Homocysteine Is a Protein Amino Acid in Humans

IMPLICATIONS FOR HOMOCYSTEINE-LINKED DISEASE*

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Homocysteine is thought to be a non-protein amino acid. However, in vitro studies suggest that homocysteine is likely to be incorporated by indirect mechanisms into proteins in living organisms. Here I show that homocysteine is a protein amino acid in humans. Homocysteine bound by amide or peptide linkages (Hcy-protein) is present in human hemoglobin, serum albumin, and y-globulins. 1 molecule of homocysteine per 1000 or 1670 molecules of methionine was present in hemoglobin or albumin, respectively. Other proteins, such as low density lipoprotein, high density lipoprotein, transferrin, antitrypsin, and fibrinogen, contained lower amounts of Hcy-N-protein. In human plasma, levels of Hcy-N-protein represented from 0.3 to 23% of total homocysteine. Thus, Hcy-N-protein is a significant component of homocysteine metabolism in humans, possibly contributing to adverse effects of homocysteine on human cells.

It has been known for 4 decades that elevated levels of homocysteine (Hcy)1 are harmful to humans (1, 2), but over 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) and predicts mortality independently of traditional risk factors in patients with coronary artery disease (4). Plasma Hcy is also a risk factor for dementia and Alzheimer's disease (5). However, mechanisms by which Hcy can be harmful are largely unknown.

In humans Hcy is formed from methionine as a by-product of biological methylation reactions (1, 2). If not removed by transsulfuration and transmethylation reactions, Hcy becomes toxic to human cells (1, 2), possibly due to its indirect incorporation into protein by methionyl-tRNA synthetase (MetRS)-mediated mechanisms involving S-nitroso-Hcy or Hcy-thiolactone (6–11) (Fig. 1). A translational pathway includes: (a) reaction of Hcy with nitric oxide, forming S-nitroso-Hcy (12), (b) attachment of S-nitroso-Hcy to tRNAMet catalyzed by MetRS, forming S-nitroso-Hcy-tRNAMet (13), and (c) transfer of S-nitroso-Hcy from S-nitroso-Hcy-tRNAMet into growing polypeptide chains on ribosomes (13). Transnitrosylation of S-nitroso-Hcy-protein results in the formation of protein chains containing Hcy at positions normally occupied by methionine (13). A post-translational pathway involves: (a) metabolic conversion of Hcy to Hcy-thiolactone by MetRS (14–16) and (b) acylation of protein lysine residues by Hcy-thiolactone (15–17). Because N-homocysteinylation leads to protein damage, we hypothesized that this aspect of Hcy metabolism provides a plausible chemical mechanism accounting at least in part for the toxicity of Hcy in humans (6–11, 15–17).

It is not known whether protein N-homocysteinylation occurs in humans. To determine this, human blood proteins were analyzed for the presence of Hcy bound by amide or peptide linkages (Hcy-N-protein) using protein chemistry and HPLC methods. As described in this communication, Hcy is a protein amino acid in humans.

MATERIALS AND METHODS

Preparation of 1-35S-Hcy-thiolactone—[35S]Hcy-thiolactone (20,000 Ci/mol) was prepared by digestion of [35S]Met (Amersham Biosciences) with hydriodic acid (18) as described before (13) and purified by adsorption to charcoal (Sigma) and elution with 0.1 M HCl.

Human Plasma and Proteins—Normal human blood was obtained from healthy volunteers as approved by the Institutional Review Board. Blood was drawn into Vacutainer EDTA tubes and chilled on ice, and the plasma was separated by centrifugation at 2,000 × g at 4 °C for 15 min. Plasma from homocystinuric subjects was kindly provided by Helga Refsum, David Rosenblatt, Vivian Shih, Ling Yu Shih, and the late John Lindenbaum. Purified individual blood proteins were obtained from Sigma.

