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Homocystamides promote free-radical and oxidative damage to proteins

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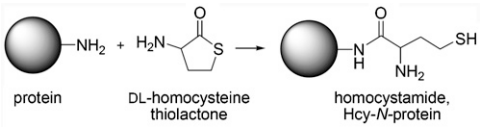
Elevated levels of homocysteine are associated with several major diseases. However, it is not clear whether homocysteine is a marker or a causative agent. The majority (*ca.* 80%) of the homocysteine present in humans is protein bound. The study of the posttranslational modification of proteins by homocysteine and its cyclic congener, homocysteine thiolactone, is emerging as an area of great current interest for unraveling the ongoing "mediator/marker controversy" [Jacobsen DW (2009) *Clin Chem* 55:1–2]. Interestingly, many of the pathologies associated with homocysteine are also linked to oxidative stress. In the current study, chemical evidence for a causal relationship between homocysteine-bound proteins and oxidative damage is presented. For example, a reproducible increase in protein carbonyl functionality occurs as a consequence of the reaction of human serum albumin with homocysteine thiolactone. This occurs at physiological temperature upon exposure to air without any added oxidants or free-radical initiators. Alpha-amino acid carbon-centered radicals, well-known precursors of protein carbonyls, are shown to form via a hydrogen atom transfer process involving thiolactone-derived homocystamides. Model peptides in buffer as well as native proteins in human blood plasma additionally exhibit properties in keeping with the homocystamide-facilitated hydrogen atom transfer and resultant carbon-centered radicals.

homocysteine | oxidation | thiyl radicals

Homocysteine (Hcy) is an accepted independent risk factor for several major pathologies including cardiovascular disease, birth defects, osteoporosis, Alzheimer's disease, and renal failure (1). Oxidative stress is also linked to declines in pulmonary, brain, circulatory, and reproductive function. It has been hypothesized that Hcy promotes oxidative stress via reactive oxygen species (ROS) generation upon disulfide bond formation. However, cysteine (Cys) also undergoes disulfide- and ROS-forming reactions, and is more abundant than Hcy, but is not generally associated with disease (2). There is thus interest in elucidating the fundamental chemistry of Hcy that is distinct from that of Cys, glutathione (GSH), and the other biological thiols. Moreover, it has been noted that evidence of Hcy-induced protein oxidation would serve as a significant alternative to measuring its ROS generation and would link Hcy to oxidative stress-related disorders (3). Extensive clinical trials, spanning decades and tens of thousands of patients, have not yet established a definitive relationship between Hcy lowering using vitamin therapy and reduced cardiovascular disease risk. This has resulted in renewed interest in examining alternative relationships between Hcy and redox status (4).

There are three main distinctive features of Hcy chemistry. First, the additional carbon in the Hcy side chain allows it to exist as a stable five-membered ring thiolactone [homocysteine thiolactone (HTL)] which readily reacts with the ε-amine of Lys residues to form isopeptide bonds (Hcy-*N*-protein, Scheme 1) as demonstrated by the extensive studies of Jakubowski (3). Second, evidence that homocysteine easily forms disulfides with free thiol groups in proteins was originally reported by Refsum et al. (5) and further studied by others (6, 7). This was later confirmed and further studied extensively by the Jacobsen group (1), who showed that the higher pK_a (10.0) of the Hcy sulfhydryl as compared to that of Cys leads to the formation of thermodynamically stable mixed disulfide bonds to protein Cys residues (Hcy-*S*-protein) (1). Third, the Hcy thiyl radical can undergo a kinetically favored hydrogen atom transfer (HAT) reaction to afford an alpha-amino carbon-centered radical (Scheme 2). This latter property of Hcy was first proposed by Zhao et al. (8) and subsequently shown in our laboratory to occur under neutral physiological conditions and in human blood plasma (9–11).



Scheme 1. The reaction of HTL with the ε-amine moiety of protein Lys residues results in a posttranslational modification known as Hcy-*N*-protein (7, 13).

