From Accuracy in Protein Synthesis to Cardiovascular Disease: The Role of Homocysteine

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Summary

The non-protein amino acid homocysteine (Hcy) enters the first step of protein synthesis and forms an aminoacyl-tRNA synthetase-bound homocysteinyl adenylate (Hcy-AMP). Direct incorporation of Hcy into tRNA and protein is prevented by editing activities of aminoacyl-tRNA synthetases that convert Hcy-AMP into Hcy thiolactone. Editing of Hcy occurs in all cell types investigated, including human. *S*-Nitrosylation of Hcy prevents its editing by MetRS and allows formation of *S*-nitroso-Hcy-tRNA^{Met}, as well as incorporation of Hcy into proteins at positions specified by methionine codons. This provides an example of how the genetic code can be expanded by invasion of the metionine coding pathway by Hcy. Hcy can also be incorporated into protein post-translationally by a facile reaction of Hcy thiolactone with ε-amino groups of protein lysine residues. Hcy is present in human blood proteins, such as hemoglobin, serum albumin, and γ-globulins. Hcy thiolactonase, a component of high-density lipoprotein, minimizes protein *N*-homocysteinylation. Incorporation of Hcy into protein provides plausible chemical mechanism by which elevated levels of Hcy contribute to human cardiovascular disease.

Key words:
translational editing, *S*-nitroso-homocysteine, genetic code, protein *N*-homocysteinylation, thiolactonase/paraoxonase, high-density lipoprotein, atherosclerosis.

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1. Introduction

The function of aminoacyl-tRNA synthetases (AARSs) in protein synthesis involves matching amino acids with their cognate tRNAs according to the rules of the genetic code (1,2). It is widely acknowledged that high accuracy is essential for the function of AARSs in maintenance of the genetic code relationships. For example, the absolute selectivity of methionyl-tRNA synthetase (MetRS) assures that the AUG word in the nucleic acid language means methionine in the protein language. An AARS achieves high selectivity by preferentialy binding its cognate substrates or by editing occasionally misactivated amino acids (3-5). Amino acid selectivity of MetRS provides a plausible explanation for the toxicity of homocysteine (Hcy) to human vascular endothelium and links accuracy in protein synthesis to cardiovascular disease as outlined below.

2. Solving the selectivity problem with Hcy by editing

One of the selectivity problems in protein synthesis is discrimination against the non-protein amino acid Hcy a universal precursor of methionine. Hcy is misactivated by MetRS (6), IleRS (6), and LeuRS (7) (Table 1), forming corresponding enzyme-bound homocysteinyl adenylate (Hcy-AMP) (equation [1]) at frequencies exceeding the frequency of errors in ribosomal protein synthesis (3). LysRS and ValRS misactivate Hcy at lower frequencies (Table 1) (8,9).

$$\text{AARS} + \text{Hcy} + \text{ATP} \Leftrightarrow \text{AARS} \cdot \text{Hcy} \sim \text{AMP} + \text{PP}_i \qquad [1]$$

Table 1

Misactivation and editing of Hcy by *E. coli* AARSs

AARS	Misactivation			Editing	
	k_{cat} (s^{-1})	K_m (mM)	Selectivity	k_{cat} (s^{-1})	Selectivity
MetRS	87	5.2	0.0054	1.2	72
IleRS	14	1.5	0.0025	1.5	114
LeuRS	6	2.1	0.0083	2.0	50
ValRS 1.6	1.6	10	0.0002	0.2	10
LysRS				>1.2	35

Misactivation was assayed by the Hcy-dependent ATP/PP_i exchange reaction. Editing was assayed by the formation of Hcy thiolactone. Selectivity in activation is obtained by dividing catalytic efficiency (k_{cat}/K_m) for Hcy by catalytic efficiency for a cognate amino acid in the ATP/PP_i exchange reaction. Selectivity in editing is obtained by dividing the rate of Hcy thiolactone formation by the rate of hydrolysis of the cognate adenylate (reprinted with permission from refs. 3 and 9).

Misactivated Hcy is subsequently edited or rejected (Table 1) as a result of an intramolecular reaction between the side chain thiolate and the activated carboxyl of Hcy; Hcy thiolactone and AMP are the products (equation [2]) (6-8). Editing prevents direct incorporation of Hcy into tRNA (Table 2) and protein (Table 3).

