Cross-talk between Cys\textsuperscript{34} and Lysine Residues in Human Serum Albumin Revealed by N-Homocysteinylation\textsuperscript{*}

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Rafal Glowacki\textsuperscript{†‡§} and Hieronim Jakubowski\textsuperscript{†‡¶}

From the \textsuperscript{†}Department of Microbiology and Molecular Genetics, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, International Center for Public Health, Newark, New Jersey 07103 and \textsuperscript{‡}Institute of Bioorganic Chemistry, Polish Academy of Sciences, 61-704 Poznan, Poland

Protein N-homocysteinylation involves a post-translational modification by homocysteine (Hcy)-thiolactone. In humans, about 70% of circulating Hcy is N-linked to blood proteins, mostly to hemoglobin and albumin. It was unclear what protein site(s) were prone to Hcy attachment and how N-linked Hcy affected protein function. Here we show that Lys\textsuperscript{225} is a predominant site of N-homocysteinylation in human serum albumin in vitro and in vivo. We also show that the reactivity of albumin lysine residues, including Lys\textsuperscript{225}, is affected by the status of Cys\textsuperscript{34}. The disulfide forms of circulating albumin, albumin-Cys\textsuperscript{34}-S-S-Cys and albumin-Cys\textsuperscript{34}-S-S-Hcy, are N-homocysteinylated faster than albumin-Cys\textsuperscript{34}-SH. Although N-homocysteinylation of albumin-Cys\textsuperscript{34}-SH and albumin-Cys\textsuperscript{34}-S-S-Cys yield different primary products, subsequent thiol-disulfide exchange reactions result in the formation of a single product, N-(Hcy-S-S-Cys)-albumin-Cys\textsuperscript{34}-SH. We also show that N-homocysteinylation affects the susceptibility of albumin to oxidation and proteolysis. The data suggest that a disulfide at Cys\textsuperscript{34} of albumin promotes conversion of N-(Hcy-SH)-albumin-Cys\textsuperscript{34}-SH to a proteolytically sensitive form N-(Hcy-S-Cys)-albumin-Cys\textsuperscript{34}-SH, which would facilitate clearance of the N-homocysteinylated form of mercaptoalbumin.

Since the 1960s, it has been known that elevated levels of homocysteine (Hcy),\textsuperscript{1} resulting from mutations in genes encoding Hcy-metabolizing enzymes, are harmful to humans (1, 2). During the past decade it has been established that even a mild increase in Hcy level is a risk factor for cardiovascular disease and stroke in humans (3, 4) and predicts mortality independently of traditional risk factors in patients with coronary artery disease (5). Plasma Hcy is also a risk factor for neurodegenerative disorders, such as dementia and Alzheimer’s disease (6). In tissue cultures, Hcy does not support growth and induces apoptotic death in human endothelial cells (7). Animal and cell culture studies have shown that Hcy induces cell death and potentiates amyloid \(\beta\)-peptide toxicity in neurons (8).

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\textsuperscript{†} Present address: Dept. of Environmental Chemistry, University of Lodz, 90-236 Lodz, Poland.

\textsuperscript{‡} To whom correspondence should be addressed. Tel.: 973-972-4483 (ext. 28733); Fax: 973-972-8982; E-mail: jakubows@umdnj.edu.

\textsuperscript{§} The abbreviations used are: Hcy, homocysteine; DTT, dithiothreitol; N-Hcy-albumin, albumin containing Hcy bound by an amide linkage; S-Hcy-albumin, albumin containing Hcy bound by a disulfide linkage; HPLC, high performance liquid chromatography; IAA, iodoacetamide; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

In humans, Hcy, formed from dietary methionine as a by-product of cellular methylation reactions, is detoxified by folic acid- and vitamin B\textsubscript{12}-dependent re-methylation to methionine (2) or vitamin B\textsubscript{6}-dependent trans-sulfuration to cysteine (1). Whereas Hcy is formed in all human organs, most of its detoxification occurs in the liver and kidneys. Detoxification of Hcy in human vascular tissues and skin occurs only by re-methylation; enzymes of the trans-sulfuration pathway are not expressed in these tissues (9).

Hcy is perhaps the most reactive amino acid in biological systems (1, 2). In addition to re-methylation to methionine or trans-sulfuration to cysteine (via cystathionine), Hcy is also metabolically converted to Hcy-thiolactone, S-nitroso-Hcy, AdoHcy, Hcy-containing disulfides, or homocysteic acid, each of which has been implicated in the pathology of hyperhomocysteinemia (1, 10).