Alpha-amino carbon radical formation in Hcy (Scheme 2) occurs under the same conditions that promote thiyl radical-mediated disulfide formation (8–11). This is due to the thiyl radical's participation in the HAT mechanism. Importantly, HAT is kinetically favored for Hcy compared to all other biological thiols due to the five-membered ring transition state shown in Scheme 2. The $^{\alpha}$C-H bond of Hcy is weaker than its S-H bond when the amino group is uncharged (8). Moreover, we have shown, in collaboration with Crowe and coworkers, that the HAT process for Hcy still proceeds effectively in mildly acidic media (12).

This unique property of Hcy has allowed its highly selective detection in neutral buffer and in human blood plasma using mildly oxidizing chromogens such as methyl viologen (11–13) ($E_{red} = -0.448$ V) (14). This is because the $^{\alpha}$C amino acid radical is a relatively potent reducing agent whereas the thiyl radical is oxidizing (9). Alpha-amino carbon radical reduction potentials have been reported as E° $(NH_2 = CHR^+/NH_2CHR^{\cdot}) = -1.9$ V vs. normal hydrogen electrode (15).

Because homocystamide thiyl radicals should also be predisposed to a kinetically favored HAT five-membered ring transition state, we propose that homocystamides can serve as sites promoting free-radical-induced changes to protein structure via $^{\alpha}$C amino acid radical formation as depicted in Scheme 3. Herein, evidence is presented for a mechanism of enhanced protein carbon radical formation and oxidation consistent with Hcy's unique chemical properties.

Author contributions: R.M.S. designed research; M.S.-V., J.O.E., S.L., and G.K.S. performed research; M.S.-V. and J.O.E. analyzed data; and M.S.-V. and R.M.S. wrote the paper.

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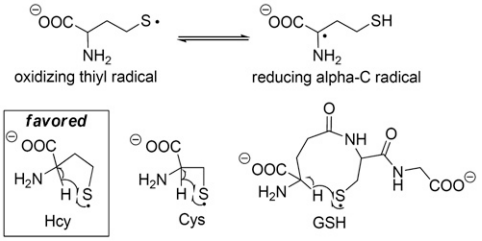


Scheme 2. Hcy thiyl radical undergoes a kinetically favored intramolecular HAT to form a reducing alpha-amino carbon radical. Captodative stabilization of the radical renders the alpha C-H bond weaker than the S-H bond by *ca.* 4 Kcal/mol. The rate constant for the kinetically favored sulfur-to-carbon-H atom transfer for Hcy is $>10^5$ s^{-1} [at pH 10.5 (8)].

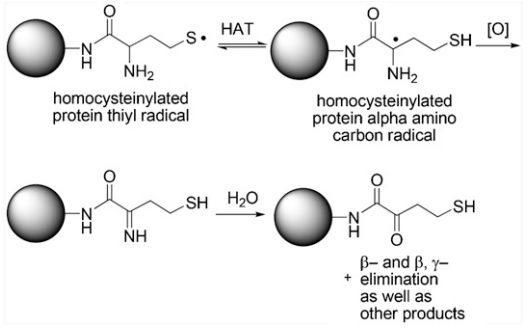
Results

Homocystamides Promote $^{\alpha}$C Radical Formation in Human Serum Albumin and Directly in Proteins in Human Blood Plasma. To investigate the effect of protein oxidation due to homocystamide incorporation, human serum albumin (HSA) was modified with an average of 7–8 D,L-homocystamides/mol via reaction with homocysteine thiolactone, following the standard procedures developed by Jakubowski (7) (*SI Appendix*). Unbound low molecular weight thiols were removed by gel filtration after disulfide reduction.

Methyl viologen (as mentioned earlier) has previously been used as a probe to detect $^{\alpha}$C radicals during pulse radiolysis (8, 17) and to detect Hcy with outstanding selectivity due to the kinetically favored $^{\alpha}$C radical formation (9–11). It was thus employed in the current work to help determine that HSA-bound homocystamides promote $^{\alpha}$C protein radical formation.

