ACTA ABP BIOCHIMICA POLONICA

Vol. 58, No. 2/2011
149–163
on-line at: www.actabp.pl

Review

Quality control in tRNA charging — editing of homocysteine

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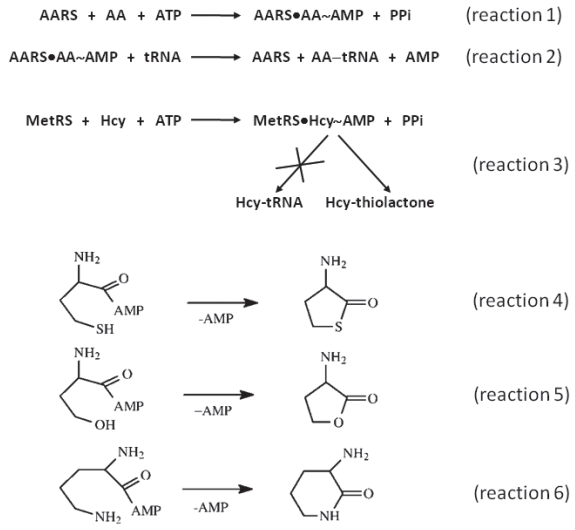
All living organisms conduct protein synthesis with a high degree of accuracy maintained in the transmission and flow of information from a gene to protein product. One crucial "quality control" point in maintaining a high level of accuracy is the selectivity by which aminoacyl-tRNA synthetases furnish correctly activated amino acids, attached to tRNA species, as the building blocks for growing protein chains. When differences in binding energies of amino acids to an aminoacyl-tRNA synthetase are inadequate, editing is used as a major determinant of enzyme selectivity. Some incorrect amino acids are edited at the active site before the transfer to tRNA (*pre-transfer editing*), while others are edited after transfer to tRNA at a separate editing site (*post-transfer editing*). Access of natural non-protein amino acids, such as homocysteine, homoserine, or ornithine to the genetic code is prevented by the editing function of aminoacyl-tRNA synthetases. Disabling editing function leads to tRNA mischarging errors and incorporation of incorrect amino acids into protein, which is detrimental to cell homeostasis and inhibits growth. Continuous homocysteine editing by methionyl-tRNA synthetase, resulting in the synthesis of homocysteine thiolactone, is part of the process of tRNA aminoacylation in living organisms, from bacteria to man. Excessive homocysteine thiolactone synthesis in hyperhomocysteinemia caused by genetic or nutritional deficiencies is linked to human vascular and neurological diseases.

Keywords: tRNA synthetase editing, homocysteine, protein synthesis, protein modification, cardiovascular disease, neurodegeneration

Received: 24 March, 2011; accepted: 02 June, 2011;
available on-line: 6 June, 2011

INTRODUCTION

The charging of a tRNA with an amino acid, catalyzed by an aminoacyl-tRNA synthetase (AARS)* (Ibba & Soll, 2000; Fersht, 2000; Ribas de Pouplana & Schimmel, 2001) fulfills two important functions in protein synthesis: information transfer and chemical activation. The information transfer function pairs off an amino acid with its cognate tRNAs according to the rules of the genetic code, thereby translating the nucleic acid language into the protein language. For example, methionyl-tRNA synthetase (MetRS) translates the AUG word in the nucleic acid language as methionine in the protein language. Similarly, LysRS translates the words AAA and AAG in the nucleic acid language as lysine in the protein language, and so on. The chemical activation function generates a high-energy ester bond between the carboxyl group of an amino acid (AA) and a hydroxyl of the 3'-terminal adenosine of tRNA in a two-step reaction with an aminoacyl adenylate as an intermediate (reaction 1 and 2).



High accuracy in the tRNA aminoacylation reaction, essential for maintaining unambiguous genetic code relationships and crucial for cellular homeostasis, depends on the ability of an AARS to faithfully select its cognate tRNA and amino acid. Faithfull selection of a cognate tRNA is achieved by preferential binding, which is facilitated by structural variation between tRNAs (Schulman, 1991). In contrast, the structural variation between amino acids is not sufficient and an AARS often selects two or more similar amino acids (Baldwin & Berg, 1966). In those cases, faithful selection of a cognate amino acid depends on an additional a 'quality control' step, in which non-cognate amino acids are rejected or edited by an AARS. The term 'quality control', first used in relation to the tRNA aminoacylation reaction in 1992 (Jakubowski & Goldman, 1992), is now commonly used to denote error-correcting mechanism(s) in protein biosynthesis and DNA replication (Lindahl & Wood, 1999; Ibba & Soll, 1999; Ling *et al.*, 2009).

Our understanding of the molecular basis of amino acid selection and editing by AARSs continues to ex-

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Abbreviations: AARS, aminoacyl-tRNA synthetase (e.g., MetRS, methionyl-tRNA synthetase, etc.); ADMA, asymmetric dimethyl arginine; Blmh, bleomycine hydrolase; CBS, cystathionine β-synthase; Hcy, homocysteine; *N*-Hcy-protein, protein containing Hcy linked by an isopeptide bond to ε-amino group of a lysine residue; MTHFR, methylenetetrahydrofolate reductase; Pon, paraoxonase

pand. The new data support the original notion that amino acid editing reactions are idiosyncratic, i.e., that the physical chemical nature of the misactivated amino acid dictates how it is edited. Enormous progress has been made in unraveling structural determinants and mechanisms of editing. That quality control is a part of the tRNA aminoacylation *in vivo*, first demonstrated for editing of homocysteine (Hcy) by MetRS (Jakubowski, 1990; Jakubowski, 1991), has subsequently been generalized to other AARSs. New evidence suggests that living organisms have the ability to modulate editing and selectivity of AARSs in response to physiological stress. Physiological consequences of editing have been documented and links to human disease discovered. These aspects of accuracy, as well as mechanism and pathophysiological consequences of Hcy editing, are the subjects of the present review. Other reviews (Schimmel, 2008; Francklyn, 2008; Ibba & Soll, 1999; Jakubowski, 2001b; Ling *et al.*, 2009; Jakubowski & Goldman, 1992; Fersht, 1986; Jakubowski, 2005a; Sankaranarayanan & Moras, 2001; Martinis & Boniecki, 2010) summarize older literature.