Table 2

AARSs catalyze synthesis of Hcy thiolactone, but not Hcy-tRNA

Reaction conditions	[^{35}S]Hcy thiolactone	[^{35}S]Hcy-tRNA
	μM/15 min	μM/15 min
MetRS	27	
MetRS, $tRNA^{fMet}$	18	<0.004
IleRS	10	
IleRS, $tRNA^{Ile}$	5	<0.004
LysRS	1.4	
LysRS, $tRNA^{Lys}$	1.1	<0.004
ValRS	0.6	
ValRs, $tRNA^{Val}$	0.7	<0.004

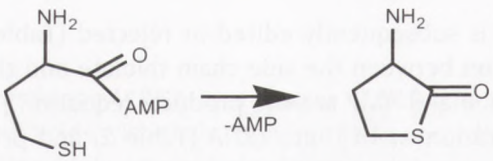
Reactions were carried out at 37°C in mixtures containing 0.1 M K-HEPES, pH 7.4, 10 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 4 units/ml yeast inorganic pyrophosphatase, 2 mM ATP, 0 or 10 μM pure *E. coli.* tRNA (amino acid acceptor activity 1100-1400 pmol/A_{260}), 0.4 μM *E. coli* AARS, and 0.2 mM [^{35}S]Hcy (10,000 Ci/mol). Hcy thiolactone was determined by two dimensional TLC. Aliquots of the tRNA aminoacylation mixtures were analyzed by precipitation with 5% trichloroacetic acid, TLC on cellulose plates, and polyacrylamide gel electrophoresis at pH 5.5 (17). In attempted tRNA aminoacylations no incorporation of [^{35}S]Hcy above background (0.004 μM/15 min) was observed at times from 5-60 min (refs. 17,18 and Jakubowski, unpublished data).

Table 3

Metabolic conversion of exogenous Hcy to Hcy thiolactone and protein Met in *E. coli* in the absence or presence of methionine, isoleucine, and leucine

Amino acid added to culture	[^{35}S]Hcy thiolactone	Protein [^{35}S]Met
	(%)	
None	100 (= 18 pmol)	100 (= 97 pmol)
Methionine	54	6.3
Isoleucine	62	103
Leucine	40	88
Methionine, Leucine	15	4
Methionine, Isoleucine	6	5
Leucine, Isoleucine	33	103
Methionine, Leucine, Isoleucine	1	10

E. coli K38 cultures in minimal medium containing indicated amino acids (0.2 mM) were labeled with 10 μM [^{35}S]Hcy for 1 h at 37°C. Hcy thiolactone was determined by two dimensional TLC. Protein Met was determined by two dimensional TLC of DTT-treated and acid-hydrolysed bacterial protein. No incorporation of [^{35}S]Hcy into protein was observed (<0.06%) under any conditions examined (ref. 22 and Jakubowski, unpublished data).



[2]

3. The molecular mechanism of Hcy editing

The Hcy editing reaction occurs in the synthetic/editing active site whose major function is to carry out the synthesis of cognate aminoacyl-tRNA, as demonstrated for MetRS (10,11) and IleRS (12). 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 (editing) sub-sites of the synthetic/editing active site (Figs. 1-3). A sub-site that binds carboxyl and α-amino groups of both cognate and non-cognate substrates is not contributing to substrate specificity.

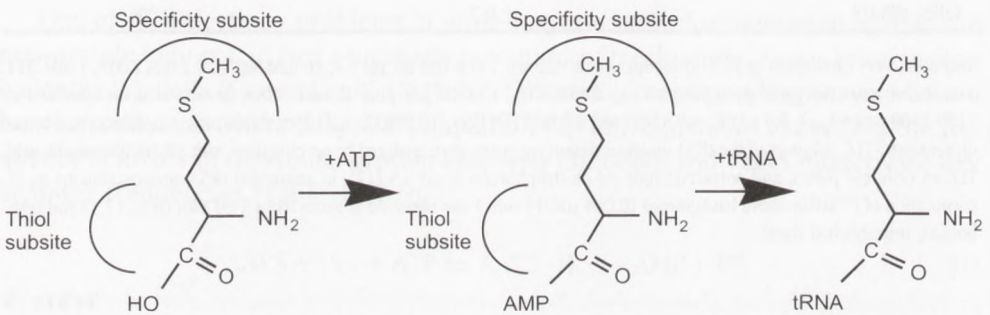


Fig. 1. Aminoacylation of $tRNA^{Met}$ with methionine catalysed by MetRS: Methionine completes the synthetic pathway because its side chain is firmly bound in the specificity sub-site of the enzyme.

