not counted as oxidized or reduced in the calculation. Cys oxidation percentage in each type of zinc finger is thus summarized in bar charts in Fig. 5 (F–I). H2O2 significantly increased oxidation of all three zinc finger apopeptide configurations (Fig. 5F). Zn(II) binding partially protected each zinc finger from oxidation by H2O2 (Fig. 5G). Cysteine oxidation in the presence of As(III) was equivalent to the apopeptide regardless of the type of zinc finger motif (Fig. 5H). When peptides were incubated with both Zn(II) and As(III), only the C2H2 PARP-1zf1 peptide, which did not bind As(III), was protected from oxidation (Fig. 5I), whereas the As(III)-binding C3H1 and C4 PARP-1zf1 motifs were oxidized. These findings demonstrate that As(III) binding to the C3H1 and C4 peptides targets these motifs for oxidation by H2O2.

Discussion

It is estimated that the human proteome contains ~3000 zinc-containing proteins serving signaling, catalytic, and structural roles (40). Zinc-coordinating cysteine residues are sensitive to oxidation, and several mechanisms have been described to account for differences in cysteine residue redox reactivity, which can vary by >6 orders of magnitude (12). The location of the cysteine within the protein, relative protein abundance, spatial relationship between a target protein and site of ROS generation, and partial oxidation under normal cell conditions have been proposed as mechanisms underlying differences in protein oxidation (12, 41). In this study, we defined a novel mechanism for selectivity of zinc finger oxidation in specific target proteins. Because As(III) binds zinc finger proteins harboring C3H1 or C4 (but not C2H2) motifs and As(III) binding fosters cysteine thiol oxidation, the subset of zinc finger proteins that bind As(III) become a sensitive target for oxidation. Therefore, arsenic binding defines which zinc finger proteins will be sensitive to oxidation by As(III)-generated ROS, and as a result, selective binding by As(III) translates into selective protein oxidation.

The results presented here, together with previous published findings, establish a model for selective oxidation of zinc finger proteins by arsenic, as illustrated in Fig. 6. This interaction-sensitization model is supported by the following evidence: (i) eliminating As(III) binding (by mutating the C3H1 zinc finger to the C2H2 zinc finger) eliminates Cys oxidation and zinc loss, and reducing ROS generation (with apocynin) mostly abrogates the arsenic effect on Cys oxidation and zinc loss (Fig. 2); and (ii) As(III) incubation with purified PARP-1 protein has no impact on its activity, but exposure to As(III) in the ROS-producing cellular environment inhibits PARP-1 activity (25). The proposed model suggests that As(III) disruption of zinc finger function is not due to a simple competition or replacement mechanism; ROS generation within the cellular environment is a critical factor. This model also provides a mechanistic basis for the arsenic binding to specific target proteins with subsequent oxidative damage. This proposed mechanism offers a novel example of redox signaling alteration by an environmental factor through selective cysteine oxidation. This mechanism was established in this study through well-designed experi-
ments testing the hypothesis using custom-synthesized peptides representing the zinc finger moiety of the target proteins and validating the hypothesis in intact cells.

This mechanism is particularly relevant to understanding arsenic carcinogenicity. Arsenic-induced oxidative DNA damage and inhibition of DNA repair through functional disruption of zinc finger DNA repair proteins are proposed mechanisms for the carcinogenic actions of arsenic. It has been challenging to reconcile the ubiquity of ROS generation in response to arsenic with evidence that zinc finger DNA repair proteins are not equally vulnerable to inhibition by arsenic. PARP-1 has long been recognized as a highly sensitive molecular target for arsenic (23, 42). Through site-directed mutations of peptide and protein, we found that As(III) preferentially binds to C3H1 and C4 zinc fingers (22), leading to loss of zinc from protein isolated from intact cells. Recent work demonstrated that the two C3H1 zinc fingers in the PARP-1 DBD have significantly different affinities for zinc (43). This characteristic of PARP-1zf1 has been proposed to possibly account for the vulnerability of PARP-1 to toxic metals such as arsenic; however, PARP-1 is

FIGURE 4. Zinc (but not As(III)) protects PARP-1zf from oxidation by H2O2. A, free thiol content measured by Ellman’s assay. Apo-PARP-1zf (1 mM; gray line) was incubated with Zn(II) (1 mM; blue line) or As(III) (1 mM; red line). Free thiol content was measured using 5,5′-dithiobis(2-nitrobenzoic acid) after oxidizing apo-peptide or metal-bound peptide with H2O2 (2.5 mM) for the indicated times. Results are shown as mean ± S.D. (n = 3). *, significantly different from the apo-PARP-1zf group (p < 0.05). B, summary of MALDI-TOF-MS analysis of disulfide formation on PARP-1zf1. Apo-, Zn(II)-bound (100 μM), or As(III)-bound (100 μM) PARP-1zf1 was exposed to H2O2 (100 μM), and iodoacetamide (1 mM) was used to react with free Cys residues. PARP-1zf1 with a disulfide bridge was detected at m/z 3509. Oxidation percentages were calculated from the relative intensity in mass spectra. Results are shown as mean ± S.D. (n = 4). *, significantly different from the H2O2 group (p < 0.05).