Table 1. Relative binding, editing, and tRNA aminoacylation (charging) by class I *E. coli* aminoacyl-tRNA synthetases
(compiled from Jakubowski & Goldman, 1992; Jakubowski, 2005)

	Isoleucyl-tRNA synthetase			Leucyl-tRNA synthetase			Methionyl-tRNA synthetase			Valyl-tRNA synthetase		
Amino Acid	Binding[a]	Editing[b]	Charging[c]	Binding[a]	Editing[b]	Charging[c]	Binding[a]	Editing[b]	Charging[c]	Binding[a]	Editing[b]	Charging[c]
Isoleucine	1 000 000	1	1 000 000	1 500		<1[d]				1 000	1	200
Leucine	1 000	6		1 000 000	1	1 000 000						
Methionine				800		<1[d]	1 000 000	1	1 000 000			
Homocysteine[f]	2 500	76	<1	8 300	25	<1	11 300	60	<1	200		<1
S-Nitroso-Hcy							76 000	1	12 000			
Ethionine							35 000	7	64 500			
Norleucine	2 160	12					5 000	5	6 700			
Norvaline	135			14 000	44		66					
Valine	8 400	43	3[d,e]	<800						1 000 000	1	1 000 000
Threonine	2 400	23	<1							4 000	180	3[d,e]
α-Aminobutyrate	940	24								5 000	58	d e
Cysteine	300	100								1 000	90	<1
Alanine	120	8.5	<1							100	48	<1
Homoserine[f]	40	5								140	1.6	

[a]Relative k_{cat}/K_m values in the ATP-PP_i exchange reaction; [b]Relative k_{cat} values in the ATP pyrophosphatase reaction; [c]Relative k_{cat} values in the tRNA aminoacylation reaction; [d]Mischarging increases for AARSs with CP1 domain mutations. [e]Fast transient tRNA mischarging observed under pre-steady state conditions (Fersht 2000, pages 239–242). [f]These amino acids are cyclized during editing.

THE ACCURACY OF AMINOACYL-tRNA SYNTHETASES

In vitro studies with purified AARSs (reviewed in (Jakubowski & Goldman, 1992; Jakubowski, 2005a)) show that TyrRS, CysRS, SerRS, AspRS, and ArgRS, exhibit essentially absolute selectivity for their cognate amino acids. MetRS, IleRS, LeuRS, ValRS, AlaRS, LysRS, ProRS, PheRS, and ThrRS are less accurate and have the ability to misactivate non-cognate amino acids (reaction 1). Misactivation occurs because there is a limited scope for structural variation among related amino acids. Pauling first recognized this problem in 1957, well before the mechanism of protein synthesis had been established (Pauling, 1957). Later studies (Jakubowski & Goldman, 1992; Jakubowski, 2005a) indicate that the initial discrimination between amino acids differing by one methyl group in their structures cannot be better than by a factor of 100 (Tables 1 and 2).

One of the major selectivity problems is the discrimination against the non-protein amino acid homocysteine (Hcy), which is misactivated by *Escherichia coli* MetRS, IleRS, LeuRS, ValRS, and LysRS. Other examples of a limited initial discrimination include: Val and Leu *vs.* Ile by IleRS; Met and Ile *vs.* Leu by LeuRS; Cys and Thr *vs.* Val by ValRS; Gly and Ser *vs.* Ala by AlaRS; ornithine (Orn) *vs.* Lys; Ser *vs.* Thr by ThrRS. However, as originally demonstrated for Val editing by IleRS (Baldwin & Berg, 1966; Eldred & Schimmel, 1972) misactivated amino acids, are generally not transferred to tRNA (Jakubowski, 1999a). The higher accuracy of the tRNA aminoacylation reaction (reaction 2), relative to the aminoacyl adenylate formation reaction (reaction 1), is a result of an editing or proofreading activity of an AARS (Jakubowski & Goldman, 1992) (Tables 1 and 2). Moreover, the editing activity contributes to functional partitioning of amino acids present in living organisms into *protein* and *non-protein* amino acids. For instance, although Hcy and Orn are misactivated by MetRS and LysRS, editing activities of those AARSs assure that Hcy and Orn remain non-protein amino acids (Jakubowski, 1999a).

Some non-cognate amino acids are transferred to tRNA, generating misacylated tRNAs;

Table 2. Relative binding, editing, and tRNA aminoacylation by class II *E. coli* aminoacyl-tRNA synthetases (compiled from (Jakubowski, 2005; Jakubowski & Goldman, 1992; Ahel *et al.,* 2002; Gruic-Sovulj *et al.,* 2007))

Aminoacyl-tRNA synthetase and amino acid	Binding[a]	Editing[b]	Charging[c]
Alanyl-tRNA synthetase			
Alanine	1 000 000	1	1 000 000
Glycine[d]	4 000	11.5	<100
Serine[d]	2 000	23	<100
Lysyl-tRNA synthetase			
Lysine		1	1 000 000
Arginine		1	600
Ornithine[e]		>13	<1
Homocysteine[e]		130	<1
Methionine		30	30
Leucine		20	16
Norleucine		50	
Norvaline		30	
Cysteine		50	4
Homoserine[e]		50	
Phenylalanyl-tRNA synthetase			
Phenylalanine	1 000 000	1	1 000 000
Tyrosine[d f]	<600	1	<100
Prolyl-tRNA synthetase			
Proline	1 000 000	1	1 000 000
Cysteine	2 700	1	2 100
Alanine[d]	36	2.5	<30
Threonyl-tRNA synthetase			
Threonine	1 000 000	1	1 000 000
Serine[d]	1 000	1	<100
Seryl-tRNA synthetase			
Serine	1 000 000	1	1 000 000
Threonine	393	17	
Cysteine	296	17	

[a]Relative k_{cat}/K_m values in the ATP-PP$_i$ exchange reaction; [b]Relative k_{cat} values in the ATP pyrophosphatase reaction; [c]Relative k_{cat} values in the tRNA aminoacylation reaction; [d]Enhanced tRNA mischarging observed with engineered synthetases; [e]These amino acids are cyclized during editing; [f]Fast transient tRNA mischarging observed under pre-steady state conditions in the yeast system (Lin *et al.,* 1984).

the misacylation reactions are 10^3–10^6-fold less efficient than the correct aminoacylation (Tables 1 and 2). The most efficient tRNA misacylations are catalyzed *in vitro* by class II AARSs: *E. coli* LysRS (Jakubowski, 1999a) which generates of one molecule of misacylated Arg-tRNA^{Lys} per 1 600 correctly acylated Lys-tRNA^{Lys} molecules, and ProRS (Ahel *et al.*, 2002) which produces one misacylated Cys-tRNA^{Pro} molecule per 480 correctly acylated Pro-tRNA^{Pro} molecules (Table 2). In these cases, misacylated tRNA can be edited 'in trans' by aminoacyl-tRNA deacylases (Wong *et al.*, 2003; Ahel *et al.*, 2003; Ruan & Soll, 2005).

Most AARSs attach only L-amino acids to tRNA (that's why only L-amino acids are present in proteins). However, although they are not constituents of proteins, D-amino acids are used for tRNA aminoacylation *in vitro,* as first described in 1967 for *E. coli* TyrRS (Calendar & Berg, 1967) and subsequently for TrpRS, AspRS, LysRS, and HisRS (Wydau *et al.*, 2009). *E. coli* TyrRS, TrpRS (Calendar & Berg, 1967), and AspRS (Soutourina *et al.*, 2000) catalyze one misacylation of cognate tRNA with a corresponding D-amino acid per 13, 145, and 4125 correct aminoacylations with cognate L-amino acid, respectively. The aminoacylation of tRNA^{Tyr} with D-tyrosine was shown to occur *in vivo* in *E. coli* (Soutourina *et al.*, 2004). Deacylation of D-aminoacyl-tRNA by specific deacylases related to the editing domain of archeal ThrRS (Dwivedi *et al.*, 2005), prevent the incorporation of D-amino acids into protein (Soutourina *et al.*, 2004, Calendar & Berg, 1967, Ferri-Fioni *et al.*, 2001).

