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Biomedicine and Diseases: Review

Molecular basis of homocysteine toxicity in humans

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Abstract. Because of its similarity to the protein amino acid methionine, homocysteine (Hcy) can enter the protein biosynthetic apparatus. However, Hcy cannot complete the protein biosynthetic pathway and is edited by the conversion to Hcy-thiolactone, a reaction catalyzed by methionyl-transfer RNA synthetase in all organisms investigated, including human. Nitrosylation converts Hcy into a methionine analogue, *S*-nitroso-Hcy, which can substitute for methionine in protein synthesis in biological systems, including cultured human endothelial cells. In humans, Hcy-thiolactone modifies proteins posttranslationally by forming adducts in which Hcy is linked by amide bonds to ε-amino group of protein lysine residues (Hcy-*εN*-Lys-protein). Levels of Hcy bound by amide or peptide linkages (Hcy-*N*-protein) in human plasma proteins are directly related to plasma 'total Hcy' levels. Hcy-*N*-hemoglobin and Hcy-*N*-albumin constitute a major pool of Hcy in human blood, larger than 'total Hcy' pool. Hcy-thiolactone and Hcy-thiolactone-hydrolyzing enzyme, a product of the *PON1* gene, are present in human plasma. Modification with Hcy-thiolactone leads to protein damage and induces immune response. Autoantibodies that specifically recognize the Hcy-*εN*-Lys-epitope on Hcy-thiolactone-modified proteins occur in humans. The ability of Hcy to interfere with protein biosynthesis, which causes protein damage, induces cell death and elicits immune response, is likely to contribute to the pathology of human disease.

Key words. Anti-Hcy-*N*-protein antibodies; atherosclerosis; high-density lipoprotein; homocysteine-thiolactone; S-nitrosohomocysteine; paraoxonase; protein *N*-homocysteinylation; thiolactonase.

Introduction

Since the 1960s, it has been known that elevated levels of homocysteine (Hcy), 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 independent 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 β-peptide toxicity in neurons [8].

Hcy is harmful not only to human cells. For example, Hcy accumulates in acetate-grown *Escherichia coli* cells and inhibits growth [9]. Accumulation of Hcy in cystathionine β-synthase-deficient yeast cells inhibits growth [10] and drastically reduces long-term cell viability [11]. The presence of exogenous Hcy has a profound inhibitory effect on the growth rate of wild-type yeast and *E. coli* cells. Growth inhibitory effects of Hcy are reversed by methionine [9, 10]. These observations suggest that Hcy interferes with fundamental biological processes common to all living cells.

In humans, Hcy, formed from dietary methionine as a by-product of cellular methylation reactions, is detoxified by folic acid-, vitamin B12-dependent remethylation to methionine [2], or vitamin B6-dependent transsulfuration 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 remethylation; enzymes of the transsulfuration pathway are not expressed in these tissues [12].

Hcy affects human tissues in several ways. For example, Hcy may promote thrombotic tendency by affecting the blood-clotting system [13–15]. Hcy stimulates vascular smooth muscle cell growth and inhibits proliferation of endothelial cells [16], possibly due to oxidative damage [17] or inhibition of methylation [18]. Acceleration of endothelial cell senescence [19], alterations in gene expression in vascular endothelial cells [7, 20–22] and increased collagen synthesis in smooth muscle cells [23, 24] induced by Hcy may also contribute to atherosclerosis. Neurotoxicity of Hcy through overstimulation of *N*-methyl-*D*-aspartate receptors [25, 26] or oxidative stress and DNA damage [27–30] was proposed to contribute to the pathogenesis of hyperhomocysteinemia. Hcy impairs endothelium-mediated nitric oxide-dependent vasodilatation [31, 32], possibly by causing accumulation of asymmetric dimethylarginine (ADMA) [33, 34], which inhibits nitric oxide synthesis [35]. In most of these studies, it is not clear whether the observed effects are due to Hcy itself or to an Hcy metabolite.

Hcy is perhaps the most reactive amino acid in biological systems [1, 2]. In addition to transmethylation to methionine or transsulfuration to cysteine (via cystathionine), Hcy can also be converted to other metabolites, such as AdoHcy, Hcy-thiolactone, Hcy-containing disulfides, homocysteic acid or *S*-nitroso-Hcy (fig. 1), that have been implicated in the pathology of hyperhomocystinemia [1].

Because of its similarity to the protein amino acid methionine, Hcy (fig. 2) can exert its biological effects by interfering with protein biosynthesis. Over the past decade protein biosynthesis-related pathways of human Hcy metabolism, involving Hcy-thiolactone and *S*-nitroso-Hcy (structures shown in fig. 2), have been discovered [36–42]. The purpose of this review is to summarize the fundamental biochemistry of these metabolites and to discuss mechanisms by which they can contribute to human disease.

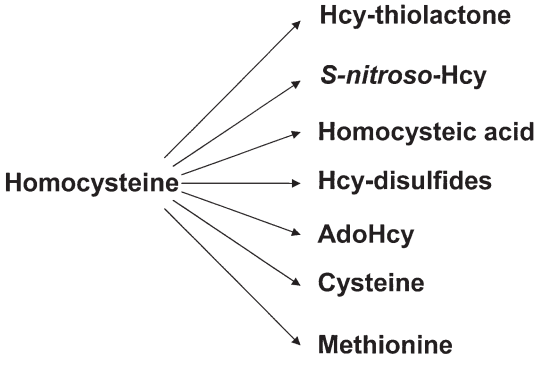


Figure 1. Metabolic conversions of homocysteine.

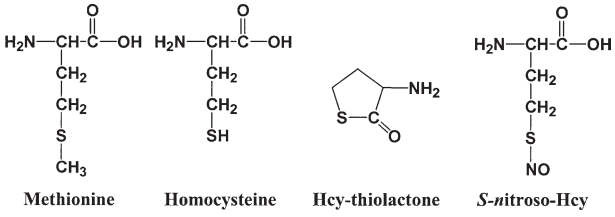
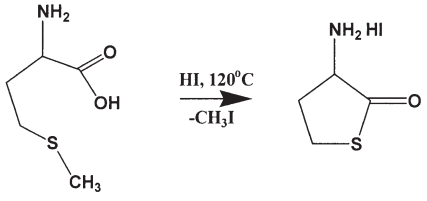


Figure 2. Chemical structures of methionine, homocysteine, Hcy-thiolactone and *S*-nitroso-Hcy.

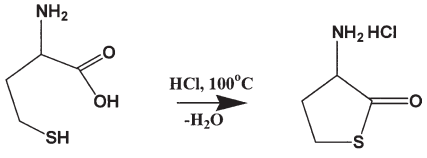
Chemical synthesis of Hcy-thiolactone

Hcy-thiolactone, a cyclic thioester of Hcy, is prepared chemically by intramolecular condensation of methionine or Hcy. For example, boiling with hydriodic acid for 4 h, a procedure originally developed for the determination of protein methionine [43], quantitatively converts methionine to Hcy-thiolactone with the liberation of methyl iodide (equation 1). The hydriodic acid digestion of [^{35}S]methionine has become a convenient method for the preparation of [^{35}S]Hcy-thiolactone for biological studies [44–50].



(1)

Intramolecular condensation of Hcy to Hcy-thiolactone occurs in the presence of hydrochloric acid (equation 2) [51]. The rate of the reaction depends on acid concentration and temperature. For example, in 0.6 N or 6 N hydrochloric acid at 100 °C, 50 % condensation occurs in 15 min or <5 min, respectively. Because Hcy-thiolactone, in contrast to Hcy, absorbs ultraviolet (UV) light (see below), the acid-dependent conversion to Hcy-thiolactone is a convenient procedure for the determination of Hcy in biological samples [11, 52].



(2)

Physicochemical properties of Hcy-thiolactone

Table 1 highlights differences in the physicochemical properties of Hcy-thiolactone, *S*-nitroso-Hcy and Hcy. The hydrochloric acid salt of Hcy-thiolactone is stable indefinitely at room temperature. Under physiological conditions (pH 7.4, 37 °C), Hcy-thiolactone (half-life of ~25 h, [44, 53]) is more stable than intermolecular aminoacyl-thioesters (e.g. methionyl-*S*-CoA hydrolyzes with a half-life of 2.25 h, [54]). In alkaline solutions Hcy-thiolactone quickly hydrolyzes to Hcy [55, 56]. For example, in 0.1 M NaOH, hydrolysis of Hcy-thiolactone is completed in 15 min at room temperature [47]. Like all thioesters [57], Hcy-thiolactone absorbs UV light with a maximum at 240 nm and $\varepsilon \sim 5000\ M^{-1}\ cm^{-1}$ in water (fig. 3), which allows its facile detection in biological samples [11, 58–60]. The pK_a of its amino group is unusually low at 7.1 [61]. Thus, under physiological conditions, Hcy-thiolactone is neutral and freely diffuses through cell membranes [10, 11, 44–46, 58–60, 62, 63]. The thioester bond of Hcy-thiolactone is highly susceptible to reactions with nucleophiles, in particular free amino groups in proteins (table 1). Under physiological conditions of pH and temperature, Hcy-thiolactone preferentially reacts with ε-amino groups of protein lysine residues [44–46], forming Hcy-ε*N*-Lys-protein (fig. 4). Other amino acid side chain groups in protein do not appreciably react with Hcy-thiolactone. In free lysine, the ε-amino group exhibits threefold greater reactivity than the α-amino group with Hcy-thiolactone (second-order rate constants 3.8 $M^{-1}\ h^{-1}$ and 1.2 $M^{-1}\ h^{-1}$, respectively; [45]). Lysine content of a protein is a major determinant of its reactivity with Hcy-thiolactone [45].