Blue solution color formation due to viologen radical appearance indeed occurs in the presence of homocystamide-enriched HSA (15 mg/mL, 2 mM) after 2 min heating at reflux (Fig. 1). These are conditions similar to those used for detecting carbon radicals derived from the unbound Hcy molecule (9–11). The control solution containing (disulfide) reduced, unmodified albumin exhibits blue color only after prolonged heating at reflux (≥twice reaction time). These observations are consistent with kinetically favored $^{\alpha}$C formation due to protein homocystamide enrichment.

A human blood plasma solution treated with HTL similarly exhibits a blue solution color in the presence of methyl viologen (2 min, reflux, 15 mg/mL proteins). Consistent with the results using isolated HSA in buffer, at least double the reaction time is needed for color formation to occur in the unmodified sample



Scheme 3. Kinetically favored HAT process involving Hcy-*N*-protein should promote carbonyl formation and multiple fragmentation products including NH_3, H_2S, ethylene, and α,β-unsaturated amides, based on direct analogy to the chemistry of free Hcy. See refs. 9, 10, and references cited therein for the chemical products resulting from the $^{\alpha}$C radical of unbound Hcy. Cooper (16) has also described analogous products upon the enzyme-catalyzed oxidation of Hcy.

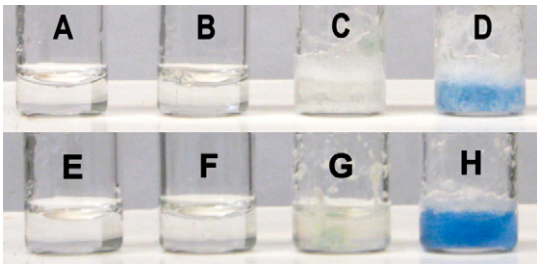


Fig. 1. Solutions heated at reflux for 2 min containing 0.5 M Tris bugger, pH 7.0, MV^{2+} (5.2×10^{-2} M). (*Upper*) (*A*) MV^{2+} blank, (*B*) HTL control solution treated in the same way as the protein samples, (*C*) HSA (15 mg/mL), and (*D*) MV^{2+} and HSA (15 mg/mL) functionalized with 7–8 homocystamide residues/mol. (*Lower*) (*E*) MV^{2+} blank, (*F*) HTL control solution treated in the same way as the protein samples, (*G*) plasma control solution, and (*H*) Hcy-*N*-protein enriched plasma.

solution. In addition, we find that fluorone black, a mildly oxidizing dye, also previously used to detect free Hcy via alpha carbon radical formation (9, 10), affords an increase in absorbance signal at room temperature in neutral buffer in the presence of HSA enriched in homocystamides (*SI Appendix*).

Homocystamides Promote Protein Carbonyl Formation. Cys residue thiyl radicals can abstract $^{\alpha}$C-H protons from proximal amino acid residues of proteins. The ensuing $^{\alpha}$C radical species react with oxygen to form superoxide and carbonyls, thereby fragmenting peptide backbones (18). In oxygen depleted environments, radical coupling and related irreversible reactions may occur as well.

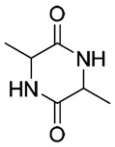
In the case of homocystamide residues, there are fewer geometric constraints for a hydrogen transfer process to occur as compared to Cys residues. The homocystamides are not embedded within the relatively rigid backbone structure and thus an initial $^{\alpha}$C-H abstraction can occur via the pathway shown in Scheme 2. Homocystamides should thus serve as relatively favored sites for protein free-radical initiation, propagation, and concomitant structural changes.

To demonstrate that protein oxidation occurs upon homocystamide enrichment, HSA functionalized with 7–8 homocystamide residues was studied via the standard colorimetric reaction with 2,4-dinitrophenyl hydrazine (2,4-DNPH). A reproducible increase (60% for HSA, as well as 24% in an analogous study using total human plasma proteins; *SI Appendix*) in the level of hydrazine-reactive functionality is observed for functionalized HSA as compared to the corresponding controls consisting of non-HTL-treated HSA which were subjected to otherwise identical processing.