Recent data suggest that the accuracy of tRNA aminoacylation *in vivo* is considerably lower than expected from *in vitro* studies. For example, in mammalian cells about 1% of Met residues used in protein synthesis are attached by MetRS to tRNAs other than tRNA^{Met} (Netzer *et al.*, 2009). Furthermore, levels of misacylated Met-tRNAs increase up to 10-fold under stress conditions, such as oxidative stress, exposure to toll-like receptor ligands, or viral infection, and the Met-misacylated tRNAs are used in protein synthesis. Although the data appear to be convincing, such extraordinarily high tRNA misacylation rates are not consistent with previous findings demonstrating much higher accuracy of tRNA selection by MetRS (Schulman, 1991). The tRNA misacylation seems to be limited to Met and was not detected with other five amino acids studied (Cys, Ile, Phe, Tyr, Val) (Netzer *et al.*, 2009). In *E. coli*, replacement of 40% Met residues by norleucine in protein increases sensitivity to oxidative stress. Norleucine-substituted cells die more rapidly than control cells when exposed to hypochlorite, hydrogen peroxide or ionizing radiation (Luo & Levine, 2009). Whether global misincorporation of Met into protein would be protective against oxidative stress in mammalian cells, as suggested by Netzer *et al.* (Netzer *et al.*, 2009), remains to be demonstrated.

THE DISCOVERY OF EDITING

An error-correcting reaction in the selection of amino acids for protein synthesis was first described for IleRS (Baldwin & Berg, 1966), which forms relatively stable enzyme-bound aminoacyl adenylates in the presence of Ile or Val and ATP. Whereas the cognate IleRS·Ile-AMP reacts with tRNA^{Ile} to form Ile-tRNA^{Ile}, the noncognate IleRS·Val-AMP is hydrolyzed in the presence of tRNA^{Ile} to Val + AMP and no Val-tRNA^{Ile} is produced. Thus, IleRS possesses an editing activity, which prevents attachment of the noncognate Val to tRNA^{Ile} (Baldwin & Berg, 1966). Specific pathways for editing were discovered much later. For example, the discovery of a deacylating activity of IleRS towards Val-tRNA^{Ile} (Eldred & Schimmel, 1972) led to a suggestion that the deacylation of mischarged tRNA is a mechanism for editing errors in amino acid selection. The first evidence for an alternative editing mechanism was obtained for a plant ValRS (Jakubowski, 1980), which catalyzes selective hydrolysis of enzyme-bound non-cognate aminoacyl adenylates at significant rates in the absence of tRNA. Subsequent studies confirmed the aminoacyl adenylate editing pathway with *E. coli* IleRS, MetRS, and ValRS, introduced the terminology of *pre-transfer* and *post-transfer editing*, and

led to a discovery that Hcy-thiolactone is a product of Hcy editing (Jakubowski & Fersht, 1981).

EDITING PATHWAYS

AARSs correct errors in amino acid selection by hydrolyzing incorrect aminoacyl adenylates (*pre-transfer editing*) (Jakubowski, 1980), by deacylating incorrect aminoacyl-tRNA (*post-transfer editing*) (Eldred & Schimmel, 1972), or by a mixture of both *pre-transfer* and *post-transfer* editing mechanisms (Jakubowski & Fersht, 1981). The contribution of a particular pathway to the overall editing activity depends on the chemical nature of the amino acid. While all AARSs that possess the editing function have the ability to hydrolyze incorrect aminoacyl adenylates, some of them (e.g., *E. coli* MetRS (Jakubowski, 2000c), LysRS (Jakubowski, 1999a), SerRS (Gruic-Sovulj *et al.*, 2007); archaeal, bacterial, and yeast ProARSs (Ahel *et al.*, 2002)) do not possess the ability to deacylate aminoacyl-tRNA. Furthermore, the ability to edit is not universal for an AARS, but depends on its cellular localization. For instance, while cytoplasmic LeuRSs have the ability to deacylate mischarged $tRNA^{Leu}$, a human mitochondrial LeuRS lacks editing activity and mischarges $tRNA^{Leu}$ with Ile *in vitro* (Lue & Kelley, 2005). The alternative editing pathways, first recognized in 1981 (Jakubowski & Fersht, 1981), and confirmed in subsequent studies (e.g., Zhu *et al.*, 2009) are illustrated in Fig. 1 and described below.

Pathway k_1 refers to the dissociation of an AARS-bound aminoacyl adenylate to give free aminoacyl adenylate which hydrolyses in solution. The best examples of the k_1 pathway are IleRS editing Cys (Jakubowski & Fersht, 1981) and PheRS editing Tyr, which is also edited by the k_3 and k_4 pathways (Lin *et al.*, 1984). A minor fraction of Ala edited by ProRS uses this pathway (Splan *et al.*, 2008).

Pathway k_2 is the tRNA-independent hydrolysis of an AARS-bound aminoacyl adenylate. This is the most commonly used editing pathway, contributing 3–100% to the overall editing of a noncognate amino acid. The editing of Hcy by MetRS, IleRS, LeuRS, ValRS (Jakubowski & Goldman, 1992), and LysRS (Jakubowski, 1999a) occurs exclusively by this pathway. ValRS and IleRS also edit Cys using this pathway (Jakubowski & Fersht, 1981). Small fractions of Thr (3%) and Val (8%), misactivated by ValRS and IleRS, respectively, are also edited by this pathway. ProRS edits Ala (Splan *et al.*, 2008), while IleRS edits Val (Dulic *et al.*, 2010) using thie pathway. The k_2 pathway is the first editing pathway demonstrated *in vivo* (Jakubowski, 1990; Jakubowski, 1991). Interestingly, the active sites of GlnRS (Gruic-Sovulj *et al.*, 2005) and SerRS (Gruic-Sovulj *et al.*, 2007), which do not possess editing activity, have an intrinsic ability to hydrolyze cognate adenylates.

Pathway k_3 refers to the tRNA-dependent hydrolysis of an AARS-bound aminoacyl adenylate without transient mischarging of tRNA. The existence of this pathway was originally suggested by experiments, which show that a $tRNA^{Val}$ variant devoid of amino acid acceptor activity partially retains the ability to stimulate editng of Thr and Cys by ValRS (Jakubowski, 1980). Subsequently, by monitoring the fate of Tyr-AMP, it was shown that the major part of the misactivated Tyr is edited by fast hydrolysis of PheRS·Tyr-AMP (Lin *et al.*, 1984). IleRS·Val~AMP is also edited by the k_3 pathway: $tRNA^{Ile}$ induces rapid hydrolysis of IleRS·Val~AMP and no mischarged $tRNA^{Ile}$ could be detected (Fersht, 1977). That transient mischarging of tRNA with Val is not required, is also supported by the finding that a DNA aptamer that binds to IleRS, but cannot be aminoacylated, also stimulates the hydrolysis of Val~AMP by IleRS (Hale & Schimmel, 1996).