In addition, the α-amino group of Hcy thiolactone is highly reactive towards aldehydes, such as pyridoxal phosphate, *o*-phthalaldehyde and streptomycin (fig. 5). For example, the reaction of Hcy-thiolactone with streptomycin (0.1 mg/mL), routinely added to standard cell culture media as an antimicrobial agent, occurs with a half-life of 10 min [46]. The second-order rate constant for the reaction is 2000 $M^{-1}\ h^{-1}$, some 400 times faster than for the reaction of Hcy-thiolactone with lysine. The reaction of Hcy-thiolactone with *o*-phthalaldehyde, generating a fluorescent adduct, offers a sensitive method for

Table 1. Physicochemical properties of Hcy-thiolactone, *S-nitroso*-Hcy, and Hcy.

Property	Hcy-thiolactone	*S-Nitroso*-Hcy	Hcy
Chemical character	aminoacyl-thioester	*S-nitroso*-thiol	mercaptoamino acid
UV/Vis light absorption	yes, a maximum at $\lambda = 240$ nm [56]	yes, three maxima at $\lambda = 230$ nm, 330 nm, and 550 nm [66]	no significant absorption at $\lambda > 220$nm
Half-life in solution at ~pH 7.4, 37 °C	~30 h [44, 53]	>1 h [67]	2 h [46, 117]
pK_a of amino group	7.1 [61]	~9.5*	~9.5*
Chemical reactivity	– resistant to oxidation – reacts with protein amino groups [44–46, 53, 80, 86–89] – susceptible to base-catalyzed hydrolysis to Hcy [43] – reacts with aldehydes [46]	– donates a nitroso group to other thiols – decomposes to disulfides [69]	– is oxidized to disulfides – reacts with nitric oxide to form *S*-nitroso-Hcy [47, 67] – forms Hcy-thiolactone in the presence of an acid [51]

* Estimate based on pK_a values for related amino acids.

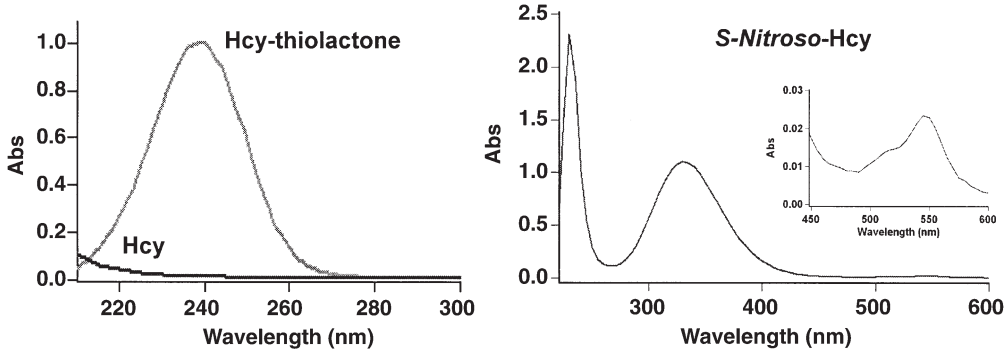


Figure 3. Absorption spectra of *L*-Hcy-thiolactone·HCl (0.2 mM) and *S*-nitroso-*L*-Hcy (1 mM) in water at room temperature. *D,L*-Hcy (0.2 mM), shown for comparison, does not appreciably absorb UV light above 220 nm.

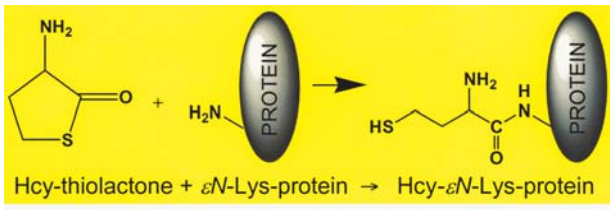


Figure 4. Hcy-εN-Lys-protein contains Hcy linked by amide bond to the ε-amino group of protein lysine residue.

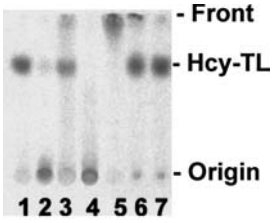


Figure 5. Reactivity of Hcy-thiolactone with aldehydes. [^{35}S]Hcy-thiolactone (10 µM) was incubated with or without 5 mM aldehyde for 30 min, pH 7.4, 23 °C. The products were separated by thin-layer chromatography (TLC). An autoradiogram of TLC separation of reaction products is shown. *Lane 1,* control, no aldehyde; *lane 2,* streptomycin; *lane 3,* pyridoxal; *lane 4,* pyridoxal 5′-phosphate; *lane 5, o*-phthalaldehyde, 2.5% ethanol; *lane 6,* all trans retinal, 75% ethanol; *lane 7,* control, no aldehyde, 75% ethanol [H. Jakubowski, unpublished data].

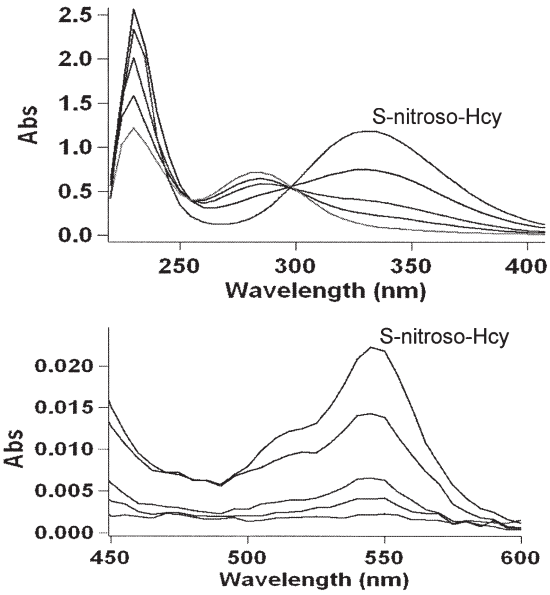


Figure 6. Decomposition of *S*-nitroso-Hcy (1 mM) in the presence of dithiothreitol (1 mM). *Upper trace* shows spectrum of *S*-nitroso-Hcy in the absence of dithiothreitol. This spectrum does not change during 1 h at room temperature. Lower traces show spectra of *S*-nitroso-Hcy taken 7 min, 14 min, 21 min and 28 min (*bottom trace*) after addition of dithiothreitol. Notice disappearance of absorption at wavelengths of 340 nm and 550 nm (characteristic for *S*-nitroso-Hcy), accompanied by a new absorption maximum at 280 nm [H. Jakubowski, unpublished data].

the determination of Hcy-thiolactone [64]. The exceptional reactivity of Hcy-thiolactone with aldehydes is most likely due to the low pK_a value of the α-amino group of Hcy-thiolactone, which is 2–3 units lower than the pK_a values for α-amino groups of amino acids (table 1).

Chemical synthesis and physicochemical properties of *S*-nitroso-Hcy

S-nitroso-Hcy can be prepared by mixing equimolar amounts of Hcy with sodium nitrite in the presence of hydrochloric acid [47, 65, 66]. *S*-Nitrosylation of Hcy is completed within 2 min at room temperature. The hydrochloride salt of *S*-nitroso-Hcy is relatively stable in solution and can be stored frozen for at least 2 weeks [47]. Like other *S*-nitroso-thiols [65], *S*-nitroso-Hcy absorbs both UV and visible light. In water, absorption maxima are at 230 nm ($\varepsilon = 2700\ M^{-1}\,cm^{-1}$), 340 nm ($\varepsilon = 1300\ M^{-1}\,cm^{-1}$) and at 550 nm ($50\ M^{-1}\,cm^{-1}$) (fig. 2). Because of the absorption of visible light (maximum at 550 mm), concentrated solutions of *S*-nitroso-Hcy are colored red.

S-nitroso-Hcy easily decomposes by transnitrosylation to other thiols, amines or hem. The transnitrosylation reactions are relatively fast [67]. For example, in the presence of dithiothreitol decomposition of *S*-nitroso-Hcy is completed within 30 min at room temperature (fig. 6). The half-life of *S*-nitroso-Hcy in human serum is about 1 h [68, 69], making it one the most stable *S*-nitrosothiols.