Because of its similarity to the protein amino acid methionine, Hcy can exert its biological effects by interfering with protein biosynthesis (10–18). For example, Hcy is metabolized to Hcy-thiolactone by methionyl-tRNA synthetase in a two-step reaction (19, 20). In the first step (Reaction 1), methionyl-tRNA synthetase catalyzes activation of Hcy with ATP, which yields methionyl-tRNA synthetase (MetRS)-bound homocysteinylladenylate.

\[
\text{MetRS + Hcy + ATP} \rightarrow \text{MetRS-Hcy} \sim \text{AMP} + \text{PP}_i
\]

\textbf{REACTION 1}

The second step (Reaction 2), in which the side chain thiolate of Hcy reacts with the activated carboxyl group of Hey, yields Hcy-thiolactone.

The energy of the anhydride bond in Hcy-AMP is conserved in the thioester bond in Hcy-thiolactone. Because of this, Hcy-thiolactone reacts with proteins, forming Hey-containing adducts, in which the carboxyl group of Hey is linked by an amide bond with e-amino group of a protein lysine residue (21–23).

Originally discovered in cultured cells (21–23), protein N-homocysteinylation is now known to occur in humans (10, 12–18). Both Hcy-thiolactone (13, 24, 25) and N-linked protein Hey (12, 16, 26, 27) have been demonstrated in human blood. About 70% of circulating Hcy is N-linked to blood proteins, mostly hemoglobin and albumin (26). A protective mechanism against protein N-homocysteinylation appears to exist in humans (28, 29).

Two major forms of albumin exist in circulation (30): albumin-Cys\textsuperscript{34}-SH, also known as mercaptoalbumin, and albumin-Cys\textsuperscript{34}-S-S-Cys (Fig. 1), accounting for about two-thirds and one-third, respectively, of total plasma albumin (31). Two minor forms, accounting for 1–2% of total albumin, also exist in circulation: albumin-Cys\textsuperscript{34}-S-S-Hcy (Fig. 1) (31) and Hcy-N-albumin (26). However, these minor forms carry >80% of...
Mechanism of Albumin N-Homocysteinylation

plasma Hcy. The ability of albumin to form a disulfide with Hcy has been examined in vitro (22, 28), and the mechanism for the formation of albumin-Cys\(^{34}\)-S-S-Cys has been proposed (32).

Molecular mechanism and functional consequences of albumin N-homocysteinylation were not known. In this study we show that Lys\(^{525}\) is a predominant site of N-homocysteinylation in human serum albumin. We also show that the status of Cys\(^{34}\) affects the reactivity of albumin lysine residues, including Lys\(^{525}\), and that N-homocysteinylation affects the susceptibility of albumin to proteolysis and oxidation.

MATERIALS AND METHODS

Preparation of \(\text{L-[35S]Hcy-thiolactone–Carryer-free} \) L-[\(35\text{S}\)]Met (5 mCi, Amersham Biosciences) was supplemented with unlabeled methionine (Sigma) to a specific activity of 20,000 Ci/mmol, lyophilized, and stored in a CentriVap concentrator, dissolved in 0.1 M potassium phosphate buffer, pH 7.4, 0.2 mM EDTA, and desalted by ultrafiltration.

Preparation of Albumin-Cys\(^{34}\)-SH and Albumin-Cys\(^{34}\)-S-S-Cys—Human serum albumin (50 mg/ml) was converted to albumin-Cys\(^{34}\)-SH by treatment with 2 mM DTT in 0.1 M potassium phosphate buffer, pH 7.4, 0.2 mM EDTA for 5 min at room temperature, diluted 10-fold with 0.1 M potassium phosphate buffer, pH 5.5, and purified by anion exchange HPLC. Fractions containing albumin-Cys\(^{34}\)-SH were concentrated to 10 mg/ml in 0.1 M potassium phosphate buffer, pH 7.4, 0.2 mM EDTA, and treated with 2-fold molar excess of Cys overnight at 37 °C. Excess Cys was removed from albumin-Cys\(^{34}\)-S-S-Cys by ultrafiltration through an Ultrafree-0.5 10-kDa cut-off membrane (Millipore) at 4 °C. The conversion of albumin-Cys\(^{34}\)-S-S-Cys to albumin-Cys\(^{34}\)-S-S-Cys was monitored by anion exchange HPLC.