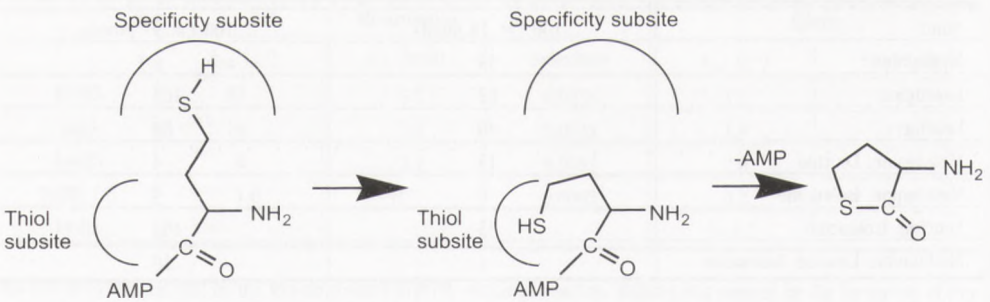


Fig. 2. Editing of Hcy by MetRS: Hcy is edited because its side chain can enter the thiol sub-site. The MetRS catalyzes cyclization of Hcy~AMP to form the Hcy thiolactone and AMP that are then released from the synthetic/editing active site of MetRS (reprinted with permission from ref. 5).

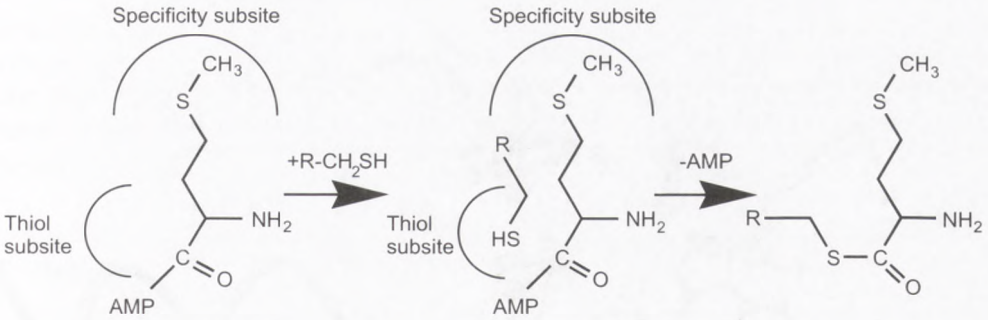


Fig. 3. Aminoacylation of thiols by MetRS. The MetRS catalyzes reaction of a thiol (R-CH_2SH) with Met-AMP to form a methionine thioester, that is then released from the synthetic/editing active site. With CoA-SH as a thiol substrate, MetRS functionally becomes methionyl-S-CoA synthetase. Similar MetRS--catalyzed reactions occur with Met-$tRNA^{Met}$ (reprinted with permission from ref. 5).

Methionine completes the synthetic pathway (Fig. 1) because its side chain is firmly bound by the hydrophobic and hydrogen bonding interactions with the specificity of sub-site residues. Crystal structure of MetRS · Met complex (13) 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 (Fig. 4). 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 non-cognate substrate Hcy, missing the methyl group of methionine, cannot interact with the specificity sub-site as effectively as cognate methionine does. This allows the side chain of Hcy to move to the thiol-binding sub-site, which promotes the synthesis of the thioester bond during editing (Fig. 2) (11). Mutations of Tyr15 and Trp305 affect Hcy/Met discrimination by the enzyme (10). Asp 52 forms a hydrogen bond with the α-amino group of the substrate methionine (Fig. 4), deduced from the crystal structure of MetRS·Met complex (13), 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 (10,11).