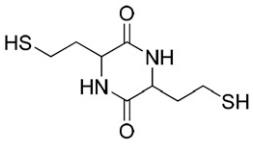
Although seemingly modest, this consistent increase in carbonyl functionality occurs spontaneously, i.e., during the standard incubation procedure between HTL and HSA at physiological temperature, 37 °C, 24 h, without the aid of any added oxidants or free-radical initiators. Moreover, hydrazine reactivity may underestimate the structural effects of homocystamide residues because $^{\alpha}$C radicals may lead to other by-products undetectable by 2,4-DNPH (as defined earlier).

Evidence for the Formation of $^{\alpha}$C Radicals from Homocystamides in Model Peptide Studies. Amino acid anhydrides, including those derived from alanine (4), glycine, and sarcosine, are well-known useful model substrates for studying peptide and protein alpha-amino acid carbon-centered radicals (19). Diketopiperazine **2** was chosen in this investigation to serve as the corresponding Hcy structure analog (we also produce **2** during the *N*-homocysteinylation reaction of Lys and Lys residues in vitro, but find its role in cross-linking proteins is minimal under these conditions; *SI Appendix*). We compared the reactions of **1**, **2**,

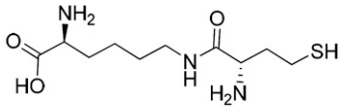
and **3** upon heating in buffer in the presence of methyl viologen.



1 **[1]**



2 **[2]**



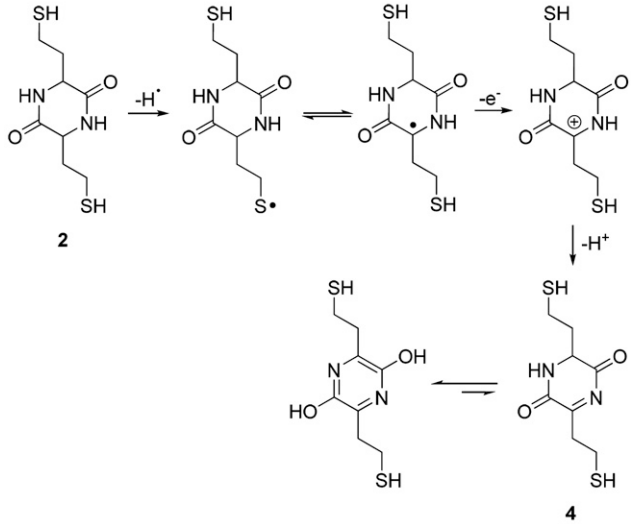
3 **[3]**

The characteristic blue radical cation viologen reduction product color appears upon heating at reflux (4 min) in neutral buffer in the presence of **2**. Importantly, HPLC product analysis confirms that 12.9% racemization of *meso*-**2** takes place upon heating in the presence of MV^{2+}. The racemization is further strong evidence for $^{\alpha}$C radical formation under these conditions. The corresponding control experiment without added methyl viologen does not result in any detectable racemization of **2** (*SI Appendix*). Racemization of *N*-Hcy-Lys, **3** was not observed under the achiral stationary phase analysis conditions.

Additionally, a neutral buffer solution of isopeptide **3**, a direct structure analog of Hcy-*N*-protein (see *SI Appendix* for full synthesis details), exhibits blue solution color within 4 min, as above. Solutions containing Hcy-Lys disulfide, reduced GSH, *N*-acetyl-HTL, Glu-Cys, or Cys-Gly, each of which cannot undergo the five-membered ring HAT transition state, do not develop any color upon heating with methyl viologen for 4 min (*SI Appendix*). Relatively harsher conditions (8–10 min at reflux) are needed for alanine anhydride (**1**)-methyl viologen solutions to develop blue color as compared to viologen solutions of **2** and **3**. This is in keeping with the relatively longer reaction times needed to reduce methyl viologen in the HSA control samples untreated with HTL.