Biological formation of Hcy-thiolactone

In living organisms, the formation of Hcy-thiolactone is a consequence of error-editing reactions of aminoacyl-transfer RNA (tRNA) synthetases [10, 11, 38–42, 50, 58–60, 62, 63]. In protein biosynthesis, the nonprotein amino acid Hcy poses a selectivity problem because of its similarity to protein amino acids methionine (fig. 2), leucine and isoleucine. Indeed, Hcy enters the first step of protein biosynthesis and forms Hcy-AMP with methionyl, leucyl- and isoleucyl-tRNA synthetases. However, misactivated Hcy is never transferred to tRNA, and thus cannot enter the genetic code. Instead, Hcy-AMP is destroyed by editing activities of these aminoacyl-tRNA synthetases [70, 71], as indicated by the side reaction in equation (3).

$$\mathrm{AARS + Hcy + ATP \Leftrightarrow AARS \cdot Hcy \sim AMP + PP_i} \quad (3)$$
$$\downarrow$$
$$\text{Hcy-thiolactone}$$

Hcy editing is universal, occurs in all organisms investigated, from bacteria [62, 70] to humans [11, 63], and prevents direct access of Hcy to the genetic code [38, 39, 42].

The molecular mechanism of Hcy editing

Although studied in several systems, the molecular mechanism of Hcy editing is best understood for *E. coli* methionyl-tRNA synthetase (MetRS) [72–74]. The Hcy editing reaction occurs in the synthetic/editing active site [72], whose major function is to carry out the synthesis of Met-tRNA. Whether an amino acid completes the synthetic or editing pathway is determined by the partitioning of its side chain between the specificity and thiol-binding subsites [73] of the synthetic/editing active site. A subsite that binds carboxyl and α-amino groups of cognate or noncognate substrates does not contribute to specificity.

Methionine completes the synthetic pathway (fig. 7) because its side chain is firmly bound by the hydrophobic and hydrogen-bonding interactions with the specificity subsite. The crystal structure of the MetRS-Met complex [74] reveals that hydrophobic interactions involve side chains of Tyr15, Trp253, Pro257 and Tyr260; Trp305 closes the bottom of the hydrophobic pocket, but is not in the contact with the methyl group of the substrate methionine. The sulfur of the substrate methionine makes two hydrogen bonds: one with the hydroxyl of Tyr260 and the other with the backbone amide of Leu13.

The noncognate substrate Hcy, missing the methyl group of methionine, cannot interact with the specificity subsite as effectively as cognate methionine does. This allows the side chain of Hcy to move to the thiol-binding subsite, which promotes the synthesis of the thioester bond during editing (fig. 8). Mutations of Tyr15 and Trp305 affect Hcy/Met discrimination by the enzyme [72]. Asp52, which forms a hydrogen bond with the α-amino group of the substrate methionine, deduced from the crystal structure of MetRS-Met complex [74], is involved in the catalysis of both synthetic and editing reactions, but does not contribute to substrate specificity of the enzyme. The substitution Asp52Ala inactivates both the synthetic and editing functions of MetRS [72, 73].

The notion of the thiol-binding subsite is supported by the ability of MetRS to edit in trans, i.e. to catalyze thioester bond formation between a thiol and the cognate methionine. With CoA-SH or cysteine as a thiol substrate, MetRS catalyzes the formation of Met-*S*-CoA thioesters [54] and Met-Cys dipeptides [73], respectively. The formation of Met-Cys dipeptide proceeds via a Met-*S*-Cys thioester intermediate, which spontaneously rearranges to the Met-Cys dipeptide. The formation of Met-Cys dipeptide is as fast as the formation of Hcy-thiolactone during Hcy editing.

Hcy-thiolactone is synthesized by methionyl-tRNA synthetase in human cells

The first indication that Hcy-thiolactone is a significant component of Hcy metabolism in mammals, including humans, came with the discovery that Hcy-thiolactone is

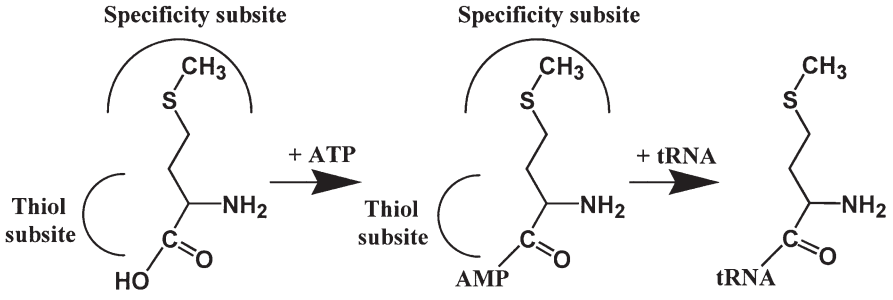


Figure 7. Aminoacylation of $tRNA^{Met}$ with methionine catalyzed by MetRS: methionine completes the synthesis pathway because its side chain is firmly bound to the specificity subsite of the enzyme and the thiol subsite is unoccupied.

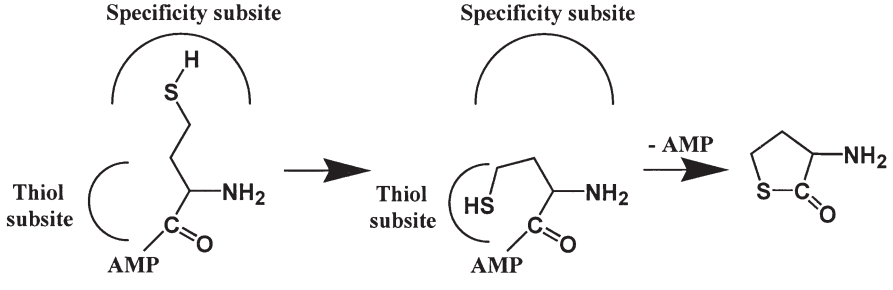


Figure 8. Editing of Hcy by MetRS: Hcy is edited because its site chain can enter the thiol subsite. The cyclization of Hcy-AMP to Hcy-thiolactone with the release of AMP in the synthetic/editing active site of MetRS is shown.

synthesized by cultured mammalian cells, such as human cervical carcinoma (HeLa), mouse adenocarcinoma (RAG) and Chinese hamster ovary (CHO) [63]. A temperature-sensitive MetRS mutant of CHO cells is unable to make Hcy-thiolactone at the nonpermissive temperature, which indicates that MetRS is involved in Hcy-thiolactone formation in CHO cells.

Subsequent work has shown that human diploid fibroblasts in which Hcy metabolism has been deregulated by mutations in the cystathionine β-synthase (CBS) gene produced more Hcy-thiolactone than wild-type fibroblasts [44]. In addition, supplementation of cultured human $CBS^{-/-}$, $CBS^{+/-}$, $CBS^{+/+}$ and human breast cancer (HTB-132) cells with the anti-folate drug aminopterin, which prevents remethylation of Hcy to methionine by methionine synthase, greatly enhanced Hcy-thiolactone synthesis in each culture. In general, human cancer cells produce more Hcy-thiolactone than normal cells [44, 63]. Further experiments with cultured human umbilical vein vascular endothelial cells (HUVECs) suggest that Hcy-thiolactone synthesis is important in human vascular tissues [46]. These experiments have shown that in the presence of physiological concentrations of Hcy, methionine and folic acid, HUVECs efficiently metabolize Hcy to Hcy-thiolactone. The extent of Hcy-thiolactone synthesis in human endothelial cells is directly proportional to Hcy, and inversely proportional to methionine concentrations, consistent with the involvement of MetRS. It should be noted that physiological levels of folic acid (26 nM) present in M199 media used in these studies are adequate for DNA synthesis and support growth of HUVECs when methionine is also present. However, these levels of folic acid are not sufficient for transmethylation of Hcy to methionine; as a result, Hcy is mostly converted to Hcy-thiolactone in these cells. Supplementation of HUVEC cultures with folic acid inhibits the synthesis of Hcy-thiolactone by lowering Hcy and increasing methionine concentrations in endothelial cells. The synthesis of Hcy-thiolactone in endothelial cell cultures is also inhibited by the supplementation with high-density lipoprotein (HDL), which carries Hcy-thiolactone hydrolyzing activity (see below).

Hcy-thiolactone is present in humans

The findings that cultured human cells, including vascular endothelial cells, have the ability to metabolize Hcy to Hcy-thiolactone suggest that Hcy-thiolactone is likely to be synthesized in vivo in a human organism. Indeed, highly selective and sensitive high-performance liquid chromatography (HPLC)- or gas chromatography/mass spectrometry (GC/MS)-based methods (table 2) have recently been developed and used successfully to demonstrate that Hcy-thiolactone is present in human plasma.

Table 2. Assay methods for determining of Hcy-thiolactone in human plasma.