Preparation of Albumin-Cys\(^{34}\)-SH—Albumin-Cys\(^{34}\)-SH (10 mg/ml) was incubated with 4-fold molar excess IAA in 0.1 M potassium phosphate buffer, pH 7.4, 0.2 mM EDTA for 1 h at 37 °C in the dark. Excess IAA was removed by ultrafiltration. The conversion of albumin-Cys\(^{34}\)-SH to albumin-Cys\(^{34}\)-SH was complete as determined by analytical thin layer chromatography. Excess hydroxy acid was removed by lyophilization, and L-[\(35\text{S}\)]Hcy-thiolactone was dissolved in deionized water at 15 μCi/ml, aliquoted, and stored at −80 °C.

Preparation of Native Albumin—Native albumin (10 mg/ml) was reduced with 25 mM DTT in 10 mM urea, 0.2 M Tris-HCl buffer, pH 8.6, 1 mM EDTA (5 h at 21 °C), and the liberated thiols were blocked with 100 mM IAA for 1 h at 37 °C in the dark. IAA-modified albumin was desalted by ultrafiltration and digested with \(1\)-tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin (enzyme/substrate ratio 1:50) in 0.1 M ammonium bicarbonate overnight at 37 °C.

Preparation of Tryptic Peptides—N-Hcy-albumin or native albumin (10 mg/ml) was reduced with 25 mM DTT in 10 mM urea, 0.2 M Tris-HCl buffer, pH 8.6, 1 mM EDTA (5 h at 21 °C), and the liberated thiols were blocked with 100 mM IAA for 1 h at 37 °C in the dark. IAA-modified albumin was desalted by ultrafiltration and digested with \(1\)-tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin (enzyme/substrate ratio 1:50) in 0.1 M ammonium bicarbonate overnight at 37 °C.

Enzyme and Peptide Mass Spectrometry—Peptide mass analyses were carried out by Dr. Hong Li on a MALDI-TOF Voyager-DE TM PRO Biospectrometry TM work station (PerSeptive Biosystems) at the New Jersey Medical School Center for Advanced Proteomics Research facility (njms.umn.edu/biochemistry/proteomics/index.htm).

RESULTS

**HCy-thiolactone Reacts Faster with Albumin-Cys\(^{34}\)-SH than with Albumin-Cys\(^{34}\)-S-S-S-Cys**—To determine whether the status of Cys\(^{34}\) in human serum albumin affects the susceptibility of that protein to N-homocysteinylation, albumin-Cys\(^{34}\)-S-S-Cys, albumin-Cys\(^{34}\)-S-S-Hcy, and albumin-Cys\(^{34}\)-SH (Fig. 1), major forms of circulating albumin (30, 31), were prepared as described under “Materials and Methods” and incubated with [\(35\text{S}\)]Hcy-thiolactone. The forma...
tion of N-[35S]Hcy-albumin was monitored by precipitation with trichloroacetic acid. As shown in Fig. 2, albumin-Cys34-S-S-Cys and albumin-Cys34-S-S-Hcy were N-homocysteinylated at similar rates. However, both disulfide forms of albumin were N-homocysteinylated 47 ± 5% faster than mercaptoalbumin, albumin-Cys34-SH. Similar results were obtained when corresponding forms of albumin were prepared in situ by treatments with 5-fold molar excess Cys, Hcy, or DTT. These results suggest that the reactivity of albumin lysine residues depends on the status of Cys34.

Thiol-Disulfide Exchange in N-Homocysteinylated Albumin-Cys34-S-S-Cys Leads to Formation of Free Sulfhydryl at Cys34—Albumin-Cys34-S-S-Cys or albumin-Cys34-SH was modified with equimolar amounts of Hcy-thiolactone and analyzed by anion exchange HPLC. Unmodified albumin-Cys34-S-S-Cys was eluted from the HPLC column at 13.2 min (Fig. 3A, broken trace). After 4 h of modification, about 50% of albumin-Cys34-S-S-Cys was N-homocysteinylated and eluted from the column as a new peak at 12.6 min, before the peak of unmodified form (Fig. 3A, thin trace). After 20 h of modification, all albumin-Cys34-S-S-Cys disappeared, and only a single peak of N-homocysteinylated albumin-Cys34-S-S-Cys at 12.6 min was observed (Fig. 3A, thick trace). Both native and N-homocysteinylated albumin-Cys34-SH were eluted from the anion exchange HPLC column at 12.6 min (not shown).