The k_3 pathway is intrinsic to the active site of LeuRS and is revealed in a LeuRS variant (missing the CP1 domain), which retains the ability to hydrolyze Ile-AMP and prevents $tRNA^{Leu}$ mischarging with Ile. Furthermore, a 2'dA variant of $tRNA^{Leu}$, devoid of amino acid acceptor activity, retains the ability to stimulate Ile editing by LeuRS (Boniecki *et al.*, 2008).

Pathway k_4 is the deacylation of an enzyme-bound misacylated tRNA. The misacylation-deacylation pathway was rigorously proven for the editing of Thr and α-aminobutyrate by ValRS (Fersht, 2000). Misacylated Thr-$tRNA^{Val}$ and α-aminobutyryl-$tRNA^{Val}$ form transiently as intermediates with the expected kinetics in pre-steady state experiments (Fersht & Kaethner, 1976; Fersht & Dingwall, 1979). In the k_4 pathway the 3' end of the mischarged tRNA is translocated from the synthetic active site to the editing site for deacylation (Ling *et al.*, 2009). A significant fraction of Thr misactivated by ValRS is also edited by the k_2 and k_3 pathways (Lin *et al.*, 1984). A minor fraction of misactivated Tyr (5%) is transferred to $tRNA^{Phe}$ and the resulting Tyr-$tRNA^{Phe}$ is rapidly deacylated (Lin *et al.*, 1984). The k_4 pathway was also proposed as a mechanism for editing Val by IleRS, Ser by ThrRS, Gly and Ser by AlaRS, Ile and Met by LeuRS, and Ala by ProRS (Ling *et al.*, 2009); however, there is no evidence for transient formation of mischarged tRNA intermediates by any of these AARS.

THE CHEMISTRY OF EDITING

Editing usually occurs by hydrolysis of an incorrect AA~AMP or AA-tRNA, which affords a free amino acid. However, some amino acids are modified as a result of editing. For example, MetRS, IleRS (Jakubowski & Fersht, 1981), LeuRS, ValRS (Jakubowski & Goldman, 1992), and LysRS (Jakubowski, 1997a) misactivate Hcy and form AARS-bound Hcy~AMP (reaction 3). The Hcy~AMP intermediate is destroyed in a tRNA-independent intramolecular reaction in which the side chain thiolate of Hcy displaces the AMP group from the carboxylate of the activated Hcy, form-

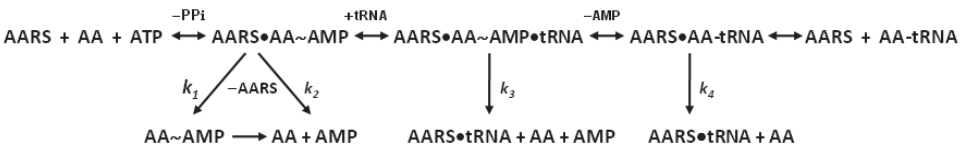

Figure 1. Editing pathways during tRNA aminoacylation
A cognate amino acid proceeds through the aminoacylation pathway, indicated by the double-headed arrows, affording correctly charged AA-tRNA. A noncognate amino acid enters the aminoacylation pathway but is rejected at points indicated by single-headed arrows; as a result, continuous hydrolysis of ATP to AMP occurs, a diagnostic feature of editing (Jakubowski & Goldman, 1992).

Table 3. *E. coli* AARSs catalyze the synthesis of Hcy-thiolactone but not Hcy-tRNA

AARS and tRNA tested	[^{35}S]Hcy-thiolactone formed, μM	Hcy-tRNA formed, μM
MetRS	27	
MetRS, $tRNA^{fMet}$	18	<0.004
IleRS	10	
IleRS, $tRNA^{Ile}$	5	<0.004
ValRS	1.4	
ValRS, $tRNA^{Val}$	1.1	<0.004
LysRS	0.6	
LystRS, $tRNA^{Lys}$	0.7	<0.004

Reactions were carried out in the reactions mixtures containing 0.1 M K-Hepes buffer, pH 7.4, 10 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 4 units/ml yeast inorganic pyrophosphatase, 2 mM ATP, 0.2 mM [^{35}S] Hcy (10 000 Ci/mol), 0.4 μM *E. coli* AARS, in the absence and presence of 10 μM indicated *E. coli* tRNA (amino acid acceptor activity 1100-1400 pmol/A_{260}). In tRNA aminoacylation assays, no incorporation of [^{35}S]Hcy above background (0.004 μM) was observed at times from 0 to 60 min (Jakubowski, 2002).

ing Hcy-thiolactone (Jakubowski, 1997a, Jakubowski & Fersht, 1981) as a product (reaction 4). tRNA essentially does not affect the Hcy editing reaction and is not mischarged with Hcy (Table 3) (Jakubowski, 2002a). One mol of Hcy-thiolactone forms per one mol of ATP hydrolyzed to AMP (Jakubowski, 1997a).

In analogous reactions, homoserine (Hse) is converted to Hse-lactone (reaction 5) by LysRS (Jakubowski, 1997a), IleRS, or ValRS (Jakubowski & Goldman, 1992), whereas ornithine (Orn) is converted to Orn-lactam (reaction 6) by LysRS (Jakubowski, 1999a). In the Orn editing reaction one mol of Orn-lactam is formed per one mol of ATP hydrolyzed to AMP (Jakubowski, 1999a).

EDITING, THIOL AMINOACYLATION, AND THE ORIGIN OF tRNA AMINOACYLATION

AARSs have the ability to aminoacylate thiols, including CoA and pantetheine, which are known to participate in non-ribosomal peptide synthesis. With CoA-SH and L-cysteine, which are the most active thiol substrates, these reactions yield aminoacyl-S-CoA thioesters and aminoacyl-Cys dipeptides, respectively. The thiol aminoacylation reactions are catalyzed by class I (Arg-, Cys-, Ile-, Met-, and ValRS) (Jakubowski, 1996b; Jakubowski, 1998; Jakubowski, 2000a; Jakubowski, 1996a; Jakubowski, 1995b) and class II AARSs (Asp-, Ser-, and LysRS) (Jakubowski, 1999a, Jakubowski, 1997a). The chemistry of these reactions mimics the chemistry of Hcy editing, while their rates approach the rate of Hcy-thiolactone formation during Hcy editing (Jakubowski, 1996b). The thiol aminoacylation reactions catalyzed by AARSs are also analogous to the amino acid activation by the multienzyme complexes in the present-day non-ribosomal peptide synthesis (Jakubowski, 2000a; Jakubowski, 1998).

The ability of the present-day AARSs to aminoacylate CoA and pantetheine appears to be a relic from ancestral AARSs (Jakubowski, 1998; Jakubowski, 2000a), which may have functioned as aminoacyl:CoA ligases in non-ribosomal peptide synthesis, before acquiring the ability to aminoacylate tRNA. Indeed, recent data of Mocibob *et al.* (Mocibob *et al.*, 2010) support this scenario. They found that several bacteria express homologs of SerRS which have the ability to aminoacylate phosphopantetheine prosthetic group of carrier proteins with Gly or Ala, but are unable to aminoacylate tRNA. Both SerRS homolog and acyl carrier proteins are encoded in the same region of the bacterial chromosome (Mocibob *et al.*, 2010). However, although they catalyze the same reaction as adenylation domains of non-ribosomal peptide synthetases, the cellular function of these new amino acid:carrier protein ligases is unknown.