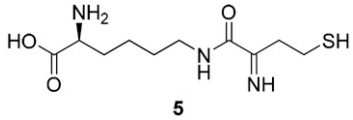
Each of the results described above is consistent with the hypothesis that the redox and HAT chemistry of Hcy-*N*-protein is analogous to that of unbound Hcy molecules. However, methyl viologen is an unnatural oxidant. In order to further study the scope of the $^{\alpha}$C radical forming process, the reactions of **2** with biological oxidants H_2O_2, peroxynitrite, and HOCl were studied. Because H_2O_2, peroxynitrite, and HOCl exist in the extracellular matrix and promote Cys residue thiyl radical formation, they are likely to react with plasma protein homocystamides (20).

We find that the oxidation of **2** in neutral buffer at 37 °C is dependent on the type of biological oxidant and on the oxidant : substrate molar ratio used. In the case of each of the three oxidants, only one equiv is enough to afford detectable consumption of **2**. At a **2** : oxidant ratio of 1 : 1, HOCl and peroxynitrite afford approximately the same distribution of products (*SI Appendix*) which include mixed disulfides as well as **4** (Scheme 4). HOCl and peroxynitrite are, as expected, stronger oxidants as compared to H_2O_2. At 2 and 5 equiv of peroxynitrite and HOCl, respectively, **4** predominates in the reaction mixtures. When up to 10 equiv of H_2O_2 are used, *ca.* 29% **2** is still present in the reaction mixture as monitored by HPLC and liquid chromatography–mass spectroscopy (LC/MS).



Scheme 4. Proposed mechanism for the formation of compound **4** from diketopiperazine **2**.

Other relatively polar products, which did not afford detectable MS signals to date, as well as mixed disulfides, were observed in these reactions (*SI Appendix*). Importantly, compound **4** elutes as an HPLC peak well resolved from *meso* and *racemic* **4** and exhibits a mass spectrum clearly showing parent ion difference (loss) of two protons. The formation of **4** from **2** must occur via the $^{\alpha}$C radical pathway (Scheme 4).



5 **[4]**

The oxidation of isopeptide **3** with hypochlorite, hydrogen peroxide, and peroxynitrite was also evaluated. Reaction mixture products were analyzed by LC/MS electrospray ionization (ESI) in positive mode. The oxidation with hypochlorite and peroxynitrite consumed compound **3** completely when an equimolar amount of oxidant was used and produced a well-resolved peak with an m/z $(M + H^+)$ of 262, corresponding to **5** (*SI Appendix*). Conversely, the reaction with hydrogen peroxide (5 equiv) did not go to completion. These results are consistent with those observed for the mechanism of oxidation of **2**: the formation of **5** occurs via the HAT process.

Discussion

In his landmark paper published in 2002, Jakubowski (21) showed that Hcy is a protein amino acid in humans and that Hcy-*N*-protein is a significant component of Hcy metabolism in humans (21). Values in the literature for levels of *N*-homocysteinylated protein in human plasma determined in healthy individuals have since been reported by three independent research groups to date, and are in excellent agreement, ranging from 0.35–0.51 μM (22). Levels correlating with disease reported to date range from hemodialysis patients exhibiting double the level of Hcy-*N*-protein compared to a control group (23) to a 24-30-fold elevation in Hcy-*N*-protein in methylenetetrahydrofolate reductase or cystathionine β-synthase (CBS) deficiency (3).

N-homocysteinylation targets include fibrinogen, low-density lipoprotein, high-density lipoprotein, albumin, hemoglobin, and ferritin. Plasma levels of prothromobogenic Hcy-*N*-fibrinogen are elevated in human CBS deficiency, which accounts for

increased susceptibility to thrombogenesis observed in CBS-deficient humans. Moreover, an autoimmune response is caused by Hcy-*N*-proteins, which has been shown to be clearly associated with stroke and coronary artery disease (3). Despite this evidence, Hcy-*N*-protein pathophysiology has been recently characterized as unproven (24).