Altered chromatographic behavior of albumin-Cys34-S-S-Cys after modification with Hcy-thiolactone could be due to two alternative mechanisms. First, blocking an ε-amino group of lysine residue(s) by N-homocysteinylation may have changed the charge of albumin, thus weakening its binding to the anion exchange column. However, this seems unlikely because similar modification of albumin-Cys34-SH did not affect its elution position from the column. Instead, N-homocysteinylation of albumin-Cys34-S-S-Cys may have caused regeneration of a free thiol at Cys34 as a result of thiol-disulfide exchange between a free thiol of newly incorporated Hcy and the disulfide at Cys34 (Reaction 3).

$$\text{Hcy-thiolactone} + \text{albumin-Cys}^{34-}\text{S-S-Cys} \rightarrow \text{N-(Hcy-SH)-albumin-Cys}^{34-}\text{S-S-Cys}$$

$$\text{N-(Hcy-S-S-Cys)-albumin-Cys}^{34-}\text{S-S-Cys} \rightarrow \text{N-(Hcy-S-S-Cys)-albumin-Cys}^{34-}\text{SH}$$

**REACTION 3**

This interpretation is supported by an observation that regeneration of a disulfide at Cys34 by incubation of N-(Hcy-S-S-Cys)-albumin-Cys34-SH with 2-fold molar excess of cysteine also restored its stronger binding to the anion exchange column (Fig. 3B).

To examine more directly whether N-(Hcy-SH)-albumin-
Cys\textsubscript{34}-S-S-Cys and \textit{N}-(Hcy-S-S-Cys)-albumin-Cys\textsubscript{34}-S-S-Cys are formed in significant amounts during \textit{N}-homocysteinylation of albumin-Cys\textsubscript{34}-S-S-Cys, \textit{[35S]}Hcy-thiolactone was used. If formed, \textit{N}-(\textit{[35S]}Hcy-SH)-albumin-Cys\textsubscript{34}-S-S-Cys and \textit{N}-(\textit{[35S]}Hcy-S-S-Cys)-albumin-Cys\textsubscript{34}-S-S-Cys would co-elute with albumin-Cys\textsubscript{34}-S-S-Cys from the anion exchange HPLC column. However, as shown in Fig. 3\textit{C}, only a single peak of radioactivity (at 12.6 min), corresponding to \textit{N}-(\textit{[35S]}Hcy-S-S-Cys)-albumin-Cys\textsubscript{34}-SH, was observed. There was no distinct \textit{35S}-radioactivity peak at the elution position of albumin-Cys\textsubscript{34}-disulfide (at 13.2 min). This result demonstrates that a single species, \textit{N}-(\textit{[35S]}Hcy-S-S-Cys)-albumin-Cys\textsubscript{34}-SH, is predominant during \textit{N}-homocysteinylation of albumin-Cys\textsubscript{34}-S-S-Cys; other products are not observed. The absence of \textit{N}-(\textit{[35S]}Hcy-SH)-albumin-Cys\textsubscript{34}-S-S-Cys suggests that its conversion to \textit{N}-(\textit{[35S]}Hcy-S-S-Cys)-albumin-Cys\textsubscript{34}-SH is much faster that the \textit{N}-homocysteinylation reaction. The absence of \textit{N}-(\textit{[35S]}Hcy-S-S-Cys)-albumin-Cys\textsubscript{34}-S-S-Cys suggests that the thiol-disulfide exchange between \textit{N}-(\textit{[35S]}Hcy-S-S-Cys)-albumin-Cys\textsubscript{34}-SH and albumin-Cys\textsubscript{34}-S-S-Cys is not favored thermodynamically.

