Protein lysine-N ζ alkylation and O-phosphorylation mediated by DTT-generated reactive oxygen species

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Abstract: Reactive oxygen species (ROS) play crucial roles in physiology and pathology. In this report, we use NMR spectroscopy and mass spectrometry (MS) to demonstrate that proteins (galectin-1, ubiquitin, RNase, cytochrome c, myoglobin, and lysozyme) under reducing conditions with dithiothreitol (DTT) become alkylated at lysine-N ζ groups and O-phosphorylated at serine and threonine residues. These adduction reactions only occur in the presence of monophosphate, potassium, trace metals Fe/Cu, and oxygen, and are promoted by reactive oxygen species (ROS) generated via DTT oxidation. Superoxide mediates the chemistry, because superoxide dismutase inhibits the reaction, and hydroxyl and phosphoryl radicals are also likely involved. While lysine alkylation accounts for most of the adduction, low levels of phosphorylation are also observed at some serine and threonine residues, as determined by western blotting and MS fingerprinting. The adducted alkyl group is found to be a fragment of DTT that forms a Schiff base at lysine N ζ groups. Although its exact chemical structure remains unknown, the DTT fragment includes a SH group and a $-\text{CHOH}-\text{CH}_2-$ group. Chemical adduction appears to be promoted in the context of a well-folded protein, because some adducted sites in the proteins studied are considerably more reactive than others and the reaction occurs to a lesser extent with shorter, unfolded peptides and not at all with small organic molecules. A structural signature involving clusters of positively charged and other polar groups appears to facilitate the reaction. Overall, our findings demonstrate a novel reaction for DTT-mediated ROS chemistry with proteins.

Keywords: NMR; mass spectrometry; reactive oxygen species; alkylation; phosphorylation

Abbreviations: Cys-CAM, carboxyamidomethylcysteine; DTT, dithiothreitol; Gal-1, galectin-1; MS, mass spectrometry; NMR, nuclear magnetic resonance spectroscopy.

Additional Supporting Information may be found in the online version of this article.

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Introduction

Maintaining protein sulfhydryls in the reduced state is crucial to the function of many proteins. The compound 1,4-dithiothreitol (DTT) is the most common reducing agent used to achieve this. Reduction of the disulfide bond occurs via two sequential thiol-disulfide exchange reactions in which the protein disulfide bond is reduced to two thiolate S^- (or SH) groups and the DTT thiolates become oxidized intramolecularly to form a six-membered ring. However, oxidation of DTT can also generate a DTT radical, along with various reactive oxygen species (ROS) in which molecular oxygen is reduced to superoxide radical, hydrogen peroxide, and in the presence of trace metals like copper and iron, to hydroxyl radical.^{1,2}

ROS are normal products of cellular metabolism^{3,4} and have roles in cell signaling and homeostasis⁵ and function, for example, in apoptosis, host defense, and redox (oxidative) signaling.^{6,7} In wound repair and blood homeostasis, platelets release ROS to recruit platelets to sites of injury. ROS also provide a link to adaptive immunity via recruitment of leukocytes. ROS, and its by products like hydrogen peroxide, are also involved in inflammatory responses, for example, ischemic injury and cardiovascular disease, including stroke and heart attack. ROS can also lead to harmful effects for example, damage of DNA and RNA,⁸ and oxidation of polyunsaturated fatty acids (lipid peroxidation) and amino acids in proteins.^{9,10} Normally, superoxide dismutase (SOD) and catalase (along with e.g., lactoperoxidases, glutathione peroxidases and peroxidoreductins, and various antioxidants (like vitamin C, vitamin E, uric acid, and glutathione) ameliorate damaging effects from superoxide and hydrogen peroxide by converting them into oxygen and water.¹¹ However, residual ROS persist in cells, and excessive amounts of ROS can result in deleterious effects,¹² including for example, cancer^{13,14} and cognitive dysfunction.¹⁵ γ -radiation and various therapeutic agents also mediate their effects through production of ROS.^{16,17}

Here, we report that ROS generated by DTT oxidation can mediate protein phosphorylation and Schiff base adduction of a DTT-derived alkyl fragment to lysine N_ϵ groups. Because many researchers use DTT in various preparations and reactions, it is likely that unwanted by-products are being produced which could cloud experimental results and interpretations. In this regard, it is important to characterize what occurs to proteins in the presence of DTT, as well as the chemistry of DTT-mediated reactions. We find that there are specific conditions under which DTT-mediated protein modifications occur, and these include the presence of ROS (normally produced via DTT redox reactions), monophosphate, potassium, and trace metals.

Results

When ^{15}N -labeled galectin-1 (Gal-1) stands overnight at low concentration in 20 mM potassium

Figure 1. NMR data on modified and unmodified Gal-1 and ubiquitin. **(A)** ^{15}N - ^1H HSQC spectra (overlaid) of 0.4 mM ^{15}N -labeled Gal-1 acquired before (red cross peaks) and after (blue cross peaks) overnight incubation at low protein concentration (10 μM) in 20 mM potassium phosphate, pH 7.3, with 8 mM dithiothreitol (DTT) at 37°C, followed by buffer exchange with standard buffer and concentrating the sample back up to a protein concentration of 0.4 mM. Many cross-peaks in modified Gal-1 vis-à-vis the unmodified protein are shifted and/or appear as multiple peaks and are labeled as such. S38 is one of the phosphorylated residues. **(B)** ^1H DIPSI spectrum (60 ms mixing time) of modified ubiquitin (green cross peaks) overlaid with that of 4 mM unmodified ubiquitin (red cross peaks). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

phosphate, $\sim\text{pH}$ 7, with 8 mM dithiothreitol (DTT) at 37°C, we observed that HSQC spectra acquired before (red cross peaks) and after (blue cross peaks) this time period are significantly different [Fig. 1(A)]. Some native state resonances lose intensity and/or are chemically shifted, and new resonances appear. Spectral changes occur much more slowly upon incubation at 4°C, yet they still occur in a similar fashion. Formation of multiple resonances for many residues indicates that Gal-1 is somehow being modified chemically and/or conformationally altered while standing in this solution. Spectral changes are not observed in the presence of other reducing agents (i.e., TCEP: tris(2-carboxyethyl)-

Figure 2. MALDI-TOF MS data on modified proteins. MS data have been acquired on Gal-1 (A), ubiquitin (B), and cytochrome c (C) as a function of incubation time in 20 mM potassium phosphate, pH 7.3, with 8 mM dithiothreitol (DTT) at 30°C. Time points are shown for 0, 2, 6, 10, and 22 h and are indicated at the right of each MS trace. Unmodified protein is the left most m/z peak in each series. m/z peaks are labeled with +1 to +6 to indicate the number of Δ mass 116 groups adducted on each protein on average over time. Smaller m/z peaks result are attributable to the protein plus adhering salts and/or the cinnamic acid matrix used in acquiring MALDI-TOF MS data, as well as phosphorylation as discussed in the text.

phosphine, or BME: β -mercaptoethanol), in the absence of DTT or in the presence of DTT at pH values below its thiol $pK_a \sim 7$ where DTT is inactive.^{1,2} In this regard, the protein is being modified specifically by chemistry associated with the DTT redox reaction.

Prior to incubation in the reactive DTT solution, Gal-1 shows the presence of a large m/z peak in MALDI-TOF MS data expected for the molecular mass of the lectin (14,584 Da), along with a few other m/z peaks attributable to protein-adhering salts and/or the matrix (cinnamic acid) used in acquiring these MS data. As time goes by, new peaks of greater m/z appear, and by 22 h very little, if any, of the native Gal-1 m/z peak remains, and at least five new, relatively intense peaks appear, with each proximal pair showing a mass difference (Δ mass) of +116 Da [Fig. 2(A)]. Other MS peaks of lower intensity not present with the unmodified protein are also observed. Because these new Δ mass +116 peaks remain following denaturation with 8M urea, SDS or 10% acetic acid, we conclude that they result from covalent adduction of chemical group(s) to the protein. This is supported by the observation that the number and intensity of MS peaks change systematically over time, as demonstrated in Figure 2.

These findings are not unique to Gal-1. We have also observed formation of these same higher mass species with other proteins (ubiquitin, cytochrome c, RNase, myoglobin, lysozyme) incubated under the same solution conditions, as exemplified with ubiquitin and cytochrome c in Figure 2(B,C), respectively.

Figure 1(B) shows a 1H DIPSI spectrum of modified ubiquitin (green cross peaks) overlaid with that of unmodified ubiquitin (red cross peaks). Because ubiquitin lacks cysteines, the presence of a reducing agent like DTT is not required to keep the protein in a reduced, active state. Nevertheless, many resonances are shifted and/or appear as multiple peaks upon modification, as observed with Gal-1. Because resonances are similarly dispersed in these modified and unmodified proteins, it appears that chemical modification does not significantly alter native protein folds, at least at these levels of adduction. This will be discussed further in subsequent sections. The observation of DTT-mediated protein modification raises several questions related to residues that are modified, the nature of the modification(s), and the mechanism of action/chemistry involved in the reaction. We address these points below by focusing on the proteins ubiquitin and Gal-1.