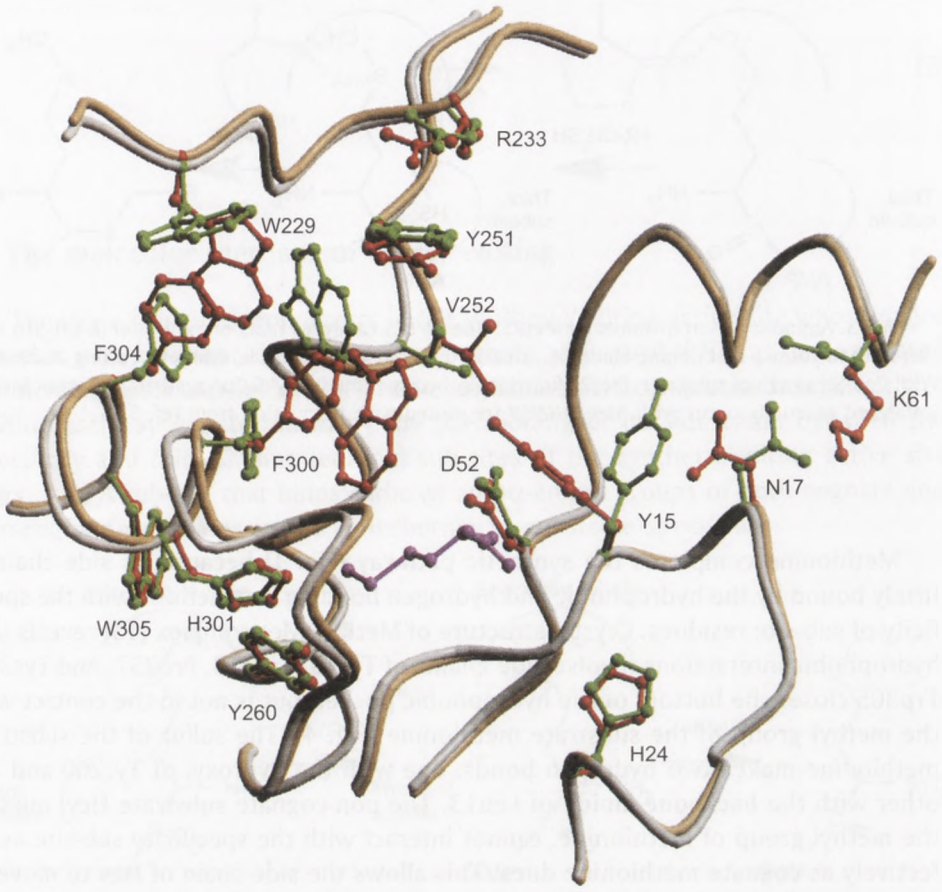


Fig. 4. The active site of MetRS: Hydrophobic and hydrogen bonding interactions provide specificity for the cognate substrate L-methionine. Superimposition of a-carbon chain backbones for the MetRS · Met complex (burly-wood) and free MetRS (light grey) shows movements of active site residues upon binding of methionne. Residue colours are red (complexed) and green (free) and L-methionine is magenta (reprinted with permission from ref. 13).

4. AARSs catalyse the synthesis of aminoacyl-S-CoA thioesters and aminoacyl-Cys dipeptides

The notion of the thiol-binding site is supported by the ability of MetRS (11) and other AARSs (8,12,14-17) to edit in trans, i.e. to catalyse thioester bond formation between a thiol and the cognate amino acid substrate (Fig. 3). For example, with CoA-SH and cysteine, that are preferred thiol substrates, MetRS catalyses the formation of Met-S-CoA thioesters and Met-Cys dipeptides. The formation of Met-Cys di-

peptide 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 editing of Hcy. The ability of AARS to catalyse formation of aminoacyl thioesters and dipeptides suggests a functional link between AARSs and non-ribosomal peptide synthesizing systems (15,16).

5. Decoding methionine codons by Hcy

A model of the synthetic/editing site of MetRS (Figs. 1-4) shows that the lack of efficient interactions of the side chain of Hcy with the specificity sub-site is a prerequisite for Hcy editing (10,11). Thus, keeping the side chain of Hcy in the specificity sub-site would prevent editing and facilitate transfer to $tRNA^{Met}$. This can be achieved by utilizing nitrosothiol chemistry. Indeed, reversible *S*-nitrosylation of Hcy prevents binding of its modified side chain to the editing sub-site and enhances binding to the specificity sub-site, which leads to the formation of *S*-nitroso-Hcy-$tRNA^{Met}$ (Fig. 5). Hcy-$tRNA^{Met}$, prepared by de-nitrosylation of *S*-nitroso-Hcy-$tRNA^{Met}$. It is the least stable aminoacyl-tRNA known, spontaneously converting to Hcy thiolactone and free tRNA with a half-life of 15 s. *S*-Nitroso-Hcy-$tRNA^{Met}$ donates *S*-nitroso-Hcy to growing protein chains on ribosomes in the bacterium *Escherichia coli* and in an *in vitro* mRNA-programmed rabbit reticulocyte protein synthesis system. Removal of the nitroso group yields proteins containing Hcy at positions normally occupied by methionine (5,18). Translationally incorporated Hcy is also present in cultured human vascular endothelial cells (Table 4), that are known to produce nitric oxide and *S*-nitroso-Hcy (19). Thus, Hcy can gain an access to the genetic code by *S*-nitrosylation-mediated invasion of the methionine-coding pathway.