Determination of Hcy-N-protein—Human plasma or a 50 mg/ml protein solution (0.2 ml) was treated with 5 mM DTT for 5 min at room temperature and ultrafiltrated through a Millipore 10-kDa cut-off membrane at 4 °C to remove free Hcy. Plasma proteins were washed on ultrafiltration devices six more times with 5 mM DTT in phosphate-buffered saline. This procedure removes >99% of the total Hcy from serum proteins. DTT-treated protein was diluted to 0.4 ml with 25 mM DTT. The samples were distributed in 0.1-ml aliquots into glass ampoules (1-ml volume) containing 0.1 ml of 12 N HCl. The ampules were sealed under vacuum, and the samples were hydrolyzed at 120 °C for up to 4 h. This procedure quantitatively converted Hcy-N-protein into Hcy-thiolactone. After hydrolysis samples were lyophilized, dissolved in 0.1 ml of 2 N ammonium bicarbonate, 1 N dipotassium phosphate supplemented with 18,000 cpm [35S]Hcy-thiolactone, and extracted with 0.4 ml of chloroform/methanol (2:1, v/v). Hcy-thiolactone was re-extracted from the organic layer with 0.1 ml of 0.1 N HCl. The acid extracts were lyophilized and dissolved in 10 µl of water.

Further purification of Hcy-thiolactone was achieved by two-dimensional thin layer chromatography on cellulose (Analtech) plates (6.7 × 5 cm) as described below (15). Hcy-thiolactone, localized on TLC plates by autoradiography using Kodak BioMax x-ray film, was extracted with water (60 µl). Final purification and quantitation was achieved by cation exchange HPLC.

Determination of Total Hcy—The principle of tHcy (19) determination involves its conversion to Hcy-thiolactone (20, 21), which is then

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1 The abbreviations used are: Hcy, homocysteine; DTT, dithiothreitol; Hcy-N-protein, Hcy bound to protein by a disulfide linkage; Hcy-S-protein, Hcy bound to protein by a disulfide linkage, equivalent to bHcy in Ref. 19; rHcy, Hcy present after reductive cleavage of disulfide bonds in sample (19); HDL, high density lipoprotein; HPLC, high pressure liquid chromatography; LDL, low density lipoprotein; MetRS, methionyl-tRNA synthetase; mAU, milliabsorbance units.
detected and quantitated by HPLC. Briefly, human plasma was treated with 5 mM DTT to convert disulfide-bound forms of Hcy to free reduced Hcy and deproteinized by ultrafiltration through Millipore 10-kDa cut-off membranes at 4 °C. The ultrafiltrate (50 μl) was lyophilized on a SpeedVac concentrator and dissolved in 6 μl of 50 mM DTT, and Hcy was converted to Hcy-thiolactone by treatment with 3 μl of 6 mM HCl for 30 min at 100 °C. After lyophilization, samples were dissolved in 50 μl of water and subjected to HPLC.

HPLC—HPLC analyses were carried out using a cation exchange PolySULFOETHYL Aspartamide column (2.1 × 200 mm, 5 μ, 300 A) from PolyLC, Inc. and System Gold Noveu HPLC instrumentation from Beckman-Coulter. Solution A (10 mM monosodium phosphate) and solution B (200 mM NaCl in 10 mM monosodium phosphate) were used as solvents. After application of sample, the column was eluted with 25% solution B for 0.5 min and a linear gradient from 25 to 80% solution B for 5 min followed by 80% solution B for 0.5 min and 3-min re-equilibration with 25% solution B.

The effluent was monitored at A240, the UV absorption maximum of Hcy-thiolactone (ε = 3,500 μM cm) (10). For each sample, the identity of the eluted material as Hcy-thiolactone was confirmed by its co-migration with an authentic Hcy-thiolactone, by its characteristic absorbance at A240, and by its sensitivity to NaOH. The detection limit was 5 pmol of Hcy-thiolactone. An example of HPLC analysis of Hcy-thiolactone content in acid hydrolysates of human plasma protein is shown in Fig. 2.

RESULTS

Serum Albumins Contain Hcy Bound via Amide or Peptide Bonds—Control experiments with proteins containing known amounts of [35S]Hcy-N-protein showed that acid hydrolysis in the presence of DTT quantitatively liberated Hcy in the form of [35S]Hcy-thiolactone. Quantities of [35S]Hcy-thiolactone released from the labeled proteins were essentially identical at various times of hydrolysis from 1 to 4 h (not shown), indicating that hydrolysis was completed within 1 h and that Hcy-thiolactone is stable under the conditions of hydrolysis. Nevertheless, monitoring in ultraviolet light at A240 showed that the amounts of Hcy-thiolactone liberated from the hydrolyzed protein were increasing as a function of time of hydrolysis (Fig. 3).