EDITING OCCURS AT THE ACTIVE SITE OR AT A SEPARATE SITE OF AN AARS

Pre-transfer editing

A model for single site editing was first reported for *E. coli* MetRS. The model is based on structure/function studies of the synthetic and editing activities of the enzyme (Kim *et al.*, 1993; Jakubowski, 1996b), is consistent with the crystal structure of MetRS·Met binary complex (Serre *et al.*, 2001), and describes how MetRS directs Met and Hcy to the synthetic and editing pathways, respectively. In the synthetic pathway, the activated carboxyl group of Met reacts with the 2'-hydroxyl of the terminal adenosine of $tRNA^{Met}$, yielding Met-$tRNA^{Met}$. In the editing pathway, the misactivated carboxyl group of Hcy reacts with the thiolate of its side chain, yielding Hcy-thiolactone. The editing occurs in the absence of $tRNA^{Met}$ (*via* editing pathway k_2, Fig. 1), which does not significantly affect the Hcy editing reaction when present (Jakubowski & Fersht, 1981; Jakubowski, 2002a) (Table 3). Whether an amino acid completes the synthetic or editing pathway is determined by the competition for its activated carboxyl group between the side chain of the amino acid and the terminal adenosine of $tRNA^{Met}$. Met completes the synthetic pathway because its side chain is firmly bound by hydrophobic and hydrogen bonding interactions in the specificity sub-site, preventing the sulfur atom of Met from competing with the 3'-terminal adenosine of $tRNA^{Met}$ for the carboxyl carbon of Met. Consistent with this model, mutations of Tyr15 and Trp 305 residues, which form the hydrophobic Met-binding pocket, affect Hcy/Met discrimination by the enzyme (Kim *et al.*, 1993). The side chain of Hcy, missing the methyl group of Met, interacts with the specificity subsite not as strongly as the side chain of Met does. This allows the side chain of Hcy to also interact with a thiol-binding sub-site, which facilitates editing (Fig. 2, upper panel). This explains why Hcy is not transferred to tRNA but is cyclized to Hcy-thiolactone. Cognate Met can also enter the editing pathway when the thiol subsite is occupied by a thiol mimicking the side chain of Hcy (Jakubowski, 1996b) (Fig. 2, middle panel).

Although direct translational incorporation of Hcy into protein is prevented by the editing function of MetRS, *S*-nitrosaylation renders Hcy resistant to editing. S-Nitroso-Hcy is so similar to Met that it is attached to $tRNA^{Met}$ by MetRS (Table 1; Fig. 2, lower panel) and incorporated translationally into protein at positions normally occupied by Met, as demonstrated in an *in vitro* rabbit reticulocyte translation system, *in vivo* in *E. coli* (Jakubowski, 2000c), and *ex vivo* in cultured human endothelial cells (Jakubowski, 2001b). Removal of the nitroso group results in proteins containing Hcy at positions normally occupied by Met. In this way, Hcy gains access to the genetic code by nitric oxide-mediated invasion of the Met-coding pathway (Jakubowski, 2005a).

A similar model accounts for Hcy editing by IleRS (Jakubowski, 1998; Jakubowski, 2000a; Jakubowski, 1995b;

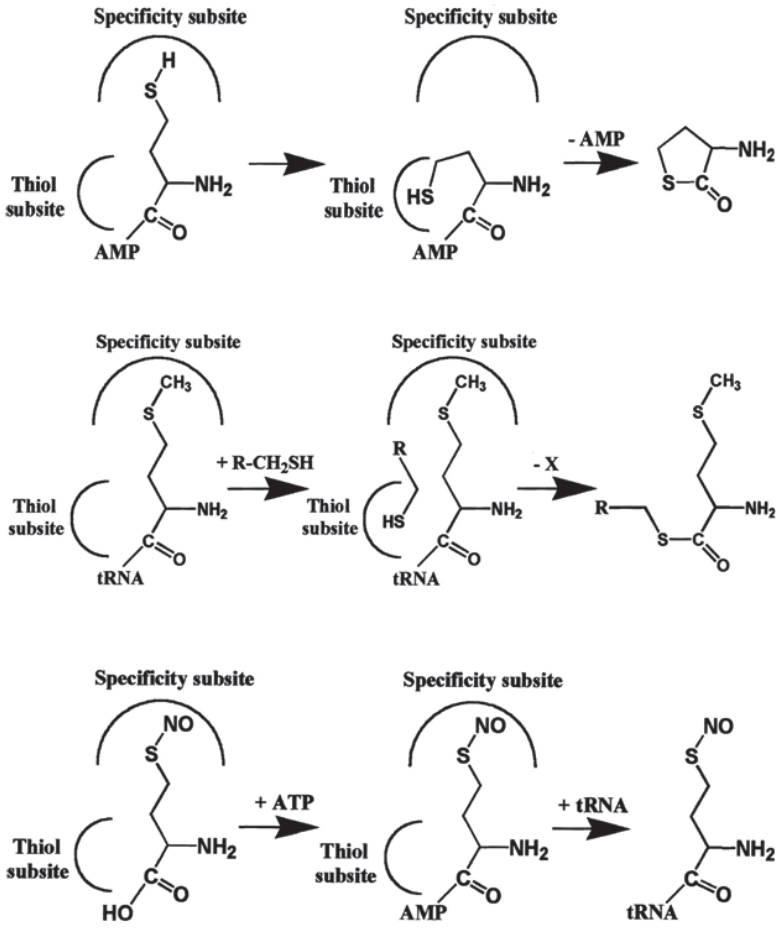

Figure 2. Editing of Hcy, aminoacylation of thiols, and synthesis of S-nitroso-Hcy-tRNAMet catalyzed by MetRS.
Upper panel: The MetRS-catalyzed cyclization of homocysteinyl adenylate to form Hcy-thiolactone and AMP, which are subsequently released from the synthetic/editing active site of MetRS. **Middle panel:** The MetRS catalyzed reaction of a thiol (mimicking the side-chain of Hcy, R-CH_2SH) with Met-$tRNA^{Met}$ to form a Met thioester, which is subsequently released from the synthetic/editing active site of MetRS. **Lower panel:** MetRS-catalyzed aminoacylation of $tRNA^{Met}$ with *S*-nitroso-Hcy. Adapted from (Jakubowski, 2001b).

Jakubowski, 1996a) and ValRS (Jakubowski, 2000a; Jakubowski, 1995b). LeuRS, which edits norvaline, Ile, and Met by pre-transfer and post-transfer mechanisms (Zhu *et al.*, 2009; Boniecki *et al.*, 2008), is also likely to edit Hcy (Jakubowski, 1995a) by a single site mechanism.