FIGURE 5. Selective As(III) binding leads to selective oxidation of C3H1 and C4 zinc finger peptides. C2H2, C3H1, and C4 PARP-1zf were mixed at equimolar concentrations to a final concentration of 100 μM and incubated with 100 μM Zn(II) (Zn) or 100 μM As(III) (As) for 30 min. H2O2 (100 μM) was added to the mixture for an additional 30 min. NEM (500 μM, 1 h) was then added to react with free thiols, introducing a +125 m/z shift for each free thiol on the mass spectra. A–E, mass spectra of the zinc finger peptide mixtures after the corresponding treatments. Peaks are assigned and labeled as shown in A. A statistical summary of the mass spectra shows the comparison of cysteine oxidation percentages in each type of zinc finger in the mixture. F, control (black bars) versus H2O2 (gray bars). H2O2 oxidized all three types of zinc fingers. G, H2O2 (gray bars) versus As(III) + H2O2 (red bars). As(III) binding did not protect any PARP-1zf motif from oxidation. H, H2O2 (gray bars) versus Zn(II) + H2O2 (blue bars). The presence of zinc protected cysteines from oxidation in each zinc finger type. I, H2O2 (gray bars) versus Zn(II) + As(III) + H2O2 (gold bars). C3H1 and C4 zinc fingers were selectively oxidized in the presence of As(III) and Zn(II), whereas C2H2 zinc fingers was protected from oxidation. The graphs represent mean ± S.D. (n = 3). *, significantly different from the previous bar in the same group (p < 0.05).
not unique for zinc finger disruption by As(III). The DNA repair protein XPA has a single C4 zinc finger, and arsenite-dependent zinc loss (22, 44) and cysteine oxidation (Fig. 1, B and C) in the cellular environment are equivalent to those in PARP-1. Neither As(III) binding to zinc finger peptides nor As(III)-dependent zinc loss from protein was detected in APTX or SP-1, both C2H2 proteins (22). The cumulative findings support a mechanism that involves both As(III) interaction with a target protein and As(III)-mediated ROS generation.

Based on the results presented here, it appears that As(III)-induced selective oxidation of cysteines in zinc finger proteins involves several coordinated steps. As(III) displacement of zinc from certain zinc finger configurations eliminates zinc protection of the zinc finger cysteine residues, thus rendering these cysteines vulnerable to attack by As(III)-generated ROS. Cysteine oxidation further disrupts As(III) binding to the zinc finger motif (Fig. 3A), thereby releasing the As(III) to interact with another target protein. Because the released As(III) would be free to interact with another target protein and then repeat this cycle, it effectively functions in a catalyst-like manner for oxidation of targeted proteins. The bind-and-release model could replace it with an oxidation-vulnerable arsenic–sulfur bond. A key step in this mechanism is the selective arsenite binding to C3H1 and C4 zinc fingers (22). This step is critical because it disrupts the important zinc–cysteine interaction, which protects cysteine thiol from oxidation, and then replaces it with an oxidation-vulnerable arsenic–sulfur bond. Compared with naked cysteine, the vulnerability of the arsenic–sulfur bond to oxidation is thermodynamically similar (Fig. 5H), but kinetically favored (Fig. 4A). This explains why cysteines on zinc finger motifs of PARP-1 are so sensitive to oxidation in the presence of low concentrations of As(III), despite the abundant free cysteine residues available in the proteome and high concentrations of antioxidants in the cellular environment. This mechanism of arsenic binding-enhanced cysteine oxidation could help to answer the challenging question posed by Hartwig (41) regarding how the specificity required for the activation or inactivation of the redox signaling pathway is achieved by the low concentration of ROS generated by metals under realistic environmental conditions.

In summary, the findings presented here reveal a novel mechanism whereby generation of ROS within a cell results in selective protein oxidation through sensitization of a target protein to zinc finger cysteine oxidation by the ability of that zinc finger motif to interact with arsenic. This work provides an example of how an environmental insult such as arsenic may alter protein oxidation profiles and disrupt redox-sensitive proteins in an important physiological process such as DNA repair.

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