Extrapolating the amounts of Hcy-thiolactone to zero time of hydrolysis yielded actual concentrations of Hcy-N-protein.

The continuous increase in Hcy-thiolactone appears to be due to a slow conversion of protein methionine to Hcy-thiolactone. Because methionine is converted to Hcy-thiolactone by the treatment with hydroiodic acid at 135 °C (18), treatment with HCl is also likely to elicit a similar conversion to a small extent. I found that, when 5 mM methionine was treated with 6 N HCl at 135 °C, Hcy-thiolactone formed at a rate of 2 μM/h, some 1,000-fold slower than in the presence of hydroiodic acid (not shown). At 120 °C the rate of conversion of methionine to Hcy-thiolactone was 0.2 μM/h (Fig. 3), similar to rates of Hcy-thiolactone release from proteins containing similar amounts of methionine (Fig. 3). Because protein is completely hydrolyzed to amino acids within 1 h, the slowly released Hcy-thiolactone is derived mostly from free methionine liberated from protein rather than directly from protein-bound methionine.

To further confirm that the slow increase in Hcy-thiolactone during hydrolysis with HCl is due to the presence of methionine, various serum albumins, containing 0 to 6 methionines per protein molecule, were analyzed. Before acid hydrolysis, >99% of disulfide bond-bound Hcy has been removed from proteins by extensive treatments with DTT. The quantities of Hcy-thiolactone released from human serum albumin, which contains 6 methionine residues, increased as a function of time of hydrolysis from 1 to 4 h. Similar slopes of Hcy-thiolactone versus time plots were observed in experiments with 50 mg/ml human serum albumin and an equivalent concentration (5 mM) of methionine. Smaller increases in Hcy-thiolactone were observed with sheep serum albumin, which contains 4 methionine residues. As expected, amounts of Hcy-thiolactone essentially did not change with time in experiments with pig and rabbit serum albumin, which contain 0 and 1 methionine residue, respectively. These observations confirm that the slow increase in Hcy-thiolactone released from protein is due to methionine. Extrapolation of the released amounts of Hcy-thiolactone to zero time indicates that 50 mg/ml solutions of human and sheep serum albumin contained 2.1 and 1.6 μM Hcy-N-protein, respectively. Pig and rabbit serum albumin at 50 mg/ml contained 0.8 and 1.8 μM Hcy-N-protein, respectively (Table I).

Albumin from each animal organism tested was found to contain Hcy-N-protein (Table I). The highest levels of Hcy-N-protein were present in human serum albumin (2.8 μM), and the lowest were in pig albumin (0.6 μM). The levels of Hcy-N-protein did not correlate with the levels of Hcy-S-albumin (Hcy bound to albumin by a disulfide linkage, bHcy in Ref. 19) in these albumins (Table I).

Human Blood Proteins Contain Hcy Bound via Amide or Peptide Bonds—To determine whether Hcy is present in some or all proteins, individual purified human blood proteins were subjected to analyses. In absolute values, the highest amounts of

![Fig. 1. Metabolism of Hcy in a human endothelial cell (7, 8).](image)

Hcy arises from methionine as a by-product of cellular transmethylation reactions. When the remethylation to methionine is impaired, for example by inadequate folate supply, Hcy is metabolized to Hcy-thiolactone by MetRS. Hcy-thiolactone freely diffuses out and into the cell and reacts with protein lysine residues or is hydrolyzed in serum to Hcy thiolactone. Hcy forms a mixed disulfide with serum albumin (Hcy-S-protein), which is then incorporated translationally into protein following formation of S-nitroso-Hcy-tRNA catalyzed by MetRS (13). Hcy-N-protein contains Hcy in amide or peptide bonds.