– HPLC (cation exchange) with UV multiwavelength detection [11]
– HPLC (reverse-phase C30) with post-column OPA-derivatiza tion and fluorescence detection [64]
– GC/MS, pre-column derivatization with heptafluorobutyric acid anhydride [75]

The HPLC-based method exploits unique physicochemical properties of Hcy-thiolactone to achieve its separation, identification and quantification [11]. After initial plasma sample workup to remove major interfering contaminants, Hcy-thiolactone is subjected to HPLC on a reverse-phase C18 column or cation exchange PolySulphoethyl A column. Because Hcy-thiolactone is mostly neutral at physiological pH of 7.4, it is retained on a C18 column equilibrated with phosphate-buffered saline, pH 7.4. Elution of Hcy-thiolactone from a C18 column was achieved with a 1–5% gradient of acetonitrile or methanol. However, at pH <6 Hcy-thiolactone is positively charged and thus not retained on a C18 column, as expected. Free Hcy is not retained on a C18 column under any of the pH conditions examined. Because of its positive charge at pH <6, Hcy-thiolactone is retained on a cation exchange PolySulphoethyl A column equilibrated with dilute solutions of monosodium phosphate, pH 5. Elution of Hcy-thiolactone from the cation exchange column is achieved by increasing salt concentration. Detection is by monitoring effluent at multiple wavelengths, including A_{240}, the UV absorption maximum of Hcy-thiolactone ($\varepsilon = 5000\ M^{-1}\,cm^{-1}$). The detection limit is 5 pmol Hcy-thiolactone. Incorporating post-column reaction with *o*-phthalaldehyde followed by fluorescence detection [64] increases the sensitivity of Hcy-thiolactone detection from 5 pmol to 10 fmol [H. Jakubowski, unpublished data].

Using HPLC with UV detection, Hcy-thiolactone has been quantified in normal human plasma [11]. Three subjects had from 70 to 200 nM Hcy-thiolactone. Three other subjects had <20 nM Hcy-thiolactone. Hcy-thiolactone represented from <0.25 to 1.4% of total Hcy levels in these subjects.

An alternative HPLC-based method involves isocratic separation of Hcy-thiolactone on a reverse-phase C30 column at pH 2.0, followed by post-column derivatization with *o*-phthalaldehyde and fluorescence detection [64]. The detection limit is 200 fmol Hcy-thiolactone. So far, this method has been used only for the determination of Hcy-thiolactone in cultures of human hepatoma Hep G2 cells.

The GC/MS method involves sample preparation by silica solid-phase extraction and derivatization with heptafluorobutyric anhydride [75]. The derivative is analyzed and quantified by GC/MS using deuterated Hcy-thiolac-

tone as internal standard. The detection and quantification limits are 1.7 nM and 5.2 nM Hcy-thiolactone, respectively. Two subjects analyzed by the GC/MS method had 18 and 25 nM plasma Hcy-thiolactone, about 0.2% relative to plasma total Hcy levels. These amounts of plasma Hcy-thiolactone are in keeping with data from earlier studies [11].

Attempts to detect Hcy-thiolactone in human plasma [53, 76–78] were made in the past, well before metabolism of Hcy-thiolactone in humans was deciphered and its physiological significance established. However, the sensitivity of methods used then was very poor, with a detection limit 10–50 μM Hcy-thiolactone, or >1000-fold worse then the present-day methods. Since we now know that human plasma levels of Hcy-thiolactone are 15–200 nM [11, 75], it is not surprising that the early attempts to detect Hcy-thiolactone were not successful. These attempts were sparked by an outlandish claim [77], later retracted by the authors [78], that extremely high Hcy-thiolactone concentrations, 0.032–6.7 mM in normal subjects and 47 mM in patients with coronary artery disease, are present in human plasma.

It has been reported that radioactivity cocrystallizing with Hcy-thiolactone is present in liver extracts from guinea pigs previously injected with radioactive methionine [79]. However, the nature of this cocrystallizing radioactivity is unclear [80] because putative radioactive Hcy-thiolactone could not be removed from the chloroform-methanol extract by an aqueous wash procedure (a procedure that removes authentic Hcy-thiolactone, e.g. [80, 81]) and no other experimental evidence has been provided.

Biological significance of *S*-nitroso-Hcy

S-Nitrosothiols are important mediators of nitric oxide-dependent signaling pathways [82]. Nitric oxide, produced by the endothelial nitric oxide synthase, reacts with thiols to produce *S*-nitrosothiols [83]. In humans, the concentration of plasma *S*-nitrosothiols, which circulate at low micromolar concentrations, is similar to that of plasma total Hcy [83]. Individual *S*-nitrosothiols are present in proportion to the concentration of free thiol. *S*-nitrosoalbumin, present in plasma of an average person at ~6 μM, appears to be a major plasma *S*-nitrosothiol. *S*-nitrosocysteine and *S*-nitrosoglutathione are present at ~250 nM and ~180 nM, respectively. *S*-nitroso-Hcy is also likely to exist in vivo, but has not yet been detected in humans.

Biological relevance of *S*-nitroso-Hcy has been highlighted by the discovery that cultured human vascular endothelial cells have the ability to support *S*-nitrosylation of Hcy [67, 68]. Incubation of endothelial cells, activated to stimulate the production of nitric oxide, with 1 mM Hcy results in the formation of bioactive *S*-nitroso-Hcy. In addition, an *S*-nitroso-Hcy-mediated mechanism is responsible for the occurrence of Hcy, linked by peptide bonds, in endothelial cell protein [38, 47] (see the following section).

Because *S*-nitroso-Hcy has antiplatelet activity, antiproliferative activity on smooth muscle cells, prevents leukocyte adhesion and protects against endothelial cell apoptosis, it has been suggested that the conversion of Hcy to *S*-nitroso-Hcy would be beneficial and protective against atherogenicity of Hcy [67, 68]. It has also been suggested that formation of *S*-nitroso-Hcy would stabilize nitric oxide and therefore enhance its effects. For example, in a bioassay with precontracted vessel rings, *S*-nitroso-Hcy (at 250 nM) was found to be 16-fold more potent than *S*-nitroso-Cys as a vasorelaxant [83]. In addition, *S*-nitrosylation of Hcy prevents it from forming disulfides with protein and other thiols and from cyclization to form Hcy-thiolactone in biological systems, which has also been suggested to be beneficial [67].

However, *S*-nitroso-Hcy can be detrimental because of its ability to mediate incorporation of Hcy into cellular protein (see the following). In this regard, it is known that *S*-nitroso-Hcy is toxic to rat cerebrocortical neurons [84]. The toxicity is abrogated by excess Hcy or other thiols [84], which greatly diminish effective concentration of *S*-nitroso-Hcy. Although it has been suggested that *S*-nitroso-Hcy toxicity is due to its ability to support the formation of peroxynitrite, it is not excluded that the incorporation of *S*-nitroso-Hcy into protein also plays a role.

Incorporation of *S*-nitroso-Hcy into protein

In terms of chemical structure, *S*-nitroso-Hcy is very similar to the protein amino acid methionine (fig. 2). This similarity raises a possibility that *S*-nitroso-Hcy can replace methionine in protein biosynthesis. Because is does not have a free thiol, *S*-nitroso-Hcy, in contrast to Hcy, is not expected to be edited (converted to Hcy-thiolactone) by MetRS and could therefore be transferred to tRNA. Nitric oxide-mediated incorporation of Hcy into tRNA and protein has been examined in vitro using purified bacterial MetRS, rabbit reticulocyte translation system, and cultured bacterial or human endothelial cells.

S-Nitroso-Hcy is a substrate for bacterial MetRS in the aminoacyl-adenylate formation reaction (fig. 9) [47]. Catalytic efficiency for *S*-nitroso-Hcy is intermediate between those for Hcy and methionine, i.e. *S*-nitroso-Hcy is a 10-fold better substrate than Hcy and 10-fold worse substrate than methionine. The MetRS-bound *S*-nitroso-Hcy-AMP intermediate is as stable as the MetRS-bound Met-AMP and, in contrast to the Hcy-AMP intermediate, is not edited (fig. 10B).

S-Nitroso-Hcy is also a substrate in the tRNA aminoacylation reaction catalyzed by MetRS (fig. 9) [47]. Catalytic

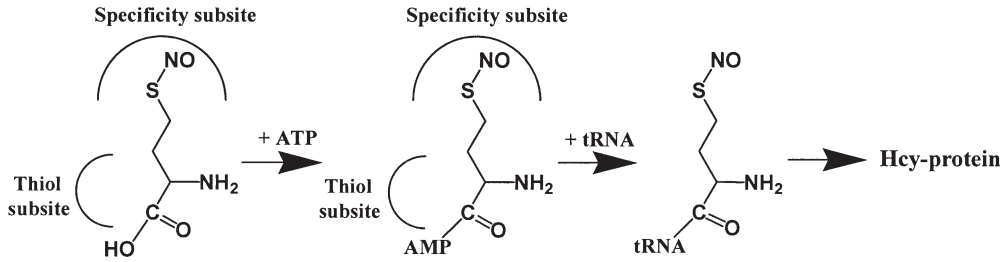


Figure 9. *S*-nitroso-Hcy-mediated incorporation of Hcy into protein. After formation of *S*-nitroso-Hcy-AMP in the active site of MetRS, the side chain of *S*-nitroso-Hcy remains in the specificity subsite. This allows the transfer of *S*-nitroso-Hcy from the adenylate to $tRNA^{Met}$. Thus formed, *S*-nitroso-Hcy-$tRNA^{Met}$ participates in ribosomal protein biosynthesis by donating *S*-nitroso-Hcy into growing polypeptide chains at positions specified by methionine codons. Removal of the nitroso group yields proteins containing Hcy linked by peptide bonds (Hcy-protein).