To determine whether the thiol-disulfide exchange occurs in \textit{trans} between different albumin molecules, preparations of \textit{N}-(Hcy-SH)-albumin-Cys\textsubscript{34}-S-S-Cys and albumin-Cys\textsubscript{34}-S-S-Cys were used. When analyzed separately by anion exchange HPLC, \textit{N}-(Hcy-SH)-albumin-Cys\textsubscript{34}-S-S-Cys (Fig. 4, \textit{thin trace}) and albumin-Cys\textsubscript{34}-S-S-Cys (Fig. 4, \textit{broken trace}) were eluted at 9.9 and 10.25 min, respectively. However, when \textit{N}-(Hcy-SH)-albumin-Cys\textsubscript{34}-SH was incubated with equimolar amounts of albumin-Cys\textsubscript{34}-S-S-Cys for 4 h at 37\textdegree C and analyzed by anion exchange HPLC, only a peak at 9.9 min was observed (Fig. 4, \textit{thick trace}) and the peak of albumin-Cys\textsubscript{34}-S-S-Cys at 10.25 min essentially disappeared. This shows that albumin-Cys\textsubscript{34}-S-S-Cys was converted to albumin-Cys\textsubscript{34}-SH. Thus, the thiol-disulfide exchange occurs in \textit{trans}.

To determine whether a thiol of Cys\textsubscript{34} in \textit{N}-(Hcy-SH)-albumin-Cys\textsubscript{34}-SH is required for the thiol-disulfide exchange, albumin was first treated with IAA to block the Cys\textsubscript{34} thiol and then \textit{N}-homocysteinylation. This yielded an albumin derivative containing a single free thiol on \textit{N}-linked Hey, \textit{N}-(Hcy-SH)-albumin-Cys\textsubscript{34}-IAA. When analyzed by anion exchange HPLC, \textit{N}-(Hcy-SH)-albumin-Cys\textsubscript{34}-S-IAA (Fig. 5, \textit{thin trace}) was eluted at 13.3 min, essentially identical to the retention time of albumin-Cys\textsubscript{34}-S-S-Cys (Fig. 5, \textit{broken trace}). However, when \textit{N}-(Hcy-SH)-albumin-Cys\textsubscript{34}-S-IAA was mixed in a 1:1
Mechanism of Albumin N-Homocysteinylation

FIG. 5. A thiol of N-linked Hcy in albumin (Alb) participates in thiol-disulfide exchange. Equimolar amounts of albumin-Cys\(^{34}\)-S-S-Cys and N-(Hcy-SH)-albumin-Cys\(^{34}\)-S-IAA (containing Cys\(^{34}\) thiol blocked with IAA) were incubated separately or together for 4 h at 37 °C and analyzed by anion exchange HPLC. Albumin-Cys\(^{34}\)-S-S-Cys (broken trace) and N-(Hcy-SH)-albumin-Cys\(^{34}\)-S-IAA (thin trace) elute at 13.3 min. A peak of albumin-Cys\(^{34}\)-SH appears at 12.9 min when both forms of albumin are mixed together (thick trace).

We next examined whether other N-homocysteinylated proteins can participate in the thiol-disulfide exchange with albumin-Cys\(^{34}\)-S-S-Cys. An experiment with N-(Hcy-SH)-transferrin and albumin is shown in Fig. 6. The preparation of albumin (solid trace) contained albumin-Cys\(^{34}\)-S-S-Cys and albumin-Cys\(^{34}\)-SH, which elute from an anion exchange HPLC column with retention times of 14.0 and 13.2 min, respectively. The retention time of transferrin was 4 min. When albumin and N-(Hcy-SH)-transferrin were mixed together and analyzed by anion exchange HPLC, the peak of albumin-Cys\(^{34}\)-S-S-Cys at 14.0 min completely disappeared (broken trace). Unmodified transferrin did not affect the elution pattern of albumin (not shown). This shows that a thiol of Cys\(^{34}\) in N-(Hcy-SH)-albumin-Cys\(^{34}\)-SH is not required for the thiol-disulfide exchange involving N-linked Hcy.

Because N-homocysteinylation results in addition of thiol group(s) to albumin, modified albumin should become more prone to formation of intermolecular aggregates after oxidation. Indeed, treatment of N-homocysteinylated albumin with H\(_2\)O\(_2\) resulted in formation of albumin aggregates (eluting as a broad peak at 18 min), which bound more strongly than monomeric forms to the DEAE column (Fig. 7A). Treatment of N-homocysteinylated and oxidized sample with DTT resulted in the disappearance of aggregates, suggesting that they were formed as a result of intermolecular disulfide bond formation. Native albumin did not form appreciable aggregates upon treatment with H\(_2\)O\(_2\) (Fig. 7B), which suggests that thiols introduced by N-homocysteinylation are required for aggregation.