Figure 3. ^1H TOCSY spectra are shown for the unmodified (A) and modified (B) ubiquitin, as discussed in the text. A number of resonances have been labeled, particularly those of lysine and arginine sidechains. The square box in spectrum B displays the region of new resonances that belong to spin systems U that are equivalent to Lys H_ϵ resonances in modified ubiquitin, and are scalar coupled to H_β , H_γ and H_δ resonances. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Modifications to ubiquitin and Gal-1

To assess which amino acid residues in ubiquitin and Gal-1 are chemically modified, we used NMR and MS fingerprinting. For NMR studies, we either followed the adduction reaction over time directly in the NMR tube or concentrated the modified protein from larger volume preparations to achieve greater signal-to-noise. In the following section, we will introduce the suffix “DTT” as nomenclature for DTT-mediated chemical modifications at specific amino acid residues, for example K6-DTT. The reason for this will become evident later.

Ubiquitin

The most significantly altered spin systems in modified ubiquitin belong to lysines, as exemplified with ^1H TOCSY spectra for the unmodified [Fig. 3(A)] and modified [Fig. 3(B)] protein. Because resonances from Lys $\text{C}\epsilon\text{H}_2$ groups are shifted the most in modified ubiquitin, and primary amines make for good reactive groups, we tentatively concluded that chemical adduction occurs at lysine N_ζ groups. Further support for chemical modification at lysine residues was had by using per-methylated-Lys ubiquitin, where MS peaks with Δmass 116 or highly shifted lysine cross peaks in NMR spectra were absent following incubation in the DTT reaction solution.

Ubiquitin has seven lysines (K6, K11, K27, K29, K33, K48, and K63). Comparison of ^{15}N - ^1H HSQC spectra for unmodified [Fig. 4(A)] and modified [Fig. 4(B)] ^{15}N -ubiquitin indicate that the most shifted and adducted lysines are K48, K63, K33, and K6.

These NMR results are consistent with those from MS fingerprinting. Relative to unmodified ubiquitin [Fig. 5(A)], trypsin-digested modified ubiquitin [Fig. 5(B)] shows the presence of three intense m/z peaks (6–7 k counts total) that we can assign to peptides 43–54, 30–42, and 55–72 with Δmass shifts of 116 Da. Each of these peptides contains a single lysine, that is, K48, K33, and K63, respectively, consistent with their N_ζ groups being chemically modified. These MS results also provide another piece of evidence supporting modification at lysine side chains. The enzyme trypsin recognizes Lys and Arg residues on the N-terminal side of cleavage sites by their positive charge and thereby hydrolyzes peptide bonds at those sites. In this regard, we noted the absence of Δmass 116-shifted peptides that otherwise could have been cleaved at those lysine-modified sites. The MS counts shown in Figure 5(B) are at the 10% intensity level to better view low intensity m/z peaks. Two of these lower intensity m/z peaks can be associated with peptide 1-11 + K6-DTT and peptide 28-42 + K29-DTT/K33-DTT. Based semi-quantitatively on MS intensities, we conclude that K6 and K29 are modified at much lower levels than are K48, K33, and K63, in line with NMR results.

Other low intensity m/z peaks are also present in these MS fingerprinting data [Fig. 5(B)]. While some of these can be associated with digested peptides from unmodified ubiquitin molecules, four of them (between 1400 and 1600 m/z) can be associated with peptides 30–42, 43–54, and 64–74 having Δmass shifts of 80. This mass shift most often indicates the presence of phosphorylated (+ PO_3)

Figure 4. Natural abundance ^{15}N - ^1H HSQC spectra are shown for unmodified (A) and modified (B) ubiquitin (4 mM protein, 20 mM phosphate, pH 7.3 and 32°C). Some resonances are labeled as discussed in the text, and cross peaks associated with residues around K48 are boxed in. Amide peaks labeled “n” indicate native peaks, whereas peaks labeled “a,” “b,” “c” indicate additional peaks that arise from alkylation of multiple primary amines of lysine and/or the N-terminal amino group. Circles indicate positions of amide resonances that are not observed at the contour level chosen or are otherwise absent from the spectrum due to for example, increased exchange rates with the solvent. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

residues.^{18,19} To confirm this, we performed western blotting that indeed demonstrated the presence of phosphoserine (pSer) and phosphothreonine (pThr) in modified ubiquitin (Fig. 6). In SDS PAGE gels, most bands for ubiquitin (Ubi) are not visible; nevertheless, pUbi bands in these westerns are clearly evident in both instances. Therefore, we conclude that S65 and T66 in modified peptides 64–74 are phosphorylated. However, in two other apparently $\Delta 80$ mass-shifted peptides (30–42 and 43–54), there are no Ser or Thr residues, and therefore other side chains may as well be phosphorylated (e.g., D32, E34, D39, E51, D52). Overall, however, our NMR and MS data do indicate that phosphorylation is a minor component of these ROS-mediated reactions.

Galectin-1

Observations with Gal-1 parallel those with ubiquitin. As we discussed above, Figure 1(a) overlays ^{15}N - ^1H HSQC spectra of unmodified (red cross peaks) and modified (blue cross peaks) ^{15}N -labeled Gal-1 [30] (incubated for 24 h at $10\ \mu\text{M}$ and then concentrated to $0.4\ \text{mM}$). As with ubiquitin, many Gal-1 cross-peaks in the modified vis-à-vis the unmodified proteins are shifted and/or appear as multiple peaks. Figure 7 shows ^{13}C HSQC spectra in D_2O for unmodified (panels A and C) and modified (panels B and D) ^{13}C -labeled Gal-1. New ^{13}C -labeled resonances appear in the Lys $\text{C}\epsilon\text{H}_2$ region of the spectrum of modified Gal-1 [Fig. 7(D)], as they did with modified ubiquitin. Most of the more significantly affected resonances either belong to lysines (especially K12, K36, and K63) or are in the vicinity of lysines (see also Supporting Information Fig. S1–S3). Moreover, $\text{C}\epsilon\text{H}_2$ resonances of these lysines in unmodified Gal-1 molecules remaining in the sample [Fig. 7(B)] are reduced in intensity as their populations decrease due to chemical modification.

Relative to MS fingerprinting with unmodified Gal-1 [Fig. 5(C)], trypsin-digestion of DTT-modified Gal-1 [Fig. 5(D)] shows the presence of five relatively intense m/z peaks. Three of these arise from peptides 100–111, 1–18 (Cys-CAM), and 29–48 (Cys-CAM) not modified with DTT fragments. Cys-CAM refers to chemical modification of cysteine residues with the carboxyamidomethyl group as explained in the Methods section. The other two are associated with modifications (Δmass shifts of 116) at K12 and K36, that is, 1–18 (Cys-CAM) and 29–48 (Cys-CAM), respectively. Several m/z peaks of lower intensity are also identified with Δmass 116-shifted peptides having single lysines, that is, peptides 100–111 with K107-DTT, 19–36 with K28-DTT, 112–129 with K127-DTT, 29–38 with K36-DTT, and CAM-49-73 with K63-DTT. There is also one m/z peak that can be associated with a double modification in peptide 1–18 (Cys-CAM). Because peptide 1–18 contains only one lysine (K12), another possible amine group that may be modified is the N-terminal amine for reasons presented in a subsequent section. Of the eight lysines in Gal-1 (K12, K28, K36, K63, K99, K107, K127, and K129), K12 and K36 are the most modified, followed by K63, K107, K28, and K127. These results are consistent with those from NMR.