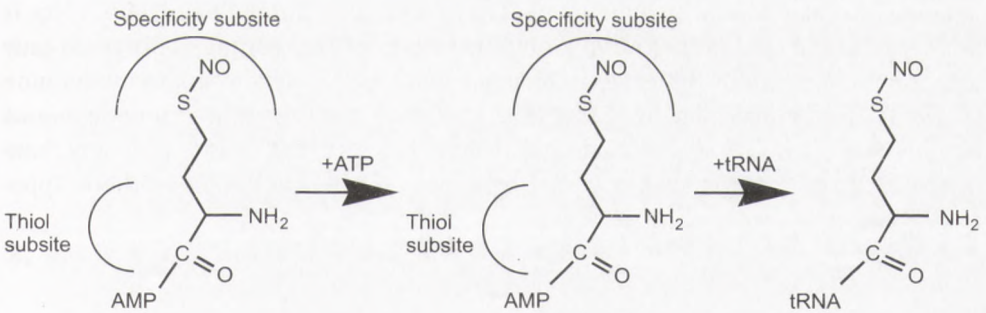


Fig. 5. Aminoacylation of $tRNA^{Met}$ with S-nitroso-Hcy catalyzed by MetRS. After formation of S-nitroso-Hcy~AMP, the side chain of S-nitroso-Hcy remains in the specificity sub-site of the synthetic/editing active site of MetRS. This allows the transfer of S-nitroso-Hcy from the adenylate to $tRNA^{Met}$ (reprinted with permission from ref. 5).

Table 4

Translational and post-translational incorporation of Hcy into HUVEC protein

Labeling conditions	Translational		Post-translational
	[^{35}S]Hcy-protein	[^{35}S]Met-protein	εN-([^{35}S]Hcy)-Lys-protein
	(%)		
[^{35}S]Hcy	37	25	38
[^{35}S]Hcy + folate, 10 μmol/L	<1	>98	<1
[^{35}S]Hcy + HDL, 1 mg/mL	68	25	7
[^{35}S]Hcy + Met, 20 μmol/L	12	76	12
Control, εN-([^{35}S]Hcy)-Lys-protein	<4	0	>96

HUVECs were maintained on Met-free M199, supplemented with dialyzed 15% fetal bovine serum, bovine endothelial cell growth factor, heparin, and 10 μmol/L [^{35}S]Hcy (50 μCi/mL), in the absence and presence of exogenous folate, HDL, or Met. Total incorporation of [^{35}S]Met and [^{35}S]Hcy into protein were determined by acid hydrolysis of DTT-treated extracellular proteins followed by TLC. Sensitivity to Edman degradation (27) was used to determine *post-translationally* incorporated Hcy (ε*N*-([^{35}S]Hcy)-Lys-protein). Control with chemically prepared ε*N*-([^{35}S]Hcy)-Lys-protein is shown in the last row of the table. Relative distribution (%) of [^{35}S]radioactivity among different chemical forms observed under each experimental condition is shown (reprinted with permission from ref. 5).

6. Hcy editing is universal

Because Hcy is a universal precursor of methionine, it is likely that all organisms had to evolve the ability to edit Hcy in order to prevent its direct incorporation into protein. Indeed, in a variety of cells examined from bacteria to human, Hcy is metabolised to thiolactone by MetRS (20-29). In the yeast *Saccharomyces cerevisiae*, both cytoplasmic and mitochondrial MetRSs edit Hcy (21,28). Editing of endogenous Hcy (made in the methionine biosynthetic pathway) by MetRS in all cell types examined, including human vascular endothelial cells, is prevented by the supplementation with *excess* methionine (27). In *E. coli*, LeuRS and IleRS, in addition to MetRS, edit exogenous Hcy (supplied to the medium) (22). As a result, editing of exogenous Hcy is not prevented by excess methionine; full inhibition of Hcy editing is observed only after supplementation with excess isoleucine and leucine, in addition to methionine (Table 3). C-Terminal domain of MetRS is important for the editing of endogenous Hcy in *E. coli* (29). These observations strongly suggest that amino acids are channelled from the methionine biosynthetic pathway to the protein biosynthetic apparatus (Fig. 6).

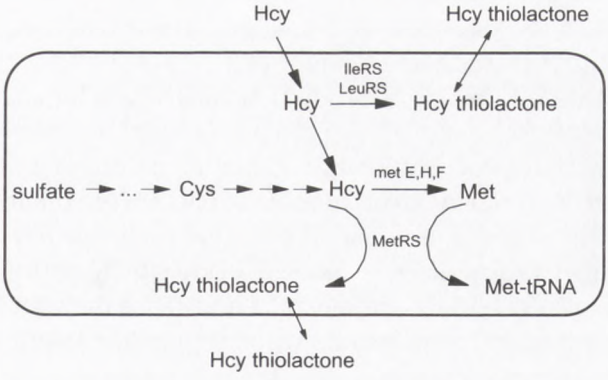


Fig. 6. Channelling of amino acids from the methionine biosynthetic pathway to protein synthesis in *Escherichia coli*. Exogenous Hcy (supplied in the media) is edited by MetRS, IleRS, and LeuRS. Endogenous Hcy (produced from sulfate in the methionine biosynthetic pathway) is edited only by MetRS (reprinted with permission from ref. 9).