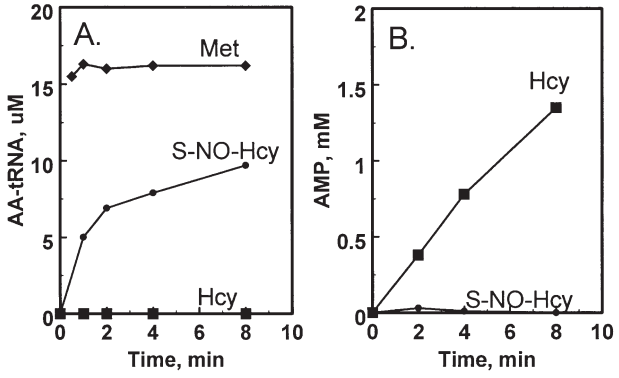


Figure 10. *S*-Nitroso-Hcy is a substrate for aminoacylation of $tRNA^{Met}$, but not for editing, catalyzed by MetRS [41]. (*A*) Incorporation of [*S*-nitroso-^{35}S]Hcy (•), [^{35}S]Hcy (■), [^{35}S]Met (♦) into $tRNA^{Met}$. (*B*) *S*-nitroso-Hcy- (•) and Hcy-dependent (■) editing activity [47].

efficiency for *S*-nitroso-Hcy is 80-fold lower than that for methionine. Both initiator and elongator tRNAs are aminoacylated with *S*-nitroso-Hcy to the same extent (60%) (fig. 10A). *S*-Nitroso-Hcy-tRNA can be easily isolated from reaction mixtures by phenol extraction. Like Met-tRNA, *S*-nitroso-Hcy-tRNA undergoes slow deacylation (half life ~ 27 min) with the release of free *S*-nitroso-Hcy at pH 7.4, 37 °C. *S*-Nitroso-Hcy-tRNA can be converted to Hcy-tRNA by a treatment with an excess thiol (e.g. 1 mM Hcy or glutathione).

Hcy-tRNA is the least stable aminoacyl-tRNA known (half-life ~ 15 s) and spontaneously decomposes with the release of Hcy-thiolactone and deacylated tRNA [47].

It has been reported that rabbit liver extracts contain an Hcy-tRNA synthetase activity, which catalyzes incorporation of Hcy into a trichloroacetic acid-precipitable material [85]. The authors also report that they were able to isolate Hcy-tRNA, which yielded mostly free Hcy upon deacylation. In view of the extremely short half-life (~ 15 s) of Hcy-tRNA, which spontaneously releases Hcy-thiolactone [47], it is unclear how this could have been achieved.

Once an amino acid is attached to tRNA, it is destined to enter ribosomal protein biosynthesis and become incorporated into protein [38, 42, 63]. Thus, facile formation of *S*-nitroso-Hcy-tRNA catalyzed by MetRS suggests that *S*-nitroso-Hcy will be incorporated into protein. Indeed, supplementation with *S*-nitroso-Hcy of cultures of recombinant *E. coli* strain expressing mouse dihydrofolate reductase (DHFR) results in incorporation of *S*-nitroso-Hcy into the DHFR protein [47]. Hcy, added to DHFR-expressing cultures, is not incorporated into protein. *S*-nitroso-Hcy-tRNA supports biosynthesis of human globin or firefly luciferase proteins in a rabbit reticulocyte cell-free translation system programmed with human globin mRNA or firefly luciferase mRNA, respectively [47]. Removal of the nitroso group by treatment with an excess thiol results in Hcy-protein (fig. 9) containing Hcy at positions normally occupied by methionine (fig. 11).

Experiments examining the metabolism of Hcy in cultures of HUVECs suggest that nitric oxide-mediated incorporation of Hcy into protein is likely to occur in the human vascular system. For example, about 50% of total Hcy incorporation into protein in HUVEC cultures is insensitive to Edman degradation [38], a procedure that liberates from protein an amino acid with a free α-amino group. This suggests that ~ 50% of total Hcy incorporation occurs into peptide bonds within a polypeptide chain, and is most likely *S*-nitroso-Hcy mediated. Hcy incorporation into protein occurs in cultures containing physio-

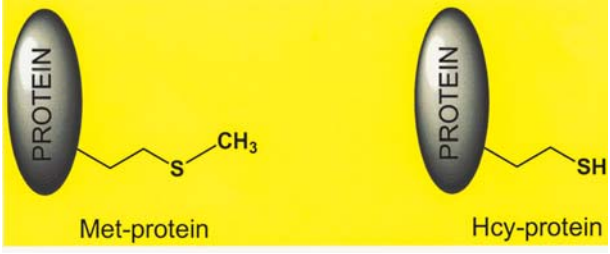


Figure 11. Hcy-protein contains Hcy linked by peptide bonds at positions normally occupied by methionine.

logical concentration, 28 nM, of folic acid. Supplementation of HUVEC cultures with 10 μM folic acid prevents incorporation of Hcy into protein [38, 46].

Hcy-thiolactone-mediated incorporation of Hcy into protein

The propensity of Hcy-thiolactone to react with primary amino groups [45] was recognized shortly after its chemical synthesis [43, 51]. Subsequently, *N*-acetylated derivative of Hcy-thiolactone was utilized for chemical modification of proteins [86, 87]. Modification with Hcy-thiolactone was used to label γ-globulins with mercury for electron microscopy applications [88, 89]. Some investigators, apparently unaware of its chemical reactivity, have been using Hcy-thiolactone instead of Hcy in biological experiments. The problems with infusing Hcy-thiolactone in experimental animals as a model of clinical homocystinuria were brought to light by Dudman et al., who showed that Hcy-thiolactone acylates bovine serum albumin [80] and human serum protein [53].

Because its biological role was discovered relatively recently, the reactivity of Hcy-thiolactone under physiological conditions remained virtually unexplored till the end of 1990s. The discovery that Hcy-thiolactone and proteins containing Hcy linked by amide or peptide bonds (Hcy-*N*-protein) are formed by cultured mammalian, including human, cells has led to a hypothesis that the chemical reactivity of Hcy-thiolactone may underlie the involvement of Hcy in the pathology of human vascular diseases [44]. This in turn prompted detailed studies of reactions of Hcy-thiolactone with proteins [45].

Hcy-thiolactone added to human serum disappears within ~ 3 h, mostly due to two reactions: formation of Hcy-*N*-protein adducts by homocysteinylation of ε-amino groups of protein lysine residues (fig. 4) [44, 45], and enzymatic hydrolysis to Hcy [48, 49], which then forms an Hcy-*S*-protein disulfide adduct, mostly with the Cys34 of serum albumin. No adducts of Hcy with other amino acid residues in serum proteins are formed. The α-amino group of N-terminal aspartate in human serum albumin does not react with Hcy-thiolactone. Each individual protein becomes *N*-homocysteinylated in proportion to its abundance in serum [45]. Second-order rate constants for reactions of Hcy-thiolactone with individual purified proteins indicate that *N*-homocysteinylation is relatively robust and goes to completion within a few hours at physiological conditions of pH and temperature. A major determinant of the reactivity of most proteins with Hcy-thiolactone is their lysine content. For proteins that vary in size from 104 to 698 amino acid residues, there is a very good correlation ($r = 0.97$) between protein lysine content and their reactivity with Hcy-thiolactone. Larger proteins, such as fibrinogen (3588 amino acid residues) and low-density lipoproteins (LDLs) (~ 5000 amino acid residues), react with Hcy-thiolactone roughly sixfold less efficiently than expected from their lysine contents.

Hcy-*N*-protein is a component of human Hcy metabolism

Evidence from tissue culture studies

The first indication that Hcy-*N*-protein is likely to be an important component of Hcy metabolism in humans came from studies of Hcy-thiolactone metabolism in human tissue cultures [44]. Proteins from normal and CBS-deficient fibroblasts and breast cancer cells have been shown to contain small amounts of Hcy-*N*-protein (0.4–2.4% relative to protein Met). When metabolic conversion of Hcy to Met was limited by the anti-folate drug aminopterin, the amounts of Hcy-*N*-protein increased.

Further experiments with cultured HUVECs provide evidence that the formation of Hcy-*N*-protein is likely to be important in human vascular tissues [46]. These experiments show that the formation of Hcy-*N*-protein occurs concomitantly with the synthesis of Hcy-thiolactone in the presence of physiological concentrations of Hcy, methionine and folic acid. Like the levels of Hcy-thiolactone, levels of Hcy-*N*-protein are directly proportional to Hcy, and inversely proportional to methionine, concentrations. Supplementation of HUVEC cultures with folic acid inhibits the synthesis of extracellular and intracellular Hcy-*N*-protein by facilitating conversion of Hcy to methionine, thereby indirectly preventing synthesis of Hcy-thiolactone. The formation of extracellular, but not intracellular, Hcy-*N*-protein in endothelial cell cultures is inhibited by supplementation with HDL, which carries an Hcy-thiolactone hydrolyzing enzyme (see below).