FIG. 6. Thiol-disulfide exchange between N-homocysteinylated transferrin and albumin-Cys\(^{34}\)-S-S-Cys. Equimolar amounts of albumin and N-(Hcy-SH)-transferrin were incubated together or separately and analyzed by anion exchange HPLC. Albumin (Alb) analyzed separately (solid trace) shows peaks of albumin-Cys\(^{34}\)-S-S-Cys (at 14.0 min) and albumin-Cys\(^{34}\)-SH (at 13.2 min). Peak of albumin-Cys\(^{34}\)-S-S-Cys disappears when albumin and N-(Hcy-SH)-transferrin are incubated together (broken trace).

FIG. 7. N-Homocysteinylation affects susceptibility of albumin to oxidation. Protein profiles from anion exchange HPLC analyses are shown. A, albumin (Alb) aggregates (4th peak eluting at 18 min, labeled N-Hcy-Alb\((ox)\)) are present in a sample of H\(_2\)O\(_2\)-oxidized N-Hcy-albumin (solid trace). After DTT treatment the peak at 18 min becomes smaller, the peak of albumin-Cys\(^{34}\)-S-S-Cys disappears (2nd peak), and the 1st peak, corresponding to albumin-Cys\(^{34}\)-SH, becomes larger (broken trace). B, minor peak is visible at 18 min on HPLC profile of H\(_2\)O\(_2\)-oxidized native albumin (solid trace). After DTT treatment the minor peak at 18 min is still visible (labeled Alb\((ox)\)), but the peak of albumin-Cys\(^{34}\)-S-S-Cys (2nd peak) disappears, and the peak of albumin-Cys\(^{34}\)-SH (1st peak) becomes larger (broken trace). Identity of the 3rd peak is not known.
Mechanism of Albumin N-Homocysteinylation

Lys, in Albumin Undergoes Preferential N-Homocysteinylation in Vitro—To identify site(s) susceptible to N-homocysteinylation, albumin was modified with Hcy-thiolactone at a molar ratio 1:1, reduced with DTT, acetamidated with IAA to block thiols, and digested with trypsin. Tryptic peptides were separated by reversed phase HPLC (Fig. 9A). A predominant N-Hcy-peptide (eluting in 32 min fraction) was further purified by cation exchange HPLC (Fig. 9C). N-Homocysteinylated peptides were detected by using trypptic digests of N-[^{35}S]Hcy-albumin in parallel experiments (Fig. 9, B and D). Purified N-Hcy-peptide was subjected to MALDI-TOF mass spectrometric analysis. A single signal at \( m/z \) of 1,302.83 (Fig. 10A) was observed on mass spectra of the N-Hcy-peptide. This mass corresponds to an acetamidated and N-homocysteinylated peptide containing Lys\(^{525} \), \( { }^{525} \text{KQTLVELVK}^{534} \) (calculated mass of the derivatized peptide is 1,302.8; the site of N-homocysteinylation is in \textit{boldface}). This peptide was also observed on mass spectra of unpurified trypptic digests of N-Hcy-albumin (not shown).

Lys\(^{525} \) in albumin-Cys\(^{34} \)-S-S-Cys was N-homocysteinylated significantly faster than the same lysine residue in N-Hcy-albumin-Cys\(^{34} \)-SH. By analyzing trypptic digests on a reversed phase C18 HPLC column (Fig. 9B), we found that 9.22 \( \pm \) 1.13% (n = 5) and 4.91 \( \pm \) 0.92% (n = 7) of N-linked Hcy in N-[^{35}S]Hcy-albumin-Cys\(^{34} \)-S-S-Cys and N-[^{35}S]Hcy-albumin-Cys\(^{34} \)-SH, respectively, was attached to Lys\(^{525} \). Thus, the reactivity of Lys\(^{525} \) is affected by the status of Cys\(^{34} \).