7. Physical-chemical properties of Hcy thiolactone

Hcy thiolactone is an intramolecular thioester of Hcy. The hydrochloric acid salt of Hcy thiolactone is stable indefinitely at room temperature. Under physiological conditions of pH and temperature, Hcy thiolactone (half-life ~25 h, ref. 24) is more stable than inter-molecular aminoacyl thioesters, such as, for instance, methionyl-S-CoA, that hydrolyzes with a half life 2.25 h (15). In diluted NaOH at room temperature, Hcy thiolactone is hydrolysed to completion in 15 min (24). Like all thioesters (30), Hcy thiolactone absorbs ultraviolet light with a maximum at 240 nm and $\varepsilon = 3{,}500$ $M^{-1}cm^{-1}$ in water (22). The pK_a of its amino group is unusually low at 7.1 (31), a characteristic property of aminoacyl (thio)esters. Thus, under physiological conditions, Hcy thiolactone is neutral, freely diffuses through cell membranes, and is mostly present in extra-cellular media (20-29).

The energy of the anhydride bond of Hcy~AMP is conserved in an intra-molecular thioester bond of Hcy thiolactone. Because of this, Hcy thiolactone is chemically reactive towards ε-amino groups of protein lysine residues (5,9,24,26,27,32-34).

8. Protein *N*-homocysteinylation in humans

The formation of isopeptide bond between the activated carboxyl of Hcy thiolactone and the ε-amino group of protein lysine residue occurs easily under physiological conditions of pH and temperature. For instance, in human serum supplemented with Hcy thiolactone, protein *N*-homocysteinylation occurs with a

half-life of 1.5 h (32-34). Rates of *N*-homocysteinylation of individual proteins positively correlate with protein lysine content (32).

Modification with Hcy thiolactone is detrimental to proteins and results in a loss of function. For example, complete inactivation of lysine oxidase (35), MetRS and trypsin (32) occurs after incorporation of 1, 8, and 11 molecules of Hcy per molecule of protein, respectively. Micromolar concentrations of Hcy thiolactone inactivate lysine oxidase with a half-life of 4 min (35), making it a likely physiological target. *N*-Homocysteinylated proteins become prone to aggregation, particularly cytochrome c (32), and can be physiologically detrimental; for instance they can elicit an immune response, as shown by injecting rabbits with LDL modified with Hcy thiolactone (36).

Protein *N*-homocysteinylation occurs in a variety of cultured human cells. In normal and cystathionine synthase-deficient fibroblasts and in breast cancer cells, protein *N*-homocysteinylation is enhanced in the presence of the antifolate drug aminopterin (24). In cultured human umbilical vein endothelial cells (HUVECs), the extent of protein *N*-homocysteinylation is positively correlated with Hcy concentrations, and negatively correlated with concentrations of methionine, folic acid, and HDL (27). Edman degradation of *N*-homocysteinylated proteins from HUVEC cultures suggests that Hcy is incorporated *via* both iso-peptide bonds (most likely by a Hcy thiolactone-mediated mechanism) (24,32) and peptide bonds (most likely by a nitric oxide-mediated mechanism) (18).

Preliminary measurements show that *N*-homocysteinylated proteins are present in normal human plasma (5,9,26) at levels equal to about 15% of total Hcy levels. Levels of *N*-homocysteinylated protein positively correlate with Hcy levels in plasma (5). Analysis of purified individual human blood proteins (from which free and disulfide-bound Hcy has been removed by extensive treatments with DTT) indicates that protein-*N*-Hcy is present in hemoglobin, serum albumin, IgG, and transferrin. One molecule of Hcy is present per 167, 280, 280, and 1250 molecules of hemoglobin, serum albumin, IgG, and transferrin, respectively (Jakubowski, unpublished data).