Although we can not explain the presence of all low intensity m/z peaks (possibly due to alternative modifications, contamination, or a polymorphic form of Gal-1), other low intensity m/z peaks can be associated with peptides having Δmass shifts of 80 Da [Fig. 5(D)], consistent with phosphorylation,^{18,19} as observed with ubiquitin. Western blotting confirmed the presence of phosphoserine (pSer) and phosphothreonine (pThr) in modified Gal-1 (Fig. 6). Note that in the SDS PAGE gel, bands for unmodified

Figure 5. MS fingerprinting. MALDI-TOF MS fingerprinting data are shown for trypsin-digests of unmodified and modified ubiquitin (**A** and **B**) and Gal-1 (**C** and **D**). m/z peaks have been labeled with amino acid sequence numbers for each protein, and peptides having additional mass due to chemical modifications are also labeled with the suffix **-DTT** for alkylation with a fragment of DTT, or **-PO₃** for phosphorylation, along with the modified residue. The y-axis shows counts. For unmodified ubiquitin (**A**) and Gal-1 (**C**), all counts are shown. For modified ubiquitin (**B**) and Gal-1 (**D**), counts are shown to emphasize lower intensity m/z peaks. For modified ubiquitin (**B**), counts are shown up to 10% intensity, and for modified Gal-1 (**D**), counts are shown up to 80% intensity.

and modified Gal-1 appear to be of equal intensity, whereas in the western blots, only the band for pGal-1 is significant. From MS fingerprinting data, we therefore conclude that the following residues are phosphorylated: pT57 or pS62 in peptide 49–63, pS7 in peptides 1–18 and 1–20, and pS38 in peptides 29–48 and CAM-29-48. In addition, one m/z peak is consistent with peptide 1–18 having both K12-DTT and pS7. Phosphorylation of S38 is supported by our NMR data. Note in Figure 1(A) that the S38 NH cross peak is highly shifted and in the direction expected for phosphorylation of a Ser side chain.²⁰ Significant chemical shifts are also noted at neighboring residues D37 and N39 (NH and side chain amino group). In addition, Figure 8 shows ¹³C HSQC spectra for unmodified [Fig. 8(A)] and modified [Fig. 8(B)] ¹³C-labeled Gal-1. S38 C_βH₂ resonances are also highly shifted as expected with Ser phosphorylation.^{20,21} T70 C_βH shows an additional minor broad peak in the ¹³C-¹H HSQC spectrum [Fig. 8(B)], which may be explained by phosphorylation of T70. For various reasons (e.g., low intensity, resonance overlap), support from NMR for phosphorylation of other residues is inconclusive. In any event, these NMR and MS data on Gal-1, as with our results on ubiquitin, indicate that phosphorylation is only a minor component of these ROS-mediated reactions.

Aside from ubiquitin and Gal-1, our western blots also indicate that cytochrome c and myoglobin (not shown), but not RNase or lysozyme (not shown), are phosphorylated as pSer and pThr. Figure 6 shows pThr results for cytochrome c and RNase. In these instances, the SDS PAGE gel shows equally strong bands for modified and unmodified cytochrome c and RNase, whereas the western blot indicates the presence of phosphorylation in cytochrome c, but not in RNase.

Conformational changes in modified proteins

At least at relatively low levels of chemical adduction, our NMR data on Gal-1 and ubiquitin indicate the absence of major conformational changes. This conclusion is based primarily on the absence of substantial changes in chemical shifts of resonances in modified compared to unmodified proteins. The most significant chemical shift changes are noted for backbone NH groups vis-à-vis side chain groups, suggesting that structural differences appear to primarily involve average orientations and/or dynamics of the backbone. Most methyl and aromatic side chains located within the protein core, for example, are minimally affected by chemical modification, indicating that the folded structures of native ubiquitin and Gal-1 are not greatly perturbed. This is

Figure 6. SDS PAGE gels and western blots are shown for modified proteins. The top row shows results from SDS PAGE gels, and the bottom row shows corresponding western blots probed using antibodies against either pSerine (pSer) or pThreonine (pThr), as labeled. Because these results are a composite taken from several experiments, molecular weight markers indicated (bovine serum albumin ~67 kDa, ovalbumin ~47 kDa, carbonic anhydrase ~32 kDa, lysozyme 16 kDa, ubiquitin ~8 kDa) varying in their position, which depends on conditions under which the gels were run, for example, time and electric field gradient strength. Some bands in PAGE gels are not apparent, most likely due to efficient transfer onto the nitrocellulose membrane used for western blotting. Data are shown for Gal-1 and ubiquitin (Ubi), along with those for cytochrome c (Cyto-c) and RNase. Lanes are marked for proteins that have not been incubated in the DTT reaction solution, as well as those that have (labeled pGal-1, pUbi, pCyto c, and pRNase). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

exemplified in Supporting Information Figures S1–S4. Supporting Information Figures S1–S3 show ^{13}C - ^1H HSQC data ($\text{C}\alpha$ and side chain resonances) on ubiquitin (unmodified and modified), while Supporting Information Figure S4(a) shows the methyl and Lys/Arg side chain regions from ^{13}C - ^1H HSQC spectra of unmodified (red cross peaks) and modified (blue cross peaks) Gal-1. In both ubiquitin and Gal-1, chemical modification primarily affects lysine residues and those on the surface of the protein, as opposed to those within the folding core.

Nevertheless, at later incubation time points as the proteins become more chemically modified (>tetra-adducted species), we do find that the protein fold is destabilized. This conclusion is based on overall NMR signal intensity (as well as intensity in MS data) that is decreased over time, suggesting that some molecules have lost native structure, aggregate, and/or fall out of solution. Although precipitation is not apparent, it may simply not be observed due to the low protein concentrations used. For those molecules that do remain in solution, NMR resonances become broader, suggesting significant changes in internal motions and/or a shift in conformational exchange. Although the reason(s) for loss of folded structure due to these chemical modifica-

tions is (are) not fully understood, it is likely that changing electrostatics and shifting the isoelectric point of the protein due to reduced positive charge from lysine alkylation may lead to destabilization of the protein fold, especially when such modifications become extensive.

Nature of the lysine adducts

Although western blotting and MS fingerprinting show the presence of some phosphorylated residues, these are at relatively low levels, with the major product of the DTT-mediated reaction being the lysine-modified species. Here, we provide some insight into the nature of this $\Delta\text{mass } 116$ adduct.

First of all, the $\Delta\text{mass } 116$ species is most consistent with adduction of a fragment of DTT. This is supported by elemental analysis (ICP-MS) on modified Gal-1 and ubiquitin, which indicates the presence of ^{32}S that could only have come from DTT. Nevertheless, the entire DTT molecule could not be being adducted, because the mass of DTT itself is 154 Da. The $\Delta\text{mass } 116$ Da could be explained by addition of 4 carbons, 2 oxygens, 1 sulfur, and 4 hydrogens from DTT. The use of Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid, DTNB)²² indicates the presence of free sulfhydryl groups, as in parent

Figure 7. The lysine and arginine side chain region from ^{13}C constant time HSQC spectra of unmodified (**A** and **C**) (0.2 mM protein in D_2O , 25 mM KPi (pH* 7.5), 8 mM DTT, 0.2 mM EDTA, 32.5°C) and modified (**B** and **D**) ^{13}C -labeled Gal-1 (final 0.062 mM protein concentration in same D_2O buffer at 32.5°C , after incubation 10 μM protein for 23 h in 20 mM KPi (pH 7.5), 8 mM DTT, 1 mM MgCl_2 at 37°C) are shown. Various resonances including spins U from alkylated CE lysines are labeled as discussed in the text. Correlations to proton spins Z and X are not observed under these low protein concentration conditions, because carbons in the covalently attached DTT adduct are not ^{13}C -labeled. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

DTT. Using for example, modified ubiquitin (80 μM), the DTNB reaction showed the presence of free sulfhydryls at a concentration of 200 μM , indicating an average of about three SH groups per ubiquitin molecule. This molar ratio is consistent with that derived from MS data on modified ubiquitin, where all native ubiquitin was converted into fractions of 0.15 monoadducted, 0.3 diadducted, 0.4 triadducted, and 0.15 tetra-adducted ubiquitin, with the weighted average also being about 3.

Additional information as to the nature of the DTT-adduct comes from NMR studies. A natural abundance ^{13}C - ^1H HSQC spectrum of modified ubiquitin (green cross peaks), overlaid with that of the unmodified protein (red cross peaks), is shown in Supporting Information Figure S1. While some resonances are slightly or moderately shifted, others are either new or have been highly shifted. While two new resonances are attributed to EDTA (as labeled), three groups (Z, X, U) of other cross peaks

are evident. These are more clearly delineated in ^{13}C - ^1H HSQC spectral expansions shown in Figure 9. In the constant-time ^{13}C - ^1H HSQC spectrum of modified [^{13}C , ^{15}N]-Gal-1, only cross peaks for spins U are observed, whereas those for spin systems X and Z are absent. This indicates that spins U originate from ^{13}C -labeled protein and spins Z and X do not, consistent with their arising from fragments of unlabeled DTT.

^1H correlations of spins U, X and Z are indicated in Supporting Information Figure S5. These data are consistent with group U resonances belonging to highly shifted lysine $\text{C}_\epsilon\text{H}_2$ methylenes in modified ubiquitin, which would be consistent with chemical adduction at Lys N_ϵ groups. TOCSY and 2D DIPSI-HSQC spectra of modified ubiquitin also indicate that resonances in groups X and Z are spin coupled, as labeled in Supporting Information Figure S5(A). In Figure 9 and Supporting Information Figure S1, it is apparent that group X resonances

Figure 8. ^{13}C constant-time HSQC spectra (same solution conditions as in Fig. 8) of unmodified (A) and modified (B) ^{13}C -labeled Gal-1 are shown to highlight chemical shift changes of S38 C_βH_2 (boxed in) and C_αH resonances, as well as possibly T70 (boxed in), and surrounding residues, as discussed in the text. Peaks labeled “imp.” indicate non- ^{13}C -labeled, low molecular weight impurities. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://www.interscience.wiley.com).]

occur in pairs (one ^{13}C with two ^1H resonances), indicating methylene (CH_2) groups, and Z resonances apparently arise from methines (CH) bonded to some electron-withdrawing groups, most probably hydroxyls, due to their ^1H chemical shifts being around 5 ppm. Spin-coupled X and Z fragment pairs were later assigned to specific lysines by using mono-lysine ubiquitin mutants, and then associated with Lys- $\text{C}_\alpha\text{H}_2$ methylenes (U) by using NOEs between these groups.