Lys\(^{525} \) in Native Albumin Is N-Homocysteinylated—Although \textit{in vitro} data indicated that Lys\(^{525} \) is a predominant site susceptible to N-homocysteinylation, it is unclear whether this site is also N-homocysteinylated \textit{in vivo}. To determine this, native albumin was isolated from two human subjects (having elevated plasma total Hcy levels of 40–80 \( \mu \text{mol/l} \)) and analyzed for the Lys\(^{525} \) modification. To facilitate detection of N-linked Hcy, native human serum albumin was enriched in N-Hcy-containing species by using thiopropyl-Sepharose. Such enriched preparations were reduced with DTT, modified with IAA, and digested with trypsin. Putative peptide containing N-homocysteinylated Lys\(^{525} \) was purified by HPLC and subjected to mass spectrometric analysis. A peptide with a mass of 1,302.92, corresponding to the mass of Lys\(^{525} \)-homocysteinylated Lys525 (eluting in 32 min fraction) was further purified by cation exchange HPLC (Fig. 10B). The signal at \( m/z \) 1,302.92 was about 2-fold smaller for albumin

![Graph A](image1.png)  ![Graph B](image2.png)  ![Graph C](image3.png)  ![Graph D](image4.png)
Mechanism of Albumin N-Homocysteinylation

from a subject who had 40 μM tHcy compared with a subject who had 80 μM tHcy. The signal at m/z 1,302.92 was also observed in analysis of commercial native albumin but was much less intense (not shown). These data strongly suggest that Lys525 is a site of N-homocysteinylation in native albumin in vitro.

DISCUSSION

Human serum albumin is the major plasma protein (30) which is also a major target for N-homocysteinylation by Hcy-thiolactone in vitro (22) and in vivo (26). The present work identifies Lys255 as a preferential site of N-homocysteinylation in human serum albumin in vitro and in vivo, and provides evidence for specific structural alterations caused by N-homocysteinylation. The findings of this work also suggest that a disulfide at Cys34, a conserved residue in albumins from various organisms, facilitates conversion of N-homocysteinylated mercaptoalbumin to a proteolytically sensitive form.

The present data support the following mechanisms of albumin N-homocysteinylation. Hcy-thiolactone reacts with lysine residues of both major forms of circulating albumin. However, the status of Cys34 affects the rate of N-homocysteinylation; the reaction with albumin-Cys34-S-S-Cys is faster than with albumin-Cys34-SH. The reactivity of Lys255, a predominant N-homocysteinylation site, in albumin-Cys34-S-S-Cys is about 2-fold greater than in albumin-Cys34-SH. The initial product of N-homocysteinylation of albumin-Cys34-S-S-Cys (N-(Hcy-SH)-albumin-Cys34-S-S-Cys) is not observed because it undergoes a rapid thiol-disulfide exchange to form N-(Hcy-S-S-Cys)-albumin-Cys34-SH (Reaction 1). Another possible product of thiol-disulfide exchange between N-(Hcy-S-S-Cys)-albumin-Cys34-SH and albumin-Cys34-S-S-Cys, N-(Hcy-S-S-Cys)-albumin-Cys34-S-S-Cys is also not observed, suggesting that the equilibrium of the reaction is shifted far to the left. The thiol-disulfide exchange occurs in trans between different molecules of N-(Hcy-SH)-albumin-Cys34-S-S-Cys. An intramolecular thiol-disulfide exchange is unlikely, because residue Cys34 (domain IA) is located too far away from Lys255 (domain IIIB) in the structure of human serum albumin (30, 35). Facile exchange is known to occur between free reduced Hcy and albumin-Cys34-S-S-Cys (32).

N-Homocysteinylation of mercaptoalbumin affords N-(Hcy-SH)-albumin-Cys34-SH (Reaction 4), which undergoes thiol-disulfide exchange with albumin-Cys34-S-S-Cys to yield N-(Hcy-S-S-Cys)-albumin-Cys34-SH (Reaction 5).

Hcy-thiolactone + albumin-Cys34-SH ⇌ N-(Hcy-SH)-albumin-Cys34-SH

REACTION 4

N-Hcy-SH-albumin-Cys34-SH + albumin-Cys34-S-S-Cys

REACTION 5

Thus, N-homocysteinylation of a mixture of albumin-Cys34-SH and albumin-Cys34-S-S-Cys, which is present in circulation (31), leads to a single N-homocysteinylated product, N-(Hcy-S-S-Cys)-albumin-Cys34-SH. The equilibrium is strongly shifted toward N-(Hcy-S-S-Cys)-albumin-Cys34-SH because the Cys34 thiolate anion has an unusually low pKₐ of ~5 (30) and thus is more thermodynamically stable than Hcy thiolate anion. The low pKₐ of the Cys34 thiolate also makes the thiol-disulfide exchange of N-(Hcy-SH)-albumin-Cys34-SH with albumin-Cys34-S-S-Cys thermodynamically more favored than with cystine. For example, under conditions where the thiol-disulfide exchange between N-(Hcy-SH)-albumin-Cys34-SH and albumin-Cys34-S-S-Cys went to completion (Fig. 3), the exchange with cysteine was <20% complete (not shown). Thiol-disulfide exchange reactions between albumin-Cys34-SH and cysteine, homocysteine, or Cys-S-S-Hcy disulfide are known to be slow, being about 20% or less complete in 4 h (32, 36).