9. Human serum HDL minimizes protein N-homocysteinylation

Because reactions of Hcy thiolactone with proteins are harmful (32,35), the ability to detoxify the thiolactone is essential for biological integrity. Indeed, a single specific Hcy thiolactone-hydrolysing enzyme is present in mammals (5,9,32-34). Human serum Hcy thiolactonase is a 45 kD protein component of HDL and requires calcium for activity and stability. LDL is devoid of thiolactonase activity. L-Isoleucine and the anti-arthritis drug D-penicillamine non-competetively inhibit Hcy thiolactonase. Thiolactonase also hydrolyzes the organophosphate paraoxon and is identical with paraoxonase, a product of the *PON1* gene, implicated in human cardiovascular dis-

ease. Hydrolysis of Hcy thiolactone and paraoxon occurs at separate sites on the enzyme (33). Thiolactonase actitivity is absent in the *PON1* gene-knockout mice (9,33) that are also more susceptible to atherosclerosis than their wild type littermates (37). Two genetic polymorphisms in human *PON1* result in amino acid substitutions at positions 55 and 192 in PON1 proteins. Thiolactonase activity is strongly associated with *PON1* genotype in human populations (34): high activity is associated with L55 and R192 alleles, more frequent in blacks than in whites; low activity is associated with M55 and Q192 alleles, more frequent in whites than in blacks. High activity form of thiolactonase affords better protection against protein *N*-homocysteinylation than the low activity form (27,34). We have suggested that thiolactonase activity is a better predictor of Hcy-associated vascular disease than *PON1* genotype (34). Thiolactonase and paraoxonase activities are highly correlated in human populations (34). Thus, the finding that paraoxonase activity is a predictor of vascular disease (38) supports our suggestion that thiolactonase activity is physiologically relevant predictor of the disease. Very high serum levels of Hcy thiolactonase in rabbits (9,33) may account for the observation that infusions with Hcy thiolactone failed to produce atherosclerosis in these animals (39).

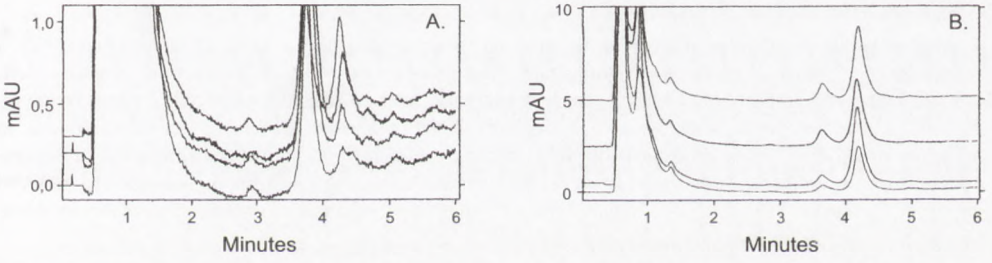


Fig. 7. Hcy is present in DTT-treated human serum protein. Free and disulfide-bound Hcy was removed from human serum by treatments with DTT and ultra-filtration through a 10 kD molecular weight cut-off membrane. DTT-treated proteins were hydrolysed with 6 N HCl in the presence of DTT for 1-4 h at 120°C. The hydrolysates were lyophylized, neutralized with ammonium bicarbonate/dipotasium phosphate, and extracted with chlorophorm/methanol. Hcy thiolactone was re-extracted from the organic layer with diluted HCl, lyophylized, and further purified by two-dimensional TLC (18,24). Hcy thiolactone was eluted with water, and subjected to HPLC on a reverse phase HAISIL Targa C18 column (2.1 · 150 mm, 3 μ, 120Å; Higgins Analytical, Inc) (5). Detection was by absorbance at 240 nm, the absorption maximum of Hcy thiolactone. *Panel A:* sample purified from normal human serum. *Panel B:* sample purified from human serum obtained from a homocysteinuric subject. HPLC profiles were obtained with proteins hydrolysed for 1h (bottom trace), 2 h, 3 h, and 4 h (top trace). The material eluting at 4.2 min co-migrates with an authentic Hcy thiolactone, has a UV absorption spectrum identical to that of Hcy thiolactone, and is sensitive to NaOH treatment as an authentic thiolactone is (Jakubowski, unpublished).

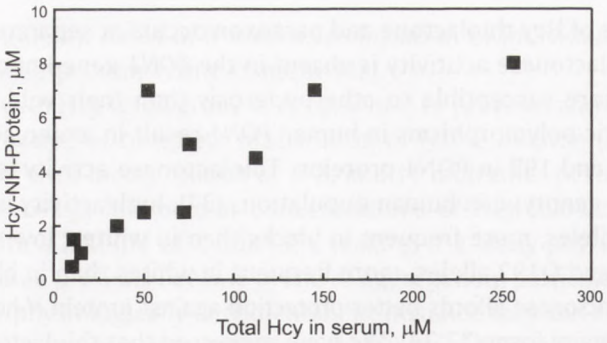


Fig. 8. Relationship between levels of Hcy-*N*-protein and Hcy in human serum. Hcy-*N*-Protein was determined as described in the legend to Fig. 7 (reprinted with permission from ref. 5).