E. coli LysRS, which is similar to MetRS in that it lacks a separate editing site, edits Orn, Hcy, and Hse at the active site. Editing by LysRS is not affected by $tRNA^{Lys}$ and no $tRNA^{Lys}$ mischarging with Orn or Hcy occurs (Jakubowski, 1999a). However, LysRS mischarges $tRNA^{Lys}$ with several other amino acids (Arg, Thr, Met, Cys, Leu, Ala, or Ser) and does not deacylate mischarged Arg-$tRNA^{Lys}$, Thr-$tRNA^{Lys}$, and Met-$tRNA^{Lys}$. Recent data suggest that MetRS catalyzes the synthesis of Met-$tRNA^{Lys}$ (and several other Met-tRNAs) *in vivo*. Indeed, mischarged Met-$tRNA^{Lys}$ has been detected in cultured human and mouse cells and Met-$tRNA^{Lys}$ levels are reported to increase up to 10-fold in response to viral infection, toll-like receptor ligands, and oxidative stress (Netzer *et al.*, 2009).

Recent data show that ProRS (Splan *et al.*, 2008), LeuRS (Boniecki *et al.*, 2008), and IleRS (Dulic *et al.*, 2010) utilize pre-transfer editing for rejection of Ala, Ile, and Val, respectively. This mechanism is predominant in archaeal and mitochondrial ProRSs that naturally do not possess the post-transfer N2 editing domain (Splan *et al.*, 2008). The CP1 post-transfer editing domain is absent in mitochondrial LeuRSs (Lue & Kelley, 2005). In, general, pre-transfer editing occurs efficiently at the active site when the rate of transfer to tRNA is slow (Dulic *et al.*, 2010; Minajigi & Francklyn, 2010) or nonexistent, as originally found for IleRS (Fersht, 1977) and MetRS (Jakubowski & Fersht, 1981).

Post-transfer editing

Post-transfer editing has been the most extensively studied aspect of quality control by AARSs within the past dozen years or so. According to a post-transfer editing model (Ling *et al.*, 2009), the 3'-end of tRNA is first mischarged at the synthetic active site and translocated to a distinct editing site, where it undergoes deacylation. Evidence that a separate site catalyzes editing was first reported for the deacylation of Val-$tRNA^{Ile}$ by *E. coli* IleRS (Schmidt & Schimmel, 1994). Extensive structural and functional studies (Ling *et al.*, 2009) show that class I IleRS, LeuRS, and ValRS share a homologous CP1 domain harboring an editing site for deacylation of mischarged tRNA (Fig. 1, editing pathway k_4). The CP1 domain is a globular insertion domain separated by a distance of 35 Å from the synthetic active site. The synthetic active site mutation eliminates initial discrimination between Val and Ile (in reaction 1) but does not affect selective deacylation of Val-$tRNA^{Ile}$ by IleRS (Schmidt & Schimmel, 1994). Furthermore, tagged mischarged Val-$tRNA^{Ile}$ cross links both to the synthetic active site and to the CP1 domain of *E. coli* IleRS, whereas tagged correctly charged Ile-$tRNA^{Ile}$ cross links only to the synthetic active site (Schmidt & Schimmel, 1995). Crystallographic studies of *T. thermophilus* IleRS (Nureki *et al.*, 1998) reveal that one Val binds at the synthetic active site in the nucleotide-binding fold domain and a second Val binds at the CP1 domain; Ile binds only at the active site. Mutations in the CP1 domain reduce the deacylase activity towards Val-$tRNA^{Ile}$ without affecting the tRNA aminoacylation reaction (Hendrickson *et al.*, 2000). The CP1 domain mutants gain the ability to mischarge tRNA. Isolated CP1 domain from IleRS and ValRS exhibit a weak but specific deacylating activity toward Val-$tRNA^{Ile}$ or Thr-$tRNA^{Val}$, respectively (Lin *et al.*, 1996).

Class II AlaRS, PheRS, ProRS, and ThrRS also possess editing domains responsible for deacylation of mischarged tRNA (Fig. 1, editing pathway k_4) (reviewed in ref. Ling *et al.*, 2009). For instance, the editing site located in the N2 domain of ThRS, the first to be described for a class II AARS, deacylates Ser-$tRNA^{Thr}$ (Dock-Bregeon *et al.*, 2000). Recent crystallographic and binding studies with nonhydrolyzable analogs, suggest

that the cognate Thr-tRNA^{Thr} is not sterically excluded from entering the editing site; instead a catalytic water molecule is excluded to avoid the hydrolysis of the cognate substrate (Hussain *et al*., 2010). A domain homologous to the N2 domain of ThrRS is present in AlaRS, which deacylates Ser-tRNA^{Ala} and Gly-tRNA^{Ala} (Beebe *et al*., 2003). A unique deacylating domain present in ProRS is responsible for the deacylation of misacylated Ala-tRNA^{Pro}, but not Cys-tRNA^{Pro} (Wong *et al*., 2003). PheRS provides another example of an idiosyncratic structural arrangement in which the synthetic active site and the editing site (responsible for deacylation of Tyr-tRNA^{Phe}) are located in different subunits, α and β, respectively (Roy *et al*., 2004).

ACCURACY OF tRNA AMINOACYLATION DEPENDS ON EDITING

Direct demonstration that editing is an important determinant of the fidelity of tRNA aminoacylation came from studies of engineered and natural variants of AARSs. For instance, an IleRS variant that is devoid of editing activity, efficiently aminoacylates tRNA^{Ile} with Val (Nureki *et al*., 1998). Editing defective variants of class I LeuRS, ValRS, and class II AlaRS, PheRS, ProRS, and ThrRS, which misacylate corresponding tRNAs, have also been generated (Ling *et al*., 2009). Furthermore, physiological stress can also affect editing, as illustrated by a recent finding showing that oxidation of a Cys residue located in the editing site impairs the editing activity of ThrRS and leads to mischarging of tRNA^{Thr} with Ser (Ling & Soll, 2010).

In many cases, tRNAs accelerate editing. However, modified forms of tRNA^{Ile}, tRNA^{Val} and tRNA^{Phe} do not stimulate editing of noncognate amino acids by corresponding AARSs, and, in contrast to native tRNA, can be efficiently misacylated (Igloi *et al*., 1978). Conversely, a 2'dA variant of tRNA^{Leu}, is devoid of aminoacylation activity, but retains the ability to stimulate pre-transfer Ile editing by LeuRS (Boniecki *et al*., 2008).

Expression of engineered editing-defective AARSs or aminoacyl-tRNA decylases has no effect on cell growth under normal conditions, but results in growth inhibition in cultures supplemented with a corresponding non-cognate amino acid (Ling *et al*., 2009). For example, *E. coli* strain harboring editing-defective ValRS, which mischarges tRNA^{Val} with threonine *in vitro,* fails to grow on media supplemented with threonine or α-aminobutyrate (Doring *et al*., 2001) whereas a strain harboring editing-defective AlaRS, which mischarges tRNA^{Ala} with Ser and Gly, fails to grow in media supplemented with Ser (Chong *et al*., 2008). However, serine toxicity is rescued by a transgene encoding the AlaXp deacylase (Chong *et al*., 2008). Oxidative stress reduces translational fidelity and delays growth in *E. coli* by impairing the editing activity of ThrRS, which leads to Ser-tRNA^{Thr} formation (Ling & Soll, 2010).