It has been hypothesized that metabolic conversion of Hcy to Hcy-thiolactone, the reactivity of Hcy-thiolactone toward proteins, and resulting protein damage contribute to pathologies associated with elevated Hcy levels in human beings (21–23). If this hypothesis is correct, it is likely that protective mechanism(s) against Hcy-thiolactone have evolved. One possible protective mechanism can be provided by Hcy-thiolactonase/paraoxonase, a component of high density lipoprotein, which detoxifies Hcy-thiolactone by hydrolyzing it to Hcy (28, 29).

Human serum albumin, because of its abundance and the ability to avidly react with Hcy and Hcy-thiolactone, is likely to serve an important dual protective role. Consistent with this hypothesis is correct, it is likely that protective mechanism(s) against Hcy-thiolactone have evolved. One possible protective mechanism can be provided by Hcy-thiolactonase/paraoxonase, a component of high density lipoprotein, which detoxifies Hcy-thiolactone by hydrolyzing it to Hcy (28, 29).

Hcy-thiolactone inducers caspase-independent vascular endothelial damage with apoptotic features (38). Thus, the conversion of Hcy into albumin-Cys34-S-S-Hcy would prevent cellular uptake of Hcy and therefore minimize the conversion to Hcy-thiolactone. Indeed, albumin-Cys34-S-S-Hcy comprises 82%, whereas reduced Hcy accounts for only 2% of total Hcy in human serum (31). In addition, albumin detoxifies a significant fraction of Hcy-thiolactone by virtue of N-homocysteinylation. Plasma pool of N-linked Hcy comprises up to 25% of total plasma Hcy in humans, with N-Hcy-albumin accounting...
for 90% of plasma N-Hcy-protein (22, 26). Both N-linked and S-linked protein Hcy are most likely detoxified in the liver where transmethylation and trans-sulfuration pathways of Hcy metabolism are the most active (1, 2). Consistent with this suggestion are our findings that N-(Hey-S-S-Cys)-albumin-Cys$^{34}$-SH, albumin-Cys$^{34}$-S-S-Cys, and albumin-Cys$^{34}$-S-S-Hcy are more susceptible to proteolysis than albumin-Cys$^{34}$-SH. Although N-(Hey-SH)-albumin-Cys$^{34}$-SH is resistant to proteolytic digestion, this form is unlikely to exist in circulation because it undergoes the thiol-disulfide exchange with albumin-Cys$^{34}$-S-S-Cys, which converts it into proteolysis-prone form, N-(Hey-S-S-Cys)-albumin-Cys$^{34}$-SH (Reaction 5). These findings suggest that a disulfide at residue Cys$^{34}$ in albumin may have an important role in facilitating proteolytic turnover of N-homocysteineylated albumin.

Different proteolytic susceptibilities of albumin-Cys$^{34}$-SH and albumin-Cys$^{34}$-S-S-Cys suggest that albumin adopts a different structure depending on the state of Cys$^{34}$. Indeed, a structural transition in albumin dependent on the state of Cys$^{34}$ has been detected by NMR spectroscopy (39). Our data suggest that N-homocysteinylation interferes with this structural transition.

Because it is a downstream metabolite that, most likely, reflects damage caused by Hcy, N-linked Hcy could be a new marker of cardiovascular risk, possibly more predictive than total Hcy. To determine this, it would be important to monitor protein N-homocysteinylation in human beings. Present methods of monitoring protein N-homocysteinylation are relatively complex and thus not very useful in a clinical setting. However, identification of Lys$^{525}$ as a predominant N-homocysteinylation site in human serum albumin opens up a way of designing new diagnostic tools for monitoring cardiovascular risk associated with elevated levels of plasma Hcy. For example, specific antibodies can be raised against N-homocysteinated peptides of albumin containing Lys$^{525}$ and used to monitor the status of albumin N-homocysteinylation in human beings.

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Rafal Glowacki and Hieronim Jakubowski

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