The mode of Hcy incorporation into endothelial cell protein has been established by using Edman degradation, a classic protein chemistry procedure which releases from proteins amino acids having free α-amino group. About half of total Hcy incorporated into protein was found to be sensitive to Edman degradation [38, 46], suggesting that Hcy incorporation is due to reaction of Hcy-thiolactone with protein lysine residues (fig. 4) [44, 45]. The presence of a fraction of Hcy-*N*-protein that is resistant to Edman degradation suggests that translational, *S*-nitroso-Hcy-mediated, incorporation of Hcy into protein (figs 9, 11) also occurs in HUVEC cultures.

Hcy-*N*-protein is present in human body

These tissue culture experiments raised a possibility that Hcy-*N*-protein is likely to occur in human body. To examine this possibility, highly selective and sensitive HPLC-based methods for the determination of Hcy-*N*-protein were recently developed [81, 90]. Initial protein

sample workup removes free and disulfide-linked Hcy by extensive treatments with dithiothreitol. One method relies on the conversion of Hcy-*N*-protein to Hcy-thiolactone, which is achieved by acid hydrolysis under reducing conditions (in the presence of dithiothreitol). Hcy-thiolactone is then purified and quantified by HPLC on a cation-exchange column with multiwavelength diode array UV detection, including A_{240} [81].

The discovery that Hcy linked by amide or peptide bond (Hcy-*N*-protein) is present in human plasma proteins was first described in 2000 [37]. Subsequent studies have shown that Hcy-*N*-protein is present in purified serum albumin from various organisms, including humans. Hcy-*N*-protein occurs in all purified individual human blood proteins examined so far [81]. The highest amounts of Hcy-*N*-protein, 0.6 and 0.36% are present in human hemoglobin, serum albumin and γ-globulin, respectively. Other serum proteins, such as HDL, LDL, fibrinogen, transferrin and antitrypsin contain from 0.04 to 0.1% Hcy-*N*-protein. Hcy-*N*-hemoglobin is present in normal blood at a concentration of 12.7 μM [81], which constitutes a major reservoir of Hcy in human blood.

The levels of Hcy-*N*-protein in individual human blood proteins correlate with the reactivity of these proteins toward Hcy-thiolactone, but whether the presence of Hcy-*N*-protein is due to Hcy-thiolactone- or *S*-nitroso-Hcy-mediated incorporation, or both, is not known. However, the presence of Hcy-*N*-protein in pig albumin [81], which does not contain methionine, strongly suggests that Hcy-thiolactone is responsible for Hcy incorporation into albumin in the pig.

Human plasma from about two dozen subjects contains from 0.1 to 13 μM Hcy-*N*-protein, which represents up to 25% of total plasma Hcy [81]. Plasma concentrations of Hcy-*N*-protein correlate positively with tHcy, suggesting that plasma tHcy level is a determinant of Hcy-*N*-protein level (fig. 12). Interestingly, in some human subjects, plasma levels of Hcy-*N*-protein are lower than expected from their tHcy content (open circles in fig. 12); this suggests that factors other than tHcy also affect plasma Hcy-*N*-protein levels [81]. A likely candidate for a determinant of plasma Hcy-*N*-protein levels, is Hcy-thiolactonase activity [48], which has been shown to affect formation of Hcy-*N*-protein in HUVEC cultures [46] and in human serum in vitro [49].

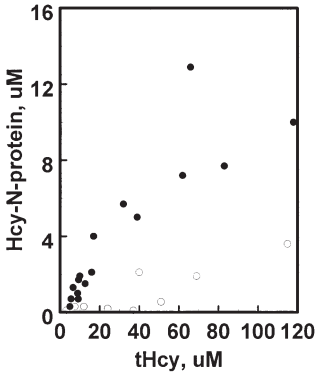


Figure 12. Plot of individual values of plasma Hcy-*N*-protein versus tHcy for human subjects [82]. See text for discussion.

An alternative method for the determination of Hcy-*N*-protein involves reaction with 4-fluoro-7-sulfamoyl-benzofurazon (ABD-F), which derivatizes Hcy, followed by acid hydrolysis to liberate the fluorescent Hcy-*S*-ABD adduct, which is then purified and quantified by HPLC on a C18 reverse-phase column with fluorescent detection [90]. Using this method, 0.51 μM Hcy-*N*-protein has been detected in 20 healthy adults, ~4% relative to plasma total Hcy. Fifteen hemodialysis patients had 0.74 μM Hcy-*N*-protein, ~2% relative to plasma total Hcy [90].

A possibility of incorporation of Hcy into protein was raised in the 1960s, shortly after the identification of CBS deficiency in humans (discussed in [91]). However, no Hcy was detected in acid hydrolysates of hair from three CBS-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 present in human hemoglobin (1 Hcy residue per 1000 methionine residues) [81] would have not been detected by using standard protein amino acid analysis methods.

It has been reported that acid hydrolysis of plasma (4 N hydrochloric acid, 110 °C, 5 h) released 958 μM Hcy in survivors of myocardial infarction and 38 μM in control subjects [92]. Other investigators could not reproduce these results and demonstrated that such apparently high Hcy levels were an artifact due to incomplete hydrolysis of serum protein and incorrect identification of peaks on chromatograms of amino acid analyses [93, 94]. The report [92] was subsequently retracted by the author [95].

Modification by Hcy-thiolactone causes protein damage

Substitution of the ε-amino group of a protein lysine residue with an Hcy residue containing a free thiol group (fig. 4) is expected to affect protein structure and function. Indeed, hemoglobin, albumin [R. Glowacki and H. Jakubowski, unpublished data] and cytochrome c [45] are sensitive to *N*-homocysteinylation; incorporation of one Hcy/mol protein induces gross structural alterations in these proteins. Hcy-*N*-cytochrome c becomes prone to aggregation due to intermolecular disulfide bond formation [45]. Hcy-*N*-hemoglobin, in contrast to unmodified hemoglobin, is susceptible to further irreversible damage

by oxidation (fig. 13). Of the two physiological forms of human albumin, Cys-*S*-albumin (containing cysteine in a disulfide linkage with Cys34 of albumin) is modified faster than mercaptoalbumin (containing Cys34 with free thiol) by Hcy-thiolactone. Hcy-thiolactone-modified and -unmodified forms of albumin exhibit different susceptibilities to proteolytic degradation by trypsin, chymotrypsin and elastase. Among many possible sites in albumin, Lys525 is a target for modification by Hcy-thiolactone.

The function of other proteins appears not to be significantly affected by incorporation of a single Hcy residue. However, incorporation of multiple Hcy residues does affect function. For example, complete loss of enzymatic activity occurs after *N*-homocysteinylation of 8 lysine residues in MetRS (33% of total lysines) or 11 lysine residues in trypsin (88% of total lysines) [45]. Extensively *N*-homocysteinylated proteins, such as myoglobin, transferin, globulins, fibrinogen, RNase A and trypsin, are prone to multimerization and undergo gross structural changes that lead to their denaturation and precipitation [45]. Chicken egg lysozyme is denatured by extensive *N*-homocysteinylation [96].

The metabolism of LDL is not significantly affected by mild *N*-homocysteinylation. Hcy-*εN*-LDL containing eight molecules of Hcy/mol LDL is taken up and degraded by leukemic L2C guinea pig lymphocyte cells in vitro to the same extent as native LDL via the high-affinity LDL-specific receptor pathway [97]. However, highly *N*-homocysteinylated LDL, in which 10 or 25% lysine residues have been modified (i.e. containing 36 and 89 mol Hcy/mol LDL), is taken up and degraded by human monocyte-derived macrophages significantly faster than native LDL [98]. Additional thiol groups present in *N*-homocysteinylated LDL (containing 125 mol Hcy/mol LDL) protect LDL lipids against oxidation [99].

Hcy-thiolactone may also inactivate enzymes by other mechanisms. For example, lysine oxidase, an important enzyme responsible for posttranslational collagen modification essential for the biogenesis of connective tissue matrices, is inactivated by micromolar concentrations of Hcy-thiolactone, which derivatizes the active site tyrosinequinone cofactor with a half-life of 4 min [100]. The inactivation of lysine oxidase by Hcy-thiolactone might play a role in skeletal abnormalities seen in homocystinuric children [1].

Hcy-thiolactone-modified proteins are immunogenic

Hcy-thiolactone-modified proteins can be physiologically detrimental and elicit an immune response, as shown by injecting rabbits with LDL [101] or keyhole limpet hemocyanin [KLH; A. Undas and H. Jakubowski, unpublished data] which has been modified with Hcy-thiolactone. Polyclonal rabbit anti-Hcy-*N*-LDL antibodies bind to extensively *N*-homocysteinylated rabbit LDL, hemoglobin and albumin, in which 40% lysine residues had been modified with Hcy-thiolactone [101]. Whether these antibodies bind to less extensively modified proteins has not been determined.