A naturally occurring variant of mouse AlaRS with Ala734Glu missense substitution in the editing domain has a slightly diminished ability to deacylate mischarged Ser-tRNA^{Ala} and Gly-tRNA^{Ala}. This editing defect was suggested to lead to the accumulation of misfolded proteins in neurons and degeneration, especially affecting Purkinje cells (Lee *et al*., 2006). It is surprising that only a two-fold slower deacylation of Ser-tRNA^{Ala} by the mutant AlaRS would lead to neurodegeneration, ataxia, and hair follicle dystrophy in a *sti* mouse. However, an alternative explanation is that the AlaRS mutation affects another, presently unknown, function of the enzyme. This suggestion is supported by findings that mutations in human GlyRS and TyrRS, which do not possess editing function, also cause neurodegeneration known as Charcot-Marie Tooth (CMT) disease (Park *et al*., 2008; Stum *et al*., 2011). Furthermore, an Arg329His missense substitution in human AlaRS, located in helical domain of the enzyme, has recently been discovered in dominant axonal CMT disease (Latour *et al*., 2010).

CLEARANCE OF MISCHARGED tRNA BY OTHER PROTEINS

As discussed in previous sections, some AARSs do not possess the deacylase activity and produce mischarged tRNA *in vitro*. Furthermore, isolated CP1 domains retain the ability to deacylate mischarged tRNA. These two findings raise a possibility that editing can be carried out by a separate protein independent of an AARS. Indeed, separate enzymes homologous to the editing domains of AlaRS, ProRS, or ThrRS that correct the mischarging errors have been identified (Ahel *et al*., 2003). For example, AlaXp, a ubiquitous protein homologous to the editing domain of AlaRS, has the ability to deacylate Ser-tRNA^{Ala} and Gly-tRNA^{Ala} *in vitro* and prevents serine toxicity in *E. coli* harboring editing-defective AlaRS (Chong *et al*., 2008).

In mouse, both AlaXp and AlaRS are required for preventing misacylation of tRNA^{Ala} with Ser. The accumulation of misfolded protein in a *sti* mouse neuroblastoma cells suggests that the deacylase activity of AlaXp is not sufficint to compensate for the small editing deficiency in AlaRS (Lee *et al*., 2006). Conversely, reducing expression of AlaXp with siRNA causes the accumulation of misfolded proteins in mouse neuroblastoma cells (Nawaz *et al*., 2011). This indicates that the editing activity of AlaRS alone is not sufficient to prevent mischarging tRNA^{Ala} with Ser and protein misfolding.

H. influenza and *E. coli* YbaK proteins are general aminoacyl-tRNA deacylases that hydrolyze *in vitro* and *in vivo* Cys-tRNA^{Pro} produced by ProRS (Ruan & Soll, 2005). Consistent with this function, deletion of *ybaK* gene suppresses a missense mutation, *thyA*:146CCA, in an *E. coli* strain which requires Cys-tRNA^{Pro} for growth in the absence of thymine. *Sulfolobus solfataricus* ThrRS-cat synthesizes both Thr-tRNA^{Thr} and Ser-tRNA^{Thr} and lacks the deacylase activity while a separate protein, ThrRS-ed, specifically deacylates Ser-tRNA^{Thr} (Korencic *et al*., 2004). Although ThrRS-ed is dispensable for growth under standard conditions, it is required for growth in serine-supplemented media.

A ubiquitous enzyme, D-Tyr-tRNA decylase (dtd) (Soutourina *et al*., 2000, Ferri-Fioni *et al*., 2001) corrects mischarging errors made by AARSs which lack the L-stereospecificity in recognition of amino acids. The dtd enzyme is not related to AARSs, exhibits a broad specificity towards D-aminoacyl-tRNAs, and does not deacylate L-aminoacyl-tRNAs. The deacylase protects *E. coli* and the yeast *S. cerevisiae* against the toxicity of D-Tyr, D-Trp, D-Asp, and D-Leu. Inactivation of the deacylase gene leads to increased toxicity of these D-amino acids. The D-aminoacyl-tRNA deacylases are widely distributed in nature and are present in bacteria, archaea, and eukaryotes (Wydau *et al*., 2009).

tRNA IDENTITY ELEMENTS REQUIRED FOR EDITING

Similar to tRNA aminoacylation, tRNA-dependent editing requires recognition of specific structural elements