Supporting Information Figure S6 shows the Z spin system region from a long-range ^{13}C - ^1H HMBC (non- ^{13}C -decoupled) spectrum of modified ubiquitin. Note that strong cross-peaks are detected between protons Z and its own Z' carbon chemical shift frequency. This can only arise from long-range proton-carbon couplings when two $\text{CH}-(\text{OH})$ spin systems are covalently linked together in a symmetric-like fashion. In addition, using protonated and deuterated DTT in both H_2O and D_2O , we found that isotope shift effects in MS data indicate that the DTT adduct contains three non-labile protons and three labile protons (e.g., OH, SH, NH groups). The three nonlabile protons are explained by the presence of three C-H groups discerned from NMR studies discussed above. Gal-1 shows similar NMR results in terms of the nature of the DTT-adduct (data not shown). Although C-OH groups in DTT can become oxidized to C=O during redox reactions,^{1,2} we could not observe signals from carbonyl groups in natural abundance 1D ^{13}C spectra of modified ubiquitin or modified ^{13}C -labeled Gal-1 (data not shown). Nevertheless, formation of carbonyl groups can not be ruled out. At this point, we surmise that the DTT-

adduct includes a SH group and a minimal $-\text{CH}_2-\text{CHOH}-$ fragment, which accounts for 1 oxygen, 1 sulfur, 2 carbon and 5 hydrogen atoms that contribute 77 Da to the MS-observed Δmass of 116 Da.

Although we do not know the precise structure or structures of the DTT-derived adduct(s), three lines of evidence indicate that it is a Schiff base formed with the Lys- N_ϵ group. For one, the protein Lys-adduct can be hydrolyzed, as expected for a Schiff base.²³ We demonstrated this by exchanging modified ubiquitin multiple times against water to remove DTT and buffer components, and then allowing the modified protein to sit in water at 37°C overnight. MS analysis demonstrated that Δmass 116 adducts were no longer observed, and the primary mass observed was that of native ubiquitin, originally present.

We also performed the same exchange protocol with modified ubiquitin in water (two separate samples with H_2O and D_2O) to promote de-alkylation by incubation overnight at 37°C . The next day, these solutions were filtered to collect fractions of both protein and eluant that contained the hydrolyzed product. MS of the protein fraction reconfirmed that dealkylation of the protein was complete during this time period in either H_2O or D_2O . High accuracy ESI MS on the eluant gave new nonbackground m/z peaks at 131 (from H_2O) and at 134 (from D_2O). The m/z difference between samples exchanged in H_2O and D_2O indicates the presence of three labile H/D groups in the adduct, consistent with results discussed above.

The second piece of evidence for Schiff base formation comes from the fact that the masses in both

Figure 9. Spectral expansions of natural abundance ^{13}C - ^1H HSQC data on more extensively modified ubiquitin, followed by ultracentrifugation over 3 kDa ultracentrifugation filters and buffer exchange into D_2O buffer, are shown here to better visualize resonance dispersion for spins X, U, and Z, as discussed in the text (see also the Supporting Information Fig. S1). Cross peaks have been labeled with numbers following these letter designations to indicate spin- or dipolar coupled grouped resonances, for example, X1 and Z1 are spin coupled in the DTT fragment and dipolar coupled to lysine $\text{C}_\alpha\text{H}_2$ resonance U1, as discussed in the text. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

cases in the above experiment are 16 amu larger than it is for Lys-adducts on the protein. This indicates that one oxygen atom has been added to the adduct upon the de-alkylation hydrolysis reaction. Hydrolysis of a Schiff base occurs via addition of an oxygen atom via carbonyl formation at the carbon attached to the Schiff base nitrogen atom which then becomes protonated as it had been initially.²³ Moreover, hydrolysis of adducts is essentially pH independent from pH 4 to pH 9, as is normally observed for hydrolysis of Schiff bases.²³

The third element of proof comes from reaction of the modified protein with the cyanoborohydride-based reducing agent NaCNBH_3 in H_2O . MS following overnight incubation with this reducing agent and the modified protein resulted in Δmass shifts of 118 instead of the Δmass shift of 116. The increase in Δmass of 2 amu indicates addition of two hydrogen atoms to each adduct. Schiff bases are known to be hydrogenated by NaCNBH_3 with the addition of protons to both N and C atoms of the Schiff base, thereby replacing the $\text{N}=\text{C}$ bond with the $\text{NH}-\text{CH}$

group.²⁴ This protonated DTT adduct also can not be hydrolyzed and remains stable. In this way, we could acquire NMR data over longer time periods than would have been possible with the initial Schiff base adduct.

We then used uniformly ^{15}N -labeled ubiquitin in an attempt to directly observe resonances consistent with a Schiff base. However, although multiple peaks were present in the amide region of modified ^{15}N -ubiquitin, no amide peaks attributable to new $^{15}\text{NH}-\text{CH}$ groups could be detected, even by lowering the pH to 4.0. One reason for this may be that modified ubiquitin precipitates slowly over time, thereby decreasing these signals in HSQC spectra. Because native ubiquitin is unaffected in this regard, only residual, nonmodified lysine N_ϵ resonances could be detected, but only at pH 4 and temperatures $<17^\circ\text{C}$ at ^{15}N chemical shifts around 32 ppm ($^1\text{J}_{\text{NH}}$ of 64.4 Hz) (Supporting Information Fig. S7). The very presence of these lysine N_ϵ peaks indicates that alkylation and subsequent hydrogenation of ubiquitin was incomplete. The chemical shifts of these Lys N_ϵ resonances agree with those reported for K27, K29, K33, K48, and K63 in ubiquitin at pH 5 and 2°C .²⁵ Resonances for K6 and K11 are absent, most likely due to high solvent exchange rates.

Attempts to correlate lysine side chain protons and protons from the Schiff base group to the lysine $^{15}\text{N}_\epsilon$ group by long-range ^{15}N - ^1H HMBC experiments were also unproductive. However, experiments on [$^{15}\text{N}-\text{H}_\epsilon$, $^{13}\text{C}-\text{C}_\epsilon$]-labeled lysine show that transfer of magnetization via long-range ^1H - ^{15}N couplings to the N_ϵ of the lysine side chain is rather inefficient. It is therefore probably not practical to measure correlations to the Schiff base N_ϵ atom or its borohydride-reduced form in larger proteins. DTT-incubated and NaCNBH_3 -treated free lysine also provided no evidence for measurable adduct formation in ^{13}C -correlated NMR spectra (Supporting Information Fig. S8).

Insight into the radical chemistry of adduction

DTT redox chemistry drives the protein adduction reactions that we observe. The rate of adduction is dependent on the concentration of DTT. Using MS data like those shown in Figure 1, we estimated the initial rate of mono-adduction (Δmass 116) K48 ubiquitin mutant ($5\ \mu\text{M}$) in 20 mM potassium phosphate, pH 7.4 and 37°C . The adduction rate increases from $2.3 \times 10^{-4}\ \text{min}^{-1}$ at 0.5 mM DTT to $4 \times 10^{-4}\ \text{min}^{-1}$ at 2 mM DTT to $7.5 \times 10^{-4}\ \text{min}^{-1}$ at 4 mM DTT which is essentially the same at 8 mM DTT. It appears that there is a threshold or steady state level of ROS that must be reached, and at $10\ \mu\text{M}$ protein Lys N_ϵ substrate, it is reached at 4 mM DTT. Adduction is also somewhat dependent on the ratio of oxidized:reduced DTT. Figure 10(A) plots the fraction of unmodified ubiquitin, along with mono-, di-,

Figure 10. (A) MALDI-TOF MS data have been acquired on ubiquitin (10 μM) as a function of time, and the fraction of unmodified ubiquitin, and mono-, di-, tri-, and tetra-adducts, were derived and plotted as a function of the ratio of oxidized:reduced DTT. The greatest level of adduction is observed when the ratio of oxidized:reduced DTT is about 4:6. (B) Using data in panel A, initial rates of production of mono-, di- and tri-adducted species were estimated and plotted versus % oxidized DTT. (C) ^1H NMR data were used to following the oxidation of DTT versus time. The fraction of oxidized DTT, calculated by integrating resonances for reduced and oxidized DTT, is plotted versus reaction time in the absence and presence of 10 μM ubiquitin. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

tri-, and tetra-adducts, as a function of the ratio of oxidized:reduced DTT, which is related to the redox potential via the Nernst equation.²⁶ The greatest level of adduction is observed when the ratio of oxidized:reduced DTT is about 4:6. This is perhaps better appreciated in Figure 10(B) which plots initial rates of production of mono-, di- and tri-adducted species versus % oxidized DTT. For all species, the rate of formation increases on going from 0 to 40% oxidized DTT, and then remains essentially constant for mono- and di-adduct species and decreases considerably for the tri-adduct species.