![Fig. 2. Cation exchange HPLC determination of Hcy-thiolactone in acid hydrolysates of human plasma protein.](image)

A, samples purified from hydrolysates of normal human plasma protein. B, samples purified from hydrolysates of human plasma protein obtained from a homocystinuric subject. HPLC profiles were obtained with proteins hydrolyzed for 1 h (bottom trace) and 2, 3, and 4 h (top traces). Hcy-thiolactone elutes at 4.2 min. An unknown major contaminant elutes at 3.7 min. Detection was by absorbance at 240 nm (in milliabsorbance units, mAU), the absorption maximum of Hcy-thiolactone. Note the different scales of the ordnates: 0–1.0 mAU in A and 0–10 mAU in B.
Hcy-N-protein, 4.2, 2.8, and 1.2 μM, were present in 50 mg/ml solutions of human hemoglobin, serum albumin, and γ-globulins, respectively (Table II). 50 mg/ml solutions of LDL, HDL, antitrypsin, and transferrin contained 0.95, 0.85, 0.65, and 0.48 μM Hcy-N-protein, respectively. Fibrinogen at 50 mg/ml contained the lowest amount of Hcy-N-protein, 0.15 μM. On a per molecule basis about 0.6% of hemoglobin molecules contained 1 molecule of Hcy in amide or peptide bonds, equivalent to 1 molecule of Hcy per 1000 molecules of protein methionine. Human serum albumin and γ-globulin contained 0.36% Hcy-N-protein. Other proteins contained from 0.04 to 0.1% Hcy-N-protein. Thus, 1 molecule of Hcy bound via amide or peptide bond is present per each 170–2500 individual protein molecules.

The levels of Hcy-N-protein in individual blood proteins appear to correlate with the reactivity of these proteins toward Hcy-thiolactone. For example, fibrinogen, found to contain about 18-fold less Hcy-N-protein than albumin (Table II), exhibits about 6-fold lower reactivity toward Hcy-thiolactone than albumin (17). Similarly, low density lipoprotein contains about 3-fold less Hcy-N-protein than albumin and exhibits about 8-fold lower reactivity toward Hcy-thiolactone than albumin (17). A major fraction of lysine residues in LDL and fibrinogen is not accessible to solvent and therefore not able to react with Hcy-thiolactone (17). A major fraction of lysine residues in LDL and fibrinogen is not accessible to solvent and therefore not able to react with Hcy-thiolactone (17).

For comparison, the levels of disulfide bond-bound Hcy in each protein (Hcy-S-protein) are shown in Table II. As expected, significant amounts of Hcy-S-protein were present in human serum albumin, about 1 molecule per 100 protein molecules. Unexpectedly, more Hcy-S-protein, about 3.4 molecules per 100 protein molecules, was detected in γ-globulin. This suggests that γ-globulin, in addition to serum albumin, is a major component of the Hcy-S-protein pool in human plasma. Other proteins contained >10-fold less Hcy-S-protein than albumin.

Relationship between Plasma Hcy-N-protein and Plasma tHcy—Fig. 4A is a plot of the individual values of plasma Hcy-N-protein versus Hcy for each human subject. B, plot of relative values of plasma Hcy-N-protein versus Hcy. Empty circles (○) indicate a subset of subjects with very low levels of Hcy-N-protein. Overall, plasma Hcy-N-protein was positively correlated with plasma tHcy. However, a subset of plasma samples, indicated by empty circles in Fig. 4A, contained much lower levels of Hcy-N-protein and the corresponding plasma tHcy values. For comparison, the levels of disulfide bond-bound Hcy in each protein (Hcy-S-protein) are shown in Table II. As expected, significant amounts of Hcy-S-protein were present in human serum albumin, about 1 molecule per 100 protein molecules. Unexpectedly, more Hcy-S-protein, about 3.4 molecules per 100 protein molecules, was detected in γ-globulin. This suggests that γ-globulin, in addition to serum albumin, is a major component of the Hcy-S-protein pool in human plasma. Other proteins contained >10-fold less Hcy-S-protein than albumin.

**DISCUSSION**

The data presented in this communication show that 1) Hcy is a protein amino acid in humans and 2) plasma levels of Hcy-N-protein are positively correlated with plasma tHcy levels. These data extend our previous *ex vivo* observations from cultured human cells to human organism and provide direct evidence that Hcy-N-protein is a significant component of Hcy metabolism in humans.
Homocysteine Is a Protein Amino Acid in Humans

A possibility of incorporation of Hcy into protein was raised in the 1960s shortly after the identification of cystathionine \(\beta\)-synthase deficiency in humans (discussed in Ref. 24). However, no Hcy was detected in acid hydrolysates of hair from three cystathionine \(\beta\)-synthase-deficient patients or brain from one of these patients. These negative results are not surprising given the low sensitivity of the standard amino acid analysis methods used in these early studies. Even the highest levels of Hcy measured in human hemoglobin in the present work (1 Hcy residue per 1000 methionine residues) would have not been detected by using standard protein amino acid analysis methods.