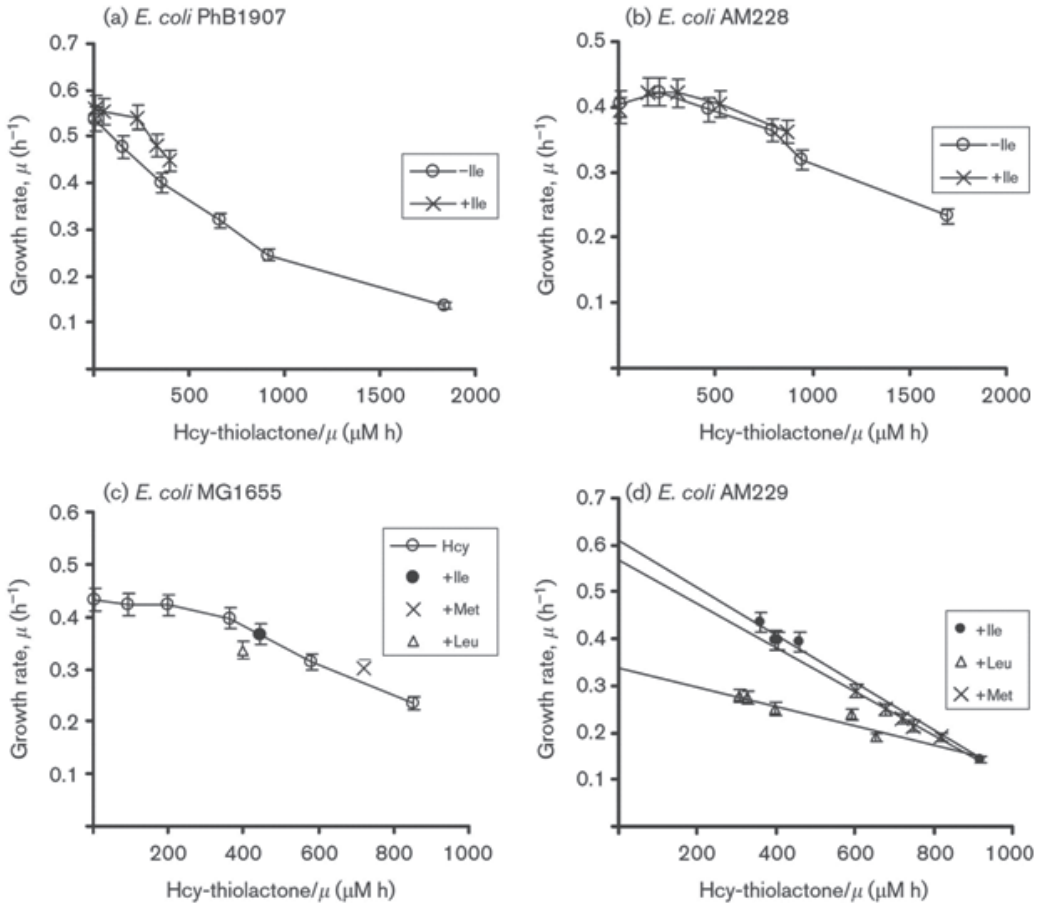

Figure 3. Growth rate is inversely related to Hcy editing
(a, b) *E. coli* strains PhB1907 (a) and AM228 (b) were grown in minimal medium supplemented with 0, 0.5, 1, 2, 4 or 8 mM DL-Hcy in the absence (○) and presence (x) of 1.6 mM Ile. **(c)** *E. coli* strain MG1655 was grown on minimal medium supplemented with 0, 0.5, 1, 2, 4 or 8 mM DL-Hcy (o) or with 8 mM DL-Hcy+0.4 mM Ile (●), Leu (△) or Met (x). **(d)** *E. coli* strain AM229 was grown in minimal medium supplemented with 8 mM DL-Hcy and 0, 0.2, 0.4, 0.8 or 1.6 mM Ile (●), Leu (△) or Met (x). Growth rates are plotted as a function of Hcy-thiolactone/growth rate. (Reprinted with permission from Sikora & Jakubowski, 2009).

in tRNA by an AARS. Some structural elements, such as 3'-terminal adenosine, are essential for both aminoacylation and editing. Other structural elements are required exclusively for aminoacylation, e.g. anticodons of $tRNA^{Ile}$ and $tRNA^{Val}$, or editing, e.g. nucleotides in the D loop (Hale *et al.*, 1997). For example, substitution of 3'-terminal adenosine by cytosine or uracil in $tRNA^{Val}$ produces a tRNA that does not stimulate Thr editing by ValRS. The 3'-modified $tRNA^{Val}$ accepts noncognate Thr in addition to the cognate Val. Removal, or substitution by an amino group, of one of the two *cis* hydroxyl groups in the 3'-terminal adenosine of $tRNA^{Val}$ or $tRNA^{Phe}$ produces variant tRNAs that do not stimulate editing and acquire the ability to accept noncognate amino acids. Replacing the D loop in $tRNA^{Ile}$ with the D loop from $tRNA^{Val}$ produces a variant of $tRNA^{Ile}$ molecule that remains fully active in the aminoacylation with Ile but no longer stimulates Val editing (Hale *et al.*, 1997). Similarly, a $tRNA^{Val}$ variant with a GAU Ile anticodon accepts Ile but does not stimulate Val editing of by IleRS. However, transplanting the D loop of $tRNA^{Ile}$ into the variant $tRNA^{Val/GAU}$ produces a molecule that stimulates Val editing.

Some AARSs use the same structural elements in tRNA both for aminoacylation and post-transfer editing, although they are recognized differently. For example, during aminoacylation the conserved G3:U70 base pair in $tRNA^{Ala}$ is recognized by N-terminal catalytic domain of *E. coli* AlaRS. However, during post-transfer editing the same base pair is recognized by a distinct domain of the enzyme (Beebe *et al.*, 2008). These observations with AlaRS are not consistent with the translocation model for post-transfer editing (Ling *et al.*, 2009). A C-terminal deletion in *Pyrococcus horikoshi* LeuRS disrupts the interaction with the elbow region of $tRNA^{Leu}$ and inactivates the aminoacylation activity but not the deacylation of Ile-$tRNA^{Leu}$ (Yao *et al.*, 2008).

HOMOCYSTEINE EDITING IS PART OF THE tRNA AMINOACYLATION PROCESS *IN VIVO*

As discussed above, Hcy is edited by MetRS, LueRS, IleRS, ValRS, and LysRS *in vitro*. Editing of Hcy, Orn, and Hse, which yield distinct reaction products (Hcy-thiolactone (reaction 4), Orn-lactam (reaction 5), Hse-lactone (reaction 6), respectively), is amenable to direct examination *in vivo*. Of these, Hcy editing has been extensively studied and is a textbook paradigm of *in vivo* error-correcting reactions. The evidence that Hcy editing is part of MetRS-catalyzed tRNA charging with Met in living organisms is summarized below:

• Hcy-thiolactone is synthesized from Hcy by MetRS in all organisms examined, including bacteria (Jakubows-

Table 4. Plasma *N*-Hcy-protein, Hcy-thiolactone, and Hcy concentrations in MTHFR- or CBS-deficient patients and unaffected individuals
Data from Chwatko *et al*., 2007, Jakubowski *et al*., 2008.

Genotype	*N*-Hcy-protein, μM (n)	Hcy-thiolactone, nM (n)	Hcy, μM (n)
MTHFR$^{-/-a}$	4.4 ± 3.4 (4)	11.8 ± 8.8 (4)	50.1 ± 15.1 (4)
MTHFR$^{-/-}$, before therapy	15.4 (1)	47.3 ± 1.7 (1)	208.0 (1)
MTHFR$^{+/-}$	1.06 ± 0.22 (6)	0.50 ± 0.29 (6)	7.8 ± 2.8 (6)
Unaffected	0.49 ± 0.08 (9)	0.20 ± 0.14 (9)	6.7 ± 1.9 (11)
CBS$^{-/-a}$	3.02 ± 2.27 (29)	14.4 ± 30.4 (14)	48.5 ± 57.5 (29)
CBS$^{-/-a}$, non-compliant	12.1 (1)		294.0 (1)

[a] *MTHFR*$^{-/-}$ and *CBS*$^{-/-}$ patients were on a Hcy-lowering therapy.

ki, 1990) yeast (Jakubowski, 1991) plants (Jakubowski & Guranowski, 2003), mice (Jakubowski *et al*., 2009), and humans (Chwatko *et al*., 2007). Because of its mostly neutral character at physiological pH (p*K* of Hcy-thiolactone is 6.7) (Jakubowski, 2006), Hcy-thiolactone accumulates in extracelluar fluids (Jakubowski, 2007).

• Hyperhomocysteinemia caused by genetic or nutritional deficiencies in Hcy or folate metabolism in *E. coli*, yeast, mice, and humans leads to increased accumulation of Hcy-thiolactone. Plasma levels of Hcy-thiolactone in cystathionine β-synthase (CBS)- or methylenetetrahydrofoate reductase (MTHFR)-deficient patients and unaffected controls are shown in Table 4. These findings establish a substrate-product relationship between Hcy and Hcy-thiolactone *in vivo*.

• MetRS mutants defective in the Met-binding site of the enzyme, in *E. coli* (Jakubowski, 1991), yeast (Jakubowski, 1991), and Chinese hamster ovary cells (Jakubowski & Goldman, 1993) are also defective in Hcy-thiolactone synthesis. In addition, increased expression of *E. coli* (Jakubowski, 1990), yeast (Jakubowski, 1991), and rice (Jakubowski & Guranowski, 2003) MetRS leads to proportional increase in Hcy-thiolactone synthesis. These observations demonstrate that MetRS is responsible for Hcy editing in all cell types.

• Exogenous Hcy (taken up from the medium) is metabolized to Hcy-thiolactone by IleRS and LeuRS, in addition to MetRS (Jakubowski, 1995a). As a result, editing of exogenous Hcy is not prevented by supplementation