DTT oxidation is stimulated by oxygen and results in production of superoxide.^{1,2,27,28} Because superoxide dismutase (SOD), which converts superoxide to molecular oxygen and hydrogen peroxide (H_2O_2), completely inhibits the adduction reaction, superoxide is crucial to the chemistry of adduction. Superoxide can form other ROS such as hydrogen peroxide and hydroxyl radical in the presence of

metals, especially iron and copper.^{28–36} Throughout a 24-h period, we found that the concentration of H_2O_2 varies between ~ 100 and $230 \mu\text{M}$ in our 8 mM DTT solution without protein. In the presence of 10 μM protein (ubiquitin), the concentration of H_2O_2 is significantly reduced at any given time point (reduced on average of 11 time points from 0.5 to 24 h by $\sim 25\%$), especially between 2 and 4 h (reduced by $\sim 50\text{--}70\%$) of the adduction reaction. Although we found that catalase has no apparent effect on the adduction reaction, this may not indicate anything because this enzyme is not an effective scavenger of H_2O_2 due to its high K_m value (1.1 M) for H_2O_2 and the relatively low levels of H_2O_2 produced.³⁷ Relatedly, the presence of protein also decreases the rate of DTT oxidation. Figure 10(C) shows that the fraction of oxidized DTT at any given time point is greater for DTT free in solution than it is for DTT in the presence of ubiquitin. DTT-generated ROS (including H_2O_2) that would otherwise promote DTT oxidation is being consumed by protein adduction.

ICP MS measurements demonstrate the presence of trace metals (16 nM ^{24}Mg , 3 nM ^{44}Ca , 1 nM ^{52}Cr , 13 nM ^{56}Fe , 0.4 nM ^{60}Ni , 3 nM ^{65}Cu , 4 nM ^{66}Zn) in our DTT solution. These metal concentrations are relative to 1.368×10^6 ppb ^{39}K in our 20 mM potassium phosphate reaction solution. Because addition of 50 μM EDTA inhibits the adduction reaction, we conclude that trace metals are also crucial to the chemistry of protein adduction. This in turn strongly suggests that DTT radicals are formed, because trace metals (especially Fe and Cu) are known to generate DTT radicals.^{1,2} By reduction of O_2 , the DTT radical can then generate superoxide, which can either dismute or react with H_2O_2 to form hydroxide and hydroxyl radical, and hydroxyl radical can also be produced by reaction of H_2O_2 with DTT thiyl radical, or H_2O_2 can stimulate thiol oxidation.^{1,2} Either way, it is likely that hydroxyl radicals are formed during the reaction. However, the steady state level of hydroxyl radical is likely to be relatively low, otherwise the protein would be subject to substantial oxidative damage due to the high reactivity and promiscuous nature of hydroxyl radicals.³⁸

The involvement of hydroxyl radical is supported by the presence of an apparent, albeit small, initial lag phase in the decrease of unmodified ubiquitin and increase in the monoadducted species at DTT^{ox}/DTT^{red} molar ratios of 0:1 and 2:8 [e.g., Fig. 10(A)]. These lag phases may reflect the requirement of superoxide and hydrogen peroxide to build up, prior to generation of hydroxyl radical,² as discussed above. They are also not apparent at higher DTT^{ox}/DTT^{red} molar ratios where hydroxyl radical may be more readily produced or present from the onset of the reaction. Moreover, they are not

apparent when we use “older” DTT solutions that sat on the shelf for several days (or longer) prior to use, which would likely allow for build up of superoxide and thereby ready production of hydroxyl radical. Furthermore, although the level of adduction shown in Figure 10(A) is maximal at about 12 h, the adduction reaction will continue further if the protein solution is replenished with fresh DTT solution which could then generate more ROS. If the protein becomes too highly chemically modified, however, it will eventually precipitate from solution.

Note also that a large molar excess of DTT is required to produce sufficient amounts of ROS to drive protein adduction. Supporting Information Figure S9 shows the time course of adduction to Gal-1 by focusing on the well-resolved ^1H NMR resonances of W68 H δ 1 [Fig. S9(A)] and DTT adduct group Z resonances [Fig. S9(B)], along with that for ^1H resonances from reduced and oxidized DTT [Fig. S9(C)]. Because these reaction rates are all essentially the same, DTT oxidation is directly correlated to protein adduction. Moreover, about 50% of reduced DTT must be oxidized to produce about 50% adduction to 10 μM of protein. Even considering adduction to only three groups on Gal-1 (tri-adducted species), this amounts to about a 1000-fold molar excess, consistent with the expected low level of ROS production.

So far, it appears that four radical players are involved in adduction chemistry: superoxide, H_2O_2 , hydroxyl radical, and DTT radical. Another is likely to be phosphoryl radical, because even in the presence of DTT, the reaction only occurs in the presence of monophosphate. It does not occur in potassium or sodium sulfate, TRIS-HCl, or potassium pyrophosphate. In fact, the rate of adduction is dependent on the concentration of phosphate. As the phosphate concentration is reduced from 20 to 0.5 mM, the reaction rate for formation of monoadducted ubiquitin is decreased from $8 \times 10^{-4} \text{ min}^{-1}$ to $1.8 \times 10^{-4} \text{ min}^{-1}$ (data not shown). This 40-fold reduction in concentration of potassium phosphate results in a fourfold reduction in reaction rate. The reaction also does not occur when potassium is absent, for example, in solutions of ammonium or sodium phosphate, whereas addition of KCl to either solution allows the reaction to proceed. The presence of Mg^{2+} or Mn^{2+} , which are generally known to interact with phosphate ions, increases the rate of adduction. In general, these concentration dependencies (e.g., DTT and monophosphate) suggest that the rate law for the adduction reaction is rather complicated and requires the simultaneous presence of a number of reactive agents.

Role for the protein chemical environment

Although we have established that lysine amine groups in well folded proteins can become alkylated

via DTT/ROS-mediated chemistry, the same reaction does not occur with primary amine groups in small organic molecules, for example, butylamine, benzylamine, ethylenediamine, diethylamine, and lysine itself. However, we do observe adduction (Δmass 116) to primary amines in small peptides: Glucagon-like peptide -1 (GLP-1)³⁹ (HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR); NF6 (MAGPHPVIVITGSHEE) (unpublished peptide); NF7 (MAGPHPVIVITGPHQE) (unpublished peptide); SC7 (KIIVKLNDGREL) and SC8 (VKLNDGRELSLD).^{40–42} MALDI-TOF MS data for these peptides incubated in the DTT reactive solution are shown for two time points (3 and 24 h) in Supporting Information Figure S10. GLP-1 has two lysines, and yet at the 24-h incubation time point, we observe only $\sim 20\%$ mono-adducted and no di-adducted species. For NF6 and NF7, we observe mono-adduction in both peptides at the level of $\sim 45\text{--}30\%$, respectively, at 24 h. Because NF6 and NF7 have no lysines, these data suggest that adduction occurs at the N-terminal primary amine groups. This assumes, of course, that ROS-mediated alkylation occurs only at primary amine groups. With SC-7 (two lysines) and SC-8 (one lysine) at 24 h, mono- and di-adduction occur at $\sim 60\text{--}70\%$ and $\sim 20\%$, respectively, in both peptides. Once again, because SC-8 has only one lysine residue, the N-terminal amine may be an additional site for ROS-mediated alkylation, as proposed with NF6 and NF7.

NMR evidence on alkylated SC-8 supports these findings. TOCSY spectra acquired pre- and post-alkylation show selective conversion from a native K2 H_α signal at 3.0 pm to a new H_α resonance at 2.53 ppm [Supporting Information Fig. S11(A)], as observed with larger proteins Gal-1 and ubiquitin. A final treatment of alkylated SC-8 with NaCNBH_3 led to the appearance of additional H_β and methyl peaks for V1 and four additional amide peaks for K2. Because K2 is adjacent to the primary amine of the N-terminus, this would explain the observation of four extra signals in a di-adducted peptide [Fig. S11(B)].