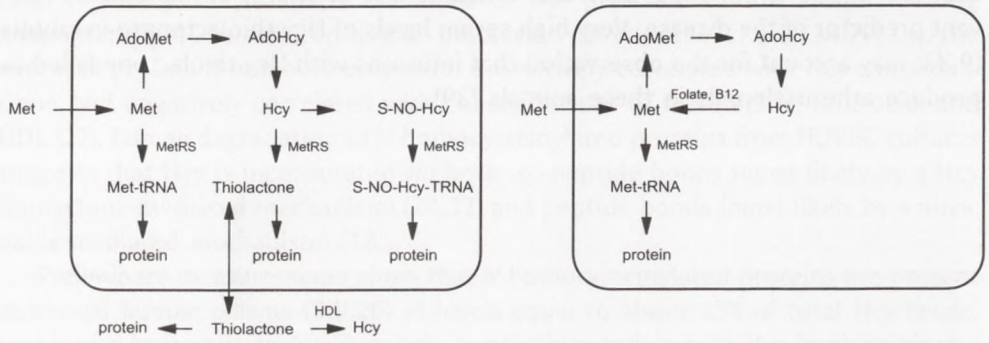


Fig. 9. Folate and the metabolism of Hcy in human vascular endothelial cells. *Right, high folate:* Hcy, a by-product of cellular methylation reactions, are efficiently re-methylated by methionine synthase. *Left, low folate:* Hcy is metabolized by MetRS to Hcy thiolactone, that diffuses from the cell, acylates lysine residues in intra- and extra-cellular proteins, or is hydrolyzed by an HDL-associated Hcy thiolactonase. In addition, nitric oxide, produced by endothelial nitric oxide synthase, allows the formation of *S*-nitroso-Hcy that is then incorporated translationally into protein following synthesis of *S*-nitroso-Hcy-tRNA^{Met} catalyzed by MetRS. Subsequent removal of the nitroso group by trans-nitrosylation leaves Hcy in protein chains (reprinted with permission from ref. 44).

10. Implications for cardiovascular disease

Elevated levels of Hcy are a risk factor for cardiovascular disease and stroke in humans (e. g., ref. 40) and predict mortality independently of traditional risk factors in patients with coronary artery disease (41). However, it is not known what aspect of Hcy metabolism can be harmful. Metabolism of Hcy in human vascular endothelial cells is summarized in Fig. 1. Toxicity of Hcy can be due to its indirect incorporation into protein by Hcy thiolactone- and *S*-nitroso-Hcy-mediated mechanisms, which would lead to protein and cell damage, a hallmark of atherosclerosis (5,44). These

mechanisms are specific for Hcy because Hcy thiolactone and *S*-nitroso-Hcy arise exclusively from Hcy. Levels of Hcy thiolactone and *N*-homocysteinylated protein depend on Hcy/Met ratios, levels of folic acid, and HDL, factors linked to cardiovascular disease. Because levels of *N*-homocysteinylated protein are positively correlated with total Hcy levels, the accumulation of *N*-homocysteinylated protein is predicted to be a risk factor for cardiovascular disease. Translational incorporation of *S*-nitroso-Hcy into protein possibly accounts for observations that atherosclerosis originates mostly at branch points in arteries (42) that are subject to mechanical stress leading to increased production of nitric oxide (43). In subjects with elevated serum Hcy levels, local concentrations of nitric oxide (43) and *S*-nitroso-Hcy are likely to be higher at arterial branch points than elsewhere. Therefore, at branch points, the incorporation of *S*-nitroso-Hcy into endothelial cell proteins, and resulting damage would be greater than at other points in arteries. Variations in HDL-associated Hcy thiolactonase, observed in human populations, affect protein *N*-homocysteinylation and may therefore play an important role in Hcy-linked human cardiovascular disease.

Abbreviations used are:

AARS, aminoacyl-tRNA synthetase (e. g., MetRS, methionyl-tRNA synthetase, other AARSs abbreviated similarly); AA, amino acid; DTT, dithiothreitol; Hcy, homocysteine; HDL, high-density lipoprotein; LDL, low-density lipoprotein; HUVEC, human umbilical vein artery endothelial cells; TLC, thin layer chromatography.

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