Polyclonal anti Hcy-*N*-protein antibodies prepared by inoculating rabbits with Hcy-*εN*-Lys-KLH recognize Hcy-*εN*-Lys-epitope on human proteins (Hcy-*εN*-Lys-hemoglobin or Hcy*εN*-Lys-albumin) containing one mol Hcy/mol protein. Specificity of anti-Hcy-*εN*-Lys-protein antibodies is demonstrated by competitive enzyme-linked immunosorbent assay (ELISA) experiments, which show that Hcy-*εN*-Lys, but not Hcy-*αN*-Lys, prevents the rabbit antibody from binding to human Hcy-*εN*-Lys-hemoglobin [A. Undas and H. Jakubowski, unpublished data]. Preimmune rabbit serum exhibits significant

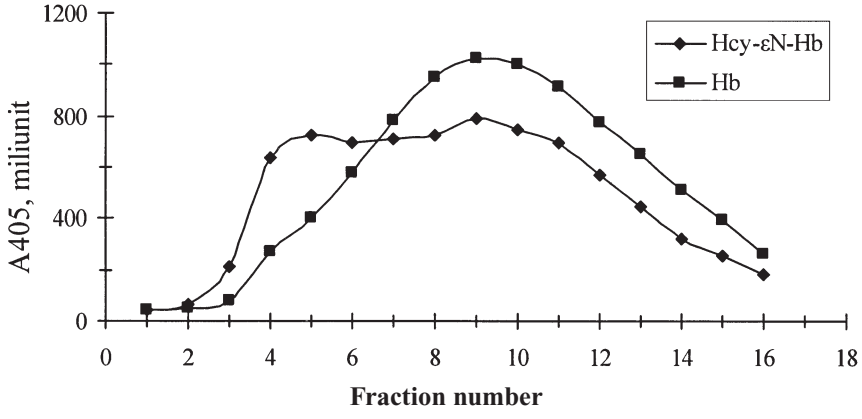


Figure 13. Hcy-*εN*-hemoglobin (Hcy-*εN*-Hb) is more susceptible to oxidation than native hemoglobin (Hb). Hcy-thiolactone-modified Hb, containing 1 mol Hcy/mol Hb, and native Hb were treated with 10-fold molar excess hydrogen peroxide for 15 min and subjected to gel filtration on a Sephacryl S-300 column. Oxidized Hcy-*εN*-Hb elutes as a high molecular weight aggregate in fractions 3–5 [R. Glowacki and H. Jakubowski, unpublished data].

titers of autoantibodies against Hcy-*εN*-Lys-protein [A. Undas and H. Jakubowski, unpublished data], which is consistent with the presence of Hcy bound by amide linkage in rabbit blood proteins [81].

Autoantibodies against Hcy-*εN*-Lys-protein are present in humans

As discussed above, Hcy-thiolactone [11], Hcy-*N*-hemoglobin and Hcy-*N*-albumin are present in human blood [81]. This suggests that autoantibodies against Hcy-*εN*-Lys-protein are also likely to be present in humans. Indeed, every human serum tested shows some titer of autoantibody against Hcy-*εN*-Lys-hemoglobin or Hcy-*εN*-Lys-albumin. Plasma levels of anti-Hcy-*εN*-Lys-protein autoantibodies appear to be positively associated with plasma tHcy levels (fig. 14**)**. Competitive ELISA experiments show that Hcy-*εN*-Lys-proteins and Hcy-*εN*-Lys itself serve as effective competitors. Unmodified proteins as well as Hcy, lysine, LysAla and Ac-*εN*-Hcy-*αN*-Lys do not compete with the human autoantibody binding [A. Undas and H. Jakubowski, unpublished data].

Interstingly, preliminary studies suggest that moderate hyperhomocysteinemia is associated with neopterin, a marker of immune activation [102]. Whether Hcy-*N*-protein, Hcy-related oxidative stress or other stimulus might be responsible for this association remains to be examined.

Hcy-thiolactone is cytotoxic

Infusion with Hcy-thiolactone was used as an early model of clinical homocystinuria in 1970s. Vascular change in response to Hcy-thiolactone infusions occurred in some animal models, but not in others. For example, baboons chronically infused with Hcy-thiolactone developed atherosclerosis [103, 104]. However, infusions with Hcy-thiolactone failed to induce atherosclerosis in rabbits [105, 106] and pigs [107]. Although unexplained at that time, these differences in sensitivity to Hcy-thiolactone could have been likely due to differences in Hcy-thiolactone metabolism among animal species. We now know that living organisms do differ in their levels and activity of Hcy-thiolactonase, a recently discovered enzymatic activity that detoxifies Hcy-thiolactone [48]. Rabbits have about 10-fold more Hcy-thiolactonase activity than an average human does [49], and therefore would be expected to be more resistant to Hcy-thiolactone than primates.

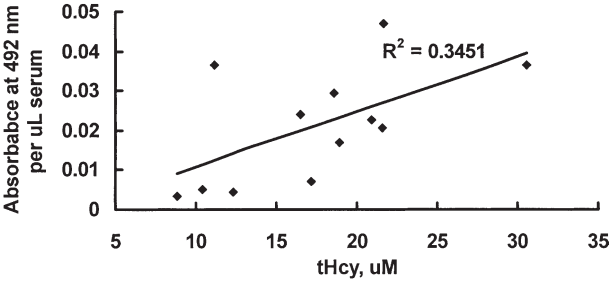


Figure 14. Plot of individual values of serum levels of anti-Hcy-*N*-protein autoantibodies versus tHcy for human subjects [A. Undas and H. Jakubowski, unpublished data].

Hcy-thiolactone is known to be acutely toxic to the central nervous system in experimental animals. Although the mechanism of its action is not entirely clear [108, 109], the toxicity of Hcy-thiolactone has been suggested to be due to its metabolism to homocysteic acid, a potent neurotransmitter [110]. However, Hcy, which is nerotoxic to cortical cultures of mixed neurons and glia from embryonic rats, is not metabolized to homocysteic acid in these cultures [26]. About 50% of neuronal cell death occurs after 6-day exposure to 0.1 mM *D,L*-Hcy [26]. Other cell culture and animal studies have shown that Hcy induces cell death, and potentiates amyloid *β*-peptide toxicity, in neurons [8, 27–30]. Whether Hcy is metabolized to Hcy-thiolactone in these experimental systems has not been examined.

Hcy-thiolactone, injected intravenously into mice and rats (in one dose over 1 min) as a possible radio-protectant in studies of tumor therapy, is extremely neurotoxic [111]. For example, at 200 mg Hcy-thiolactone/kg many mice developed immediate seizures followed by death within minutes. At 350 mg/kg, all animals developed seizures and died. At doses of 100 mg/kg or below, mice developed only mild somnolescence, and no long-term effects were observed within 30 days.

Exposure of mouse embryos to Hcy-thiolactone (0.5 mM and above) causes increased lethality, growth retardation, blisters and abnormalities of somite development [112]. *L*-Hcy, but not the *D*-form, is toxic to rat embryos; however, both *L*- and *D*-forms of Hcy-thiolactone are toxic [113]. The stereospecific embryotoxicity of *L*-Hcy is consistent with the stereospecificity of MetRS, which converts only *L*-Hcy to *L*-Hcy-thiolactone. On the other hand, embryotoxicity of both *L*- and *D*-forms of Hcy-thiolactone is consistent with identical chemical reactivity of each stereoisomer of Hcy-thiolactone towards proteins. Toxicity of Hcy to chicken embryos [25, 114] can also be due to the conversion to Hcy-thiolactone. In fact, Hcy-thiolactone, which is unlikely to be efficiently hydrolyzed to Hcy in the chicken due to lack of Hcy-thiolactonase [41, 48], is also toxic to chicken embryos [114]. These observations suggest that chemical reactivity towards components of cells is responsible for the toxicity of Hcy-thiolactone.

Tissue culture studies show that Hcy-thiolactone induces apoptotic death in human vascular endothelial [115] and promyeloid HL-60 cells [116]. Apoptosis is also induced

by Hcy, but at much higher concentrations (3 mM, [7]) than Hcy-thiolactone (0.05 mM, [115]). Hcy-thiolactone has also been shown to inhibit insulin signaling in HTC rat hepatoma cells transformed with insulin receptor [117].

Toxicity has usually been observed after acute exposure to Hcy-thiolactone concentrations far exceeding the concentrations that are present in humans. Thus, it might be unclear whether acute Hcy-thiolactone toxicity, observed within hours or days, is relevant for chronic hyperhomocysteinemia in humans, whose detrimental effects are manifested after decades of exposure. However, it is likely that the small amount of damage caused by mild elevations in Hcy-thiolactone levels could accumulate to harmful levels over extended periods of time required for the development of atherosclerosis or Alzheimer's disease.