Because not all lysine N_ϵ groups in well-folded proteins (ubiquitin, Gal-1, cytochrome c, RNase, myoglobin, lysozyme) and these smaller peptides are equally reactive (and some are not at all modified), there appears to be some environmental signature around reactive amines in peptides and proteins that promote DTT/ROS-mediated adduction. For example, while the fraction of unmodified ubiquitin decreases over time, that of monoadducted protein increases rapidly and is followed less rapidly by the appearance of diadducted species [Fig. 10(A)]. However, the fractions of tri- and tetra-adducted species increase only slightly over the same time period. Even though each of these species (mono, di, tri, tetra) could be a composite of all lysines being

Figure 11. The fraction of mono-adduct derived from MALDI-TOF MS data is plotted versus time of incubation for all mono-lysine ubiquitin mutants, along with peptide SC8. The apparent rate of adduction for each protein/peptide was determined simply by taking the linear slope of these plots.

adducted, analysis of NMR and MS fingerprinting data indicates that not all lysine sites are equally adducted. In this regard, it appears that only one or two lysines react rapidly (possibly the mono-adducted species), another two react slowly (possibly the di- and tri-adducted species), and three apparently do not react to any significant extent, at least within the first 24 h of reaction. If they all reacted equally rapidly over this time period, then higher order species would have been produced much more rapidly, and MS results would have shown only small fractions of mono- and di-adducted species at the early time points, as they would have quickly been converted into higher order species. In Figure 2, it is apparent that at the 10-h time point Gal-1 and ubiquitin both show the presence of mostly mono- and diadducted species. Cytochrome c, which has up to hexa-adducted species at the same time point [Fig. 2(C)], is perhaps an exception, most likely because this protein contains 18 lysines.

These findings stand in contrast to reactivities of lysines in ubiquitin mutants in which all but one lysine are substituted with arginines. In these mutants, adduction rates are nearly the same for all lysines, as shown in Figure 11. Mutants K11 ($3 \times 10^{-4} \text{ min}^{-1}$) and K6 ($2.8 \times 10^{-4} \text{ min}^{-1}$) show the fastest rates of adduction, followed by K33 ($2.7 \times 10^{-4} \text{ min}^{-1}$), K48 ($2.5 \times 10^{-4} \text{ min}^{-1}$), K63 ($2.1 \times 10^{-4} \text{ min}^{-1}$), and finally K27 ($1.5 \times 10^{-4} \text{ min}^{-1}$) which is the same as that for K29. If these rates held for the same residues in native ubiquitin, then all lysines would be nearly similarly adducted, and they are not. The greater selectivity for lysines in native ubiquitin vis-à-vis any of the lysine mutants can not simply be due to electrostatic differences, because lysines were substituted for arginines which at pH 7.4 should also be positively charged. There-

fore, the presence of multiple positively charged lysines (amine groups) likely plays a role in these ROS-mediated adductions. In addition, the rate of mono-adduction for native ubiquitin ($\sim 15 \times 10^{-4} \text{ min}^{-1}$) is about sixfold faster than the average rate for lysine mutants ($2.3 \times 10^{-4} \text{ min}^{-1}$). If anything, the rate of adduction should have been slower for lysines in native ubiquitin, because more ROS would have been available for the single lysine in each mutant vis-à-vis the seven lysine in native ubiquitin. Note also that the rate of lysine adduction in peptide SC8 ($6.3 \times 10^{-4} \text{ min}^{-1}$) is about twofold faster than that for the ubiquitin K11 mutant, even though reactant concentrations are essentially the same, even in terms of lysine equivalents. Because lysine in the small, unfolded peptide SC8 should be more solvent exposed than a lysine in ubiquitin, steric hinderance and/or limited reactive agent accessibility should play a role at lysine sites in folded proteins, while the greater monoadduction rate in native ubiquitin vis-à-vis any monolysine ubiquitin mutant supports a role for the presence of multiple lysines vis-à-vis arginines.

Discussion

Here, we report on DTT-mediated chemical adduction to proteins, which occurs in aqueous solutions of potassium phosphate (pH $> \sim 7$) with trace amounts of iron and copper. We found that while β -hydroxyl groups of serine and threonine residues can be phosphorylated, the majority of modifications made to the proteins studied are additions of DTT-fragments to Lys N_ϵ groups. Even though the exact structure of the DTT adduct remains unknown, we do know that it is composed of a $\text{CHOH}-\text{CH}_2-$ fragment, a SH group, and a third labile proton, most likely a second OH group. Moreover, we found that DTT fragments are covalently attached to lysine- N_ϵ groups via Schiff bases. Formation of Schiff bases at protein lysine N_ϵ groups is not unique. Lehwess-Litzmann *et al.*⁴³ recently reported the X-ray structure of a transaldolase in which a Schiff base intermediate is observed at lysine K86. Our results from western blotting and MS fingerprinting also indicate that at least some serine and threonine residues, as well as possibly other oxygen-containing side chains, become phosphorylated during the DTT-mediated reaction.

The chemistry of adduction occurs via oxidation of DTT that generates ROS which in turn drives the observed alkylation and phosphorylation reactions. Superoxide and hydroxyl radical are two of the major players, along most likely with phosphoryl radicals. In addition, because lysines are the residues most chemically modified, they should be at the heart of the reaction sites. Positively charged lysine residues would provide attractive sites for electrostatic interactions with negatively charged

phosphate groups and phosphoryl radicals. Our implication for the involvement of phosphoryl radicals emanates from the fact that the presence of monophosphate is required for these DTT-mediated reactions. Furthermore, positively charged arginines, which are themselves not apparently modified, can not substitute for lysines, as evidenced by results with per-methylated lysine and monolysine ubiquitin mutants. While positively charged Lys and Arg residues can both interact with and electrostatically stabilize phosphate groups, they exhibit different chemical properties and therefore behave quite differently towards phosphate groups. In general, a phosphate group will bind more strongly to Lys than to Arg, primarily because the stronger the base (Arg $pK_a \sim 12$ vis-à-vis Lys $pK_a \sim 10$) is, the weaker will be its interaction as a hydrogen-bond donor to an acceptor. Another difference derives from the guanidine group spreading charge over a larger volume, whereas lysine concentrates it at its $N\epsilon H_3^+$ group in a way that is particularly well suited to interact with phosphate oxygen atoms. This has been experimentally observed in various enzyme systems. For example, Goedl and Nidetzky⁴⁴ reported that while Arg507 and Lys512 are both intimately involved in α, α -trehalose phosphorlyase-mediated conversion of α, α -trehalose to α -D-glucose 1-phosphate and α -D-glucose, Lys512 is primarily required for phosphate ion binding, while Arg507 promotes catalysis through stabilization of the transition state. Martinez-Liarte *et al.*⁴⁵ also reported that inorganic phosphate binding at the active center of aspartate aminotransferase is mediated primarily by electrostatic interactions with a lysine (Lys258), and that this interaction helps stabilize the phosphate ion in its dianionic state.

It also appears that all requisite components of the adduction reaction should react simultaneously in a concerted fashion, most likely within a small volume element around protein reactive sites undergoing adduction. Two observations in particular support this proposal. First of all, these radicals are generally short lived⁴⁶ and diffusing in from solution would make for an inefficient radical-mediated reaction, and second, since DTT fragments and phosphate groups become adducted, they both must interact at some stage of the reaction with residues on the protein. Adduction reactions also appear to be enhanced by the presence of multiple lysines in the protein. With native ubiquitin that has seven lysines, the rate for DTT-fragment monoadduction ($\sim 9 \times 10^{-4} \text{ min}^{-1}$) is the greater than that for any monolysine ubiquitin mutant (range of $1.7 \times 10^{-4} \text{ min}^{-1}$ to $2.9 \times 10^{-4} \text{ min}^{-1}$). This is the case even though the net positive charge for any monolysine ubiquitin variant (all but one Lys substituted with Arg) is the same as that for native ubiquitin. Aside from supporting the idea that arginine can not sub-

stitute for lysine, it appears that maintaining the same net electrostatic character alone is not sufficient to achieve the same reaction rate, and underscores the importance of lysine groups in these adduction reactions.

However, even with the presence of multiple lysines, not all of them are equally reactive. For example, only two of the eight lysines in Gal-1 (K12 and K36) are highly reactive, followed by K28, K63, K107, and K127, with K99 and K129 showing no apparent reactivity. In ubiquitin, K33, K48, and K63 are the most reactive lysines, followed by K6 and K29. K11 and K27, which do not appear to be reactive in native ubiquitin, are however nearly equally reactive in monolysine ubiquitin variants. In this regard, the concentration of reaction components plays a role, because in both instances the amount of for example, ROS generated by DTT is the same, whereas as the concentration of lysine residues is quite different. Reactivity at a given lysine in native ubiquitin is driven in part by competition for reactive species like ROS. There are a number of possibilities that could explain this. For example, a particular lysine may not be readily accessible to reactive species, or it may be involved in a salt bridge and therefore not as reactive, or there may be competition between/among proximal lysines. Greater reactivity at specific lysines may also be attributed to spatial orientations and dense patches of positive charge from primary amines, as well as to the nature of other surrounding residues.