The levels of Hcy-N-protein present in individual human blood proteins (Table II) are roughly proportional to their abundance in human blood. This indicates that most of Hcy-N-protein in normal human blood is present in hemoglobin (about 75%), albumin (22%), and \(\gamma\)-globulins (2%). All other blood proteins contain about 1% of total Hcy-N-protein. In contrast, most of Hcy-S-protein is present in albumin (65%) and \(\gamma\)-globulin (25%); hemoglobin contains about 10% of Hcy-S-protein present in human blood.

The metabolic role of Hcy-N-protein and Hcy-S-protein in human blood is not understood. However, it is likely that formation of these Hcy addsucts with major blood proteins functions as a protective mechanism, which detoxifies Hcy and its reactive metabolites.

The findings of the present work support a hypothesis that protein N-homocysteinylated and resulting protein damage cause Hcy toxicity to human cells, especially to vascular endothelial. In previous work we have shown that Hcy-N-protein occurs in a variety of cultured human cells. In normal and cystathionine synthase-deficient fibroblasts and in breast cancer cells, Hcy-N-protein is elevated by the presence of the antifolate drug aminopterin (15). In cultured human umbilical vein endothelial cells, Hcy-N-protein level is positively correlated with tHcy and negatively correlated with methionine, folic acid, and HDL (16). As shown in the present work, similar positive correlation between Hcy-N-protein and tHcy has been found in vivo in humans (Fig. 4A). Edman degradation of Hcy-N-protein from endothelial cell cultures suggests that Hcy, in addition to being linked by amide bonds to side chains of protein lysine residues, is also linked by peptide bonds (8, 16). However, in the present work it was not possible to determine what specific mechanism(s) are responsible for the presence of Hcy in human blood proteins.

The formation of Hcy-N-protein, mediated by S-nitroso-Hcy, possibly accounts for the observations that atherosclerosis originates mostly at branch points in arteries (25) that are subject to mechanical stress leading to increased production of nitric oxide (26). Local concentrations of nitric oxide (26) and S-nitroso-Hcy (12) are likely to be higher at arterial branch points than elsewhere. Therefore, at branch points, S-nitroso-Hcy-mediated formation of Hcy-N-protein and resulting damage would be greater than at other points in arteries. This would be particularly damaging for arterial branch points in subjects with elevated serum Hcy levels.

Hcy-thiolactone is toxic to endothelial cells. For example, chronic infusions of baboons with Hcy-thiolactone cause endothelial cell injury (29). Hcy-thiolactone, but not Hcy, was found to induce gross changes in human endothelial cell morphology and to induce cell death with apoptotic features (30). These effects are likely to be caused by Hcy-thiolactone-mediated formation of Hcy-N-protein, which results in protein damage (17, 27, 28). However, the role of Hcy-N-protein in Hcy-induced toxicity has not been explored.

Hcy-thiolactonase/paraoxonase, a component of HDL, minimizes protein N-homocysteinylation in vitro (16, 22, 29) and has been implicated in human cardiovascular disease (31). We have suggested that Hcy-thiolactonase activity is a better predictor of Hcy-associated vascular disease than \(\text{PON1} \) genotype (23). Hcy-thiolactonase and paraoxonase activities are highly correlated in human populations (23). Thus, the finding that paraoxonase activity is a predictor of vascular disease (32) supports our suggestion that Hcy-thiolactonase activity is a physiologically relevant predictor of the disease. However, the relationship of Hcy-thiolactonase/paraoxonase to Hcy-N-protein in humans needs to be examined. This need is underscored by the present observation that in a subpopulation of human subjects levels of plasma Hcy-N-protein are much lower than in most subjects (Fig. 4).

In conclusion, this work shows that Hcy is a protein amino acid in humans and that plasma Hcy-N-protein is correlated with plasma tHcy. Our finding and the observations of others suggesting that Hcy-N-protein has adverse effects on physiological function do not establish a role of Hcy-N-protein in human disease. However, they underscore the importance of examining protein \(N\)-homocysteinylation in the context of human disease.

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