Possible protective mechanisms against incorporation of Hcy into protein

Living organisms possess defensive mechanisms against endogenous and exogenous toxic agents. These mechanisms involve metabolism of toxicants to facilitate their elimination form the organism. Because protein *N*-homocysteinylation is detrimental to protein function and could cause cell and tissue damage, protective mechanisms that minimize incorporation of Hcy into protein are likely to exist. The first line of defense against Hcy toxicity apparently involves its removal by transmethylation to methionine and transsulfuration to cysteine, most of which occurs in the liver [1]. Possible candidates for the second line of defense against Hcy toxicity would be serum albumin, hemoglobin and HDL.

Serum albumin, by forming Hcy-*S*-albumin disulfide, in which Hcy is linked to Cys34 of albumin [118], would limit cellular uptake of Hcy and its conversion to Hcy-thiolactone or *S*-nitroso-Hcy. In fact, Hcy-*S*-albumin represents about 80% of total plasma Hcy in normal human subjects, and plasma-free Hcy comprises only 2% total Hcy [119]. In addition, serum albumin or hemoglobin, the most abundant blood proteins, would effectively detoxify a significant fraction of Hcy-thiolactone by virtue of *N*-homocysteinylation [48], which occurs in humans. Indeed, Hcy-*N*-hemoglobin and Hcy-*N*-albumin are present in normal human blood at 12.7 μM and 2.8 μM, respectively [81]. How Hcy-*N*-protein and Hcy-*S*-protein are cleared from the circulation remains to be determined. One possibility is that Hcy-*N*-protein and Hcy-*S*-protein would be taken up and turned over by the liver, where most of total body Hcy is converted to methionine and cysteine [1].

HDL is likely to be protective because it carries Hcy-thiolactonase/paraoxonase, which hydrolyzes Hcy-thiolactone to Hcy, thereby minimizing protein *N*-homocysteinylation [46, 48, 49]. When human serum is separated into lipoprotein fractions, all Hcy-thiolactone hydrolyzing activity is found associated with HDL fraction and absent in LDL fraction [41, 48]. Purification of Hcy-thiolactonase to homogeneity can only be achieved by using nonionic detergents [48]. The Hcy-thiolactonase enzyme is present in mammals, but not in birds. *N*-Terminal sequencing of purified human Hcy-thiolactonase indicates that it is identical to paraoxonase, a product of the *PON1* gene [48]. Hcy-thiolactonase activity is absent in *PON1* knockout mice [41, 48], which also are more susceptible to atherosclerosis than their wild-type littermates [120]. Human *PON1* has several genetic polymorphisms, two of which result in amino acid substitutions at positions 55 and 192 in PON1 proteins. Hcy-thiolactonase activity is strongly associated with *PON1* genotype in human populations [49]: high Hcy-thiolactonase activity is associated with L55 and R192 alleles, more frequent in blacks than in whites; low Hcy-thiolactonase activity is associated with M55 and Q192 alleles, more frequent in whites than in blacks. The high-activity form of Hcy-thiolactonase affords better protection against protein *N*-homocysteinylation than the low-activity form [49]. A different Hcy-thiolactone hydrolyzing enzyme is present in plants [60].

Human Hcy-thiolactonase/paraoxonase itself can be inactivated by its natural substrate, Hcy-thiolactone. It is expected that the high-activity form of the enzyme would be inactivated to a lesser degree than the low-activity form. Indeed, whereas the low-activity form loses 40–60% activity, the high-activity form loses only 10–25% upon exposure to 0.01–1 mM Hcy-thiolactone [121].

Implications for human disease

Hcy editing by MetRS generates the thioester Hcy-thiolactone, which is dangerous because it reacts with proteins, even at low concentrations present in human plasma. The reaction occurs with ε-NH_2 group of a protein lysine residue, thereby adding a reactive –SH group. Such Hcy-ε*N*-Lys-protein is more sensitive to further damage by oxidation than unmodified protein. Hcy enhances oxidative stress by increasing tumor necrosis factor-α (TNF-α) expression, which in turn upregulates NAD(P)H oxidase and nitric oxide synthase [122]. Thus, these two mechanisms, Hcy-thiolactone-mediated protein *N*-homocysteinylation and Hcy-induced oxidative stress, would act synergistically to increase protein damage. A sulfhydryl group added to a protein by *N*-homocysteinylation can oxidize with other –SH groups to make disulfide-linked protein multimers and aggregates. The function of such proteins is lost. In addition, the human organism recognizes Hcy-ε*N*-Lys-proteins as foreign and

generates an immune response, which suggests how Hcy can cause immune reactions in atherosclerosis. When a human organism is chronically overwhelmed by excess Hcy, for example due to inadequate folic acid intake, protective mechanisms cannot efficiently cope with inadvertent synthesis of harmful metabolites of Hcy, which then cause damage to vascular endothelium, and the disease develops.

HDL is strongly protective against atherosclerosis, mostly due to its role in the removal of excess cholesterol from peripheral tissues [123]. HDL also protects by inhibiting lipoprotein oxidation; this is due in part to serum paraoxonase, an esterase carried on HDL [120]. HDL is also likely to protect by hydrolyzing Hcy-thiolactone; this is due to the biological Hcy-thiolactonase activity of paraoxonase [46, 48, 49]. Low serum Hcy-thiolactonase activity, and thus reduced ability to prevent protein damage by Hcy-thiolactone, is likely to contribute to Hcy-linked cardiovascular disease [124]. Our observations that Hcy-thiolactonase and paraoxonase activities are highly correlated in human populations [49, 124] suggest that paraoxonase activity can be considered a surrogate for biological Hcy-thiolactonase activity. Thus, findings that low paraoxonase activity is associated with cardiovascular disease [125, 126] and nonfatal myocardial infarction in HDL-deficiency states [127] support a suggestion that Hcy-thiolactonase activity is a physiologically relevant predictor of the disease.

Protein *N*-homocysteinylation is a novel example of protein modification that may explain the involvement of Hcy in the pathology of human disease. Other protein modifications by drugs or cellular metabolites have been implicated in human disease. For example, acetylation of proteins by aspirin is thought to be an underlying cause of aspirin intolerance [128]. Peniciloylation of proteins by the antibiotic penicillin is involved in penicillin allergy [129]. Modification of proteins by glucose is believed to underlie the pathogenesis of diabetes [130] and Alzheimer's disease [131]. Proteins modified by products of lipid oxidation are implicated in the ethiology of atherosclerosis [123, 132]. A common aspect of these modification reactions is the involvement of protein lysine residues as sites of modifications.

How can protein damage lead to cell injury, a hallmark of atherosclerosis [123]? One plausible scenario is that *N*-homocysteinylated proteins on the surface of vascular vessels will be recognized by macrophages either directly or indirectly. Macrophages will attempt to phagocytize damaged proteins on the surface of endothelial cells, which would lead to destruction of endothelial cells and damage to vascular wall. Alternatively, *N*-homocysteinylated endothelial cells will attract anti-Hcy-*N*-protein antibodies and form antigen-antibody complexes on the surface of vascular vessels. Endothelial cells coated with antibody will be recognized and then bound by macrophages through their Fc receptors. After binding, the endothelial cells will be ingested and destroyed, which would result in injury to vascular surface. If the agent initiating the injury, e.g. Hcy-*N*-protein, is present continuously, attempts to repair the damaged vascular wall will eventually lead to an atherosclerotic plaque.

Perspectives

Our understanding of fundamental mechanisms by which Hcy can be incorporated into protein has increased considerably over the past years. Perhaps the most surprising finding is the existence in human blood of a pool of Hcy-*N*-protein [81], which contains more Hcy than the tHcy pool [133]. However, there are still a number of issues that require further investigation. Although hemoglobin and albumin comprise apparently major targets for protein *N*-homocysteinylation, other protein targets most likely exist and would be worth searching for. Other interesting questions that remain to be answered relate to the turnover of Hcy-*N*-protein: how Hcy-*N*-protein is removed from the circulation and how turnover of Hcy-*N*-protein contributes to plasma ADMA levels. The presence of anti-Hcy-*N*-protein autoantibodies in humans rises an important question of how such autoantibodies are generated and what specific Hcy-*N*-proteins elicit an autoimmune response. In addition, potential protective mechanisms which minimize incorporation of Hcy into protein need to be examined. Another issue concerns the roles of Hcy-thiolactone, *S*-nitroso-Hcy, Hcy-*N*-protein, anti-Hcy-*N*-protein autoantibodies and of HDL-Hcy-thiolactonase enzyme in Hcy-associated human diseases such as cardiovascular disease, Alzheimer's disease and so on. Our preliminary data suggest that plasma tHcy is a determinant of Hcy-*N*-protein and anti-Hcy-*N*-protein autoantibodies in humans. It will be crucial for our future understanding of human disease to confirm and extend these results by examining determinants of Hcy-thiolactone, *S*-nitroso-Hcy, Hcy-*N*-protein, anti-Hcy-*N*-protein autoantibodies and of HDL-Hcy-thiolactonase in population-based studies.

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