Although the exact structural signature and its role in DTT-generated ROS reactivity are not known, we can gain some insight by analyzing the structures of ubiquitin and Gal-1. Figures 12 and 13 highlight lysine residues in Gal-1 (PDB 1gzv) and ubiquitin (PDB 1ubq), respectively. In Gal-1 for example, the most reactive lysines, K12 and K36, are proximal to each other, as well as to K127, K129, and the N-terminal amine group, and on the other side of K127 lies K28. In ubiquitin, K33, K48, and K63 are the most reactive, and all are oriented into solution and proximal to other primary amine groups. For example, K33 is proximal to K11, K27, and K29, and three of these are part of 3-turn helix 7 α ;1. K33 is also proximal to E16, D32 and E34, and K48 is proximal to E50. Although of no apparent direct relationship to the present study, K48 is a key lysine residue involved in ubiquitination.^{47,48} K63 is also proximal to many polar/charged residues, that is M1-NH₃⁺, N60, Q62, and E64, as well as to S57, S65, and T66. Reactive K6 is also oriented out into solution, and lies in the middle of three-strands (β 1, β 2, and β 5) of the five-stranded anti-parallel β -sheet with proximity to phosphorylation sites at S65 and T66. Relatively nonreactive K27 is partially internalized and therefore surrounded by many hydrophobic residues. In general,

Figure 12. X-ray crystal structure of Gal-1 (PDB access code 1gzv) in (A) with the dimer oriented in a front view, and (B) with the dimer seen from the top. Lysine residues and the N-terminal amino group of A1 are highlighted and labeled in both protein monomers. Green surface coloring display perturbed backbone amides in modified Gal-1. The blue arrows indicate the binding position of the natural ligand lactose that has removed from the view. Cysteine residues are colored yellow. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the more reactive lysines tend to be part of a group of lysines, among other polar/charge residues, whereas the less reactive lysines tend to be more isolated, potentially involved in salt bridges, and/or less solvent exposed.

Mention has been made of proximity of reactive lysines to various polar and/or charged groups, primarily because these other groups can be phosphorylated during DTT-generated ROS-mediated reactions. In Gal-1, we have identified pS7, pS38, pT57, pS62, and pT70. In ubiquitin, we have pS65 and pT66. Other hydroxylated residues are also possibly phosphorylated. In addition, some peptides in MS fingerprinting suggest that for example, carboxylated residues may be phosphorylated, for example, pD37, pE114, pD122, and/or pD124 as possibilities in Gal-1, and pD32, pE34, D39, pE50, and/or pD51 as possibilities in ubiquitin. Moreover, phosphorylation, like DTT alkylation, is not unique to Gal-1 and ubiquitin, as we have shown evidence for at least cytochrome c also being phosphorylated. Given the likelihood for the involvement of phosphoryl radicals and the promiscuous nature of ROS, reactivity at lysine residues could easily “spill over” to adjacent chemical groups at primary reaction site(s). The relatively low populations of phosphorylated residues, as estimated from MS fingerprinting data, support the idea that phosphorylation is not the primary reaction for these ROS-mediated reactions. However, proximity and ROS promiscuity may explain why some residues do become phosphorylated.

Our present working model is that ROS and DTT radicals are attracted to specific lysines due to their positive charge character and the overall chemical nature of their environment. These lysines appear to be those that are surrounded by other polar and charged

Figure 13. X-ray crystal structures ubiquitin (PDB access code 1ubq). Lysine residues are highlighted and labeled. Perturbed backbone and side chain atoms of nonlysine residues in the modified ubiquitin are shown by green surface coloring. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

groups and perhaps not involved in salt bridges. During the radical chemistry, the most reactive lysines become adducted as Schiff base-linked DTT-fragments. Some serine and threonine residues, as a consequence of their proximity to reactive lysines, are themselves phosphorylated, most likely as side reactions by the promiscuous nature of the highly reactive phosphoryl radical. It remains unknown what if any the role of phosphate or phosphoryl radical is when it comes to DTT-fragment adduction at lysine $N\epsilon H_3^+$ groups. Interestingly, the X-ray structure of a transaldolase reaction intermediate⁴³ shows similarities with our DTT-mediated reaction site. In this instance, a Schiff base is also formed at a lysine (K86) $N\epsilon$ group in the transaldolase active site. Moreover, the amino acid composition of the transaldolase active site is similar to our reaction sites. Aside from the crucial K86, the transaldolase active site contains several polar residues (Asn, Gln, Ser, and Thr) and charged groups (Arg, Asp and Glu), and bound substrate (e.g., fructose-6-phosphate) is coordinated via its hydroxyl and phosphate groups to form an extended hydrogen-bonding network comprising N108, T110, E60, N28, D6, R136, S167, and R169. In addition, the transaldolase active site and related fructose-6-phosphate aldolase contain conserved phenylalanine (F132) and tyrosine residues that appear to be critical reaction determinants. We also note that aromatic groups are also positioned near reactive lysines in ubiquitin and Gal-1. Given these compositional and Schiff base similarities, it may be that the reaction chemistries could also be similar.

Alkylation and phosphorylation reactions that we have discussed here are unique to chemistry mediated by reactions involving DTT-generated ROS. However, although these same reactions could not occur in cells due to the absence of DTT, it is conceivable that similar ROS-mediated chemistry could occur in cells. All of the requisite components, aside from DTT, are present in the reducing environment in cells. Oxygen is of course present, and in normal cells, ROS is constantly being produced at some level, usually more so in highly metabolic cells and in cells involved in pathological disorders like cancer. Intracellular inorganic phosphate is present at concentrations in the ~ 50 mM range,⁴⁹ and the intracellular concentration of K^+ is on the order of ~ 100 mM.⁵⁰ Although Fe is usually tightly bound to different proteins as cofactors or for storage (e.g., ferritin), ~ 0.2 – 3% of cellular iron is normally “iron in transit”, that is the labile iron pool.⁵¹ ROS-mediated damage to proteins and DNA in cells is well known.^{8–10,52–55} In any event, our results presented here highlight the promiscuous nature of ROS-mediated chemical adduction.

Conclusions

Because DTT-mediated chemical modifications (DTT-fragment adduction and phosphorylation) that we

report here occur to several proteins (ubiquitin, Gal-1, cytochrome c, RNase, myoglobin, lysozyme), it is likely that they occur to most, if not all, proteins. To date, 13,165 papers have been published (PubMed search) in which DTT has been used as a reducing agent. In this regard, our results should stand as a cautionary note to investigators who use DTT in their studies.

Materials and Methods

Proteins and supplies

Uniformly [¹⁵N]-labeled Gal-1 was produced as described by Nesmelova *et al.*⁵⁶ Uniformly [¹⁵N]-labeled ubiquitin was graciously provided by the Kyle Walters lab and prepared as described previously.⁵⁷ Other proteins used here were purchased from Sigma and prepared without further purification. Protein samples were made up in 20 mM potassium phosphate (containing 8 mM DTT) at pH 7.4, or as otherwise described in the text. Chemicals used in this study were commercially available analytical grade and were used as purchased without further purification. KH_2PO_4 and K_2HPO_4 were purchased from Mallinckrodt Baker; DTT was purchased from Roche Diagnostics GmbH; Ubiquitin mutants were purchased from Biomol International, LP. All other chemicals used were procured from Sigma-Aldrich. Water and acetonitrile used in these experiments were of HPLC grade and were obtained from Fisher Scientific.

NMR spectroscopy

Sample preparation. Ubiquitin and Gal-1 were prepared in 20 or 25 mM potassium phosphate (KPi) buffer with 99.9% D_2O or 10% D_2O (v/v) in 5 mm Wilmad tubes or 250 μ L Shigemi microcells. Reference samples for Gal-1 were prepared in KPi with 8 mM dithiotreitol (DTT) and 0.1–0.2 mM EDTA to sequester traces of metal ions. For real-time NMR experiments with DTT-incubated Gal-1, EDTA was first removed via multiple cycles of ultracentrifugation (Millipore 4 mL 3 kDa cutoff) and then by diluting the protein stock solution to 10 μ M protein into old ($>50\%$ oxidized) 8 mM DTT, 25 mM KPi buffer (pH 7.5), and followed by incubation of the solution for ~ 24 h at 37°C. To increase sensitivity in some NMR experiments, 50 mL solutions of 10 μ M protein were incubated with DTT overnight at 37°C, and then rapidly concentrated via ultracentrifugation to 0.4 mM in fresh KPi/DTT buffer containing 0.1 mM EDTA. The resulting concentrated Gal-1 protein solution showed no significant NMR spectral changes vis-à-vis the spectrum acquired with 10 μ M Gal-1. With [¹³C, ¹⁵N]-labeled Gal-1, the final NMR concentration was 62 μ M for concentrated, modified protein.

Similar protocols were used to prepare samples with ubiquitin, except that no DTT was used during the concentration procedure and buffer exchange

steps with modified protein. NMR samples of unlabeled and ^{15}N -labeled modified ubiquitin had protein concentrations of 4 and 0.97 mM, respectively.