In the current investigation, we have examined homocystamide chemistry. The aim was to contribute to an understanding of the chemical role of Hcy-*N*-protein, particularly in protein oxidation. Each of the examples investigated, including HSA in buffer, total plasma proteins in human plasma, cyclic peptides, and the isopeptide Hcy-*N*-lysine exhibited evidence of $^{\alpha}$C radical formation and concomitant oxidation in the presence of a variety of oxidants over a range of concentrations. Importantly, HSA displayed enhanced carbonyl formation at physiological temperature without added oxidants, simply upon homocystamide enrichment. The results described herein complement earlier studies of the properties of Hcy-*N*-protein, which showed that homocystamides render proteins more prone to oxidation as evidenced by enhanced disulfide formation (25, 26) and alteration of the redox status of cytochrome c (27).

Another natural source of thiyl radicals to consider is the well-known transition metal-catalyzed oxidation of biological thiols. For example, 20% of all Hcy in circulation is believed to undergo autooxidation, i.e., free-radical-mediated disulfide formation, in a process catalyzed by copper-bound albumin (1). Chemical evidence presented herein for homocystamides, and previously for free Hcy (5–7), shows that disulfide formation via autooxidation is accompanied by intramolecular HAT chemistry. This affords highly reactive free Hcy- and Hcy-*N*-protein-derived $^{\alpha}$C radicals under oxidative conditions that can also enhance Hcy-*S*-protein formation. The kinetically favored HAT chemistry of Hcy is thus potentially relevant to all forms of Hcy.

Conclusion

Several of the major diseases associated with elevated levels of homocysteine are also linked to oxidative stress. Elucidation of the altered redox status of hyperhomocysteinemic patients is needed as a step toward targeted therapy (4). In this study, strong evidence is presented showing that an intramolecular HAT mechanism provides chemical rationale for the sought after (2) link between protein oxidation and homocysteine.

The kinetically favored formation of $^{\alpha}$C radicals renders Hcy-*N*-protein capable of initiating oxidative damage under the natural conditions that induce thiyl radical formation. Moreover, the amount of $^{\alpha}$C radicals and their resultant products (20) are expected to reflect the total circulating Hcy (including homocystamides). This is due to the fact that (*i*) 20% conversion of all circulating Hcy undergoes autooxidation (1) and (*ii*) a large proportion [e.g., quantitative (28)] of thiyl radicals formed during autooxidation are well known to afford $^{\alpha}$C radicals under physiological conditions (8–11). Further studies involving the detection and unique chemistry of homocysteine and its various naturally occurring bound and unbound forms are ongoing in our laboratory.

Materials and Methods

Fatty free HSA and lyophilized human plasma were obtained from Sigma-Aldrich. All other reagents were obtained from commercial sources and used without further purification. Commercially available oxidized BSA was obtained from Cell Biolabs. Standard PD-10 columns (10 mL of Sephadex G-25 medium, gravity flow) were obtained from GE Healthcare. UV-visible (UV-vis) measurements were collected on a UV-Vis Cary 50 instrument (Varian, Inc.). HPLC was carried out using either a Nova Pak C_{18} 300 Å, 4 μm, 3.9 × 150 mm (Waters, USA) column or a Discovery C_{18} 300 Å, 5 μm, 2.1 × 250 mm (Supelco) on a Waters 1525 binary pump equipped with a Waters 2996 photodiode array detector. ^{1}H NMR and ^{13}C NMR were obtained on an ARX-400 Advance Bruker spectrometer. ESI [low-resolution mass spectrometry (LRMS) or high-resolution mass spectrometry (HRMS)] spectra were obtained at Mass Spectrometry Facility of Georgia State University. LC/MS analysis of oxidation reaction mixtures were carried out at the Mass Spectrometry Facility in Portland State University using a Discovery C_{18} 300 Å, 5 μm, 2.1 × 250 mm (Supelco) and/or Atlantis hyrophilic interaction liquid chromatography silica, 100 Å, 5 μm, 2.1 × 150 mm (Waters, USA) on an Accela LC system, and a Finnigan LTQ-XL linear ion trap and LTQ Orbitrap discovery mass spectrometer detectors. More specific procedures are included in *SI Appendix*.

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