For alkylation and hydrogenation of ^{15}N -labeled ubiquitin, half of the solution was incubated at 8 μM protein in 8 mM DTT (KPi, pH 7.4) at 37°C for 24 h, whereas the other half was incubated in an identical fashion without DTT. After rapid removal of DTT by ultracentrifugation (Amicon 3 kDa), the alkylated and reference ubiquitin samples were concentrated to about 2.7 mL and reacted overnight with excess NaCNBH_3 at 30 μM protein in 25 mM KPi buffer (pH 6.0) at room temperature. A final buffer exchange was used to remove the borohydride salts, and the protein solution was concentrated to 0.97 in 25 mM KPi (pH 7.42) for NMR analysis. Adjustment of the pH to 4.0 (meter) was done by adding small aliquots of diluted HCl solution.

For NMR experiments with peptide SC-8, a stock solution of 7 mM SC-8 was made in water (10% D_2O) at pH 3.7 (meter).^{40–42} Incubations were carried out with 8 mM DTT (deuterated) in KPi buffer (pH 7.5) for a period of 24 h at 37°C at both 20 and 300 μM peptide concentrations. After incubation the pH was adjusted to pH 2.8, 3.2, and/or 3.7 to detect additional resonances in the 2D DIPSI spectra. DTT was deuterated to avoid large proton signals from DTT and derived fragmentation products that otherwise interfere with modified lysine resonances resonating in the region between 2 and 4 ppm. Hydrogenation of alkylated SC-8 using NaCNBH_3 was done at a peptide concentration of 20 μM , followed by desalting of the peptide solution over a short Sephadex column to remove excess borate salts. Addition of 10% D_2O and adjustment of the pH to 3.2 yielded the final NMR sample.

NMR experiments. NMR experiments were carried out on Bruker Avance III 750, 600, 700, or 900 MHz spectrometers, the last three spectrometers being equipped with cryogenic triple resonance probes. All spectrometers had xyz pulse field gradient capabilities. Standard temperature was set to 30°C, unless stated otherwise. Chemical shifts were internally referenced to DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid). Temperature calibration was done externally by using a thin thermocouple (T2) hanging in a water-filled NMR tube inserted into the NMR probe or by making use of a Bruker ethyleneglycol-DMSO- d_6 (80%/20%) temperature calibration sample (http://scs.illinois.edu/nmr/handouts/general_pdf/NMRTempCal-ColoradoBoulderRichShoemaker.xls). The 1D ^{13}C spectra were recorded using inverse-gated proton Waltz-16 decoupling. Residual water in 1D spectra was suppressed by using gradients and excitation sculpting.⁵⁸ Typically, 1024 to 2048 scans per spectrum were recorded for the 10 μM real-time oxidation series to achieve suffi-

cient signal-to noise ratio. Gradient versions of 2D DIPSI, 2D NOESY, 2D ^{15}N - ^1H HSQC, (constant-time) ^{13}C - ^1H HSQC, 2D DIPSI- ^{13}C -HSQC, and ^{13}C - ^1H HMBC pulse sequences were employed from the standard Bruker pulse sequence library. For HSQC spectra, 2048 (^1H) $\times$ 280 (^{15}N) and 2048 (^1H) $\times$ 640 (^{13}C) data points were typically collected over spectral widths of 36 (^{15}N) and 72 ppm (aliphatic region ^{13}C). For ^{15}N - ^1H HSQC/HMBC spectra of alkylated and/or hydrogenated ^{15}N -ubiquitin, a full spectral width in the ^{15}N dimension (20–200 ppm) was used to detect Arg/Lys side chain nitrogens. The ^{15}N - ^1H HMBC experiment was run at 7 and 17°C and is a modified version of a low-pass filtered, non-decoupled experiment in which a pre-saturation step was added to further suppress the water signal.

Proton resonances of the reference 7 mM SC-8 peptide sample at pH 3.7 were sequentially assigned via 2D NOESY (mixing time 400 ms) and 2D DIPSI (mixing time 85 ms) at 850 MHz. Natural abundance ^{15}N - ^1H HSQC and ^{13}C - ^1H HSQC were subsequently used to assign the ^{15}N amide and aliphatic ^{13}C resonances of SC-8.

Processing of NMR spectra was performed using Bruker Topspin 2.1 (Rheinstetten, Germany), and Sparky software⁵⁹ was used to analyze 2D spectra. Peak integration of 1D spectra was carried out using Topspin. ^1H and ^{15}N resonance assignments for recombinant Gal-1 have already been reported.⁵⁶ Resonance assignments for ubiquitin were kindly provided by Ad Bax and VLI Research (<http://www.vli-research.com/ubshifts.htm>). Peak positions were reassigned at pH 7.3 and 32.5°C using a combination of 2D TOCSY, 2D NOESY, and natural abundance ^{15}N - ^1H HSQC, ^{13}C - ^1H HSQC and ^{13}C - ^1H HMBC heteronuclear correlation experiments. The complete list of ubiquitin assignments at physiological pH is available on request.

Mass spectrometry

MALDI-TOF. Protein and peptide samples were desalted with C-4 and C-18 Zip-Tips (Millipore), respectively, following the standard procedure. The 3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid (SA) matrix was used for MS of proteins, whereas α -cyano-4-hydroxycinnamic acid (CHCA) matrix was used for MS of peptides. The eluted protein/peptide samples were mixed (1:1 v/v) with matrix in 50% acetonitrile containing 0.1% TFA. Sample-matrix solution ($\sim 1 \mu\text{L}$) was spotted on the stainless steel MALDI target plate and allowed to dry at room temperature. Analysis of protein and peptide samples was performed on a Biflex III, Bruker Daltonics MALDI-TOF instrument over an m/z range of 5000–22,000 in reflectron mode for protein and m/z range of 500–4000 in linear mode for peptides using a pulser frequency of 4.993 kHz. Ionization was

induced by 337-nm nitrogen gas laser source with 8 μ J of energy and a repetition rate of 20 Hz. Each spectrum recorded was an average of 200–300 laser shots. The instrument was calibrated with commercially available standard protein Myoglobin *m/z* 16952.18 for protein analysis and with commercially available peptides angiotensinII *m/z*, 1046.54 and ACTH *m/z* 2465.19 for trypsin digest analysis.

For MS fingerprinting, proteins (Gal-1 and Ubiquitin) were digested with trypsin in ammonium bicarbonate solution (pH $\sim$ 8.0) using a protein:enzyme ratio of 30:1 (wt/wt) at room temperature employing sequencing grade trypsin from Promega. Free sulfhydryls in Gal-1 were alkylated with iodoacetamide to the stable S-carboxyamidomethylcysteine (CAM) adduct. Following incubation for $\sim$ 6 h, digestion was terminated by adding 10% TFA solution and MALDI analysis of the digested samples was performed.

ESI. Protein or peptide samples were first desalted employing spin column MWCO 3000 just before ESI experiment. ESI-MS spectra were recorded on QSTARXL system (AB Sciex) equipped with hybrid quadrupole TOF analyzer. Samples were dissolved in 50% acetonitrile containing 0.1% formic acid at a concentration of $\sim$ 10 μ M. The sample was injected into the ESI ion source at a flow rate of 1 μ L min $^{-1}$. Scanning was performed in positive ion mode from *m/z* 700–2200 using a pulser frequency of 4.993 kHz and pulse duration of 18 μ s. Calibration was performed using Rennin 586.9830 *m/z* $^{+3}$ and 879.9705 *m/z* $^{+2}$.

ICP MS. Thermo Scientific iCAP 6500 dual view ICP-OES (Inductively Coupled Plasma – Optical Emission Spectrometry) was used with the following parameters: power 1150 W, Neb flow 0.65 L min $^{-1}$ (PFA microconcentric nebulizer, 300 μ L), cooling gas 12 L min $^{-1}$, Aux gas 0.3 L min $^{-1}$, and integration of five replicates/sample acquired at 10 sec/replicate. Samples were diluted 20-fold prior to analysis with the addition of a Cesium matrix modifier and Yttrium as an internal standard. The buffer solution analyzed contained 20 mM potassium phosphate; the ppb of 39 K was then used to establish concentrations of trace metals as discussed in the text.

Western blotting

SDS PAGE gels and western blotting were run with several modified and unmodified proteins as discussed in the text, along with molecular weight markers bovine serum albumin $\sim$ 67 kDa, ovalbumin $\sim$ 47 kDa, carbonic anhydrase $\sim$ 32 kDa, lysozyme 16 kDa, and ubiquitin $\sim$ 8 kDa. Commercial standard SDS PAGE gels were loaded with $\sim$ 5 μ g protein in each lane, and run at an appropriate voltage until the gel front dye passed through the gel matrix.

Western electroblotting was performed using nitrocellulose membranes for the transfer step from PAGE gels and appropriate antiphospho-residue antibodies were used to probe for and identify phosphorylated proteins via chemo-luminescence, all according to manufacturer's instructions (Invitrogen). Bovine serum albumin was used to block for non-specific binding of antibodies. Western blots were processed using a BenchPro 4100 automated western blot processing system, and digital images of western blots are shown here. Some bands in PAGE gels are not apparent, most likely due to efficient transfer onto the membrane for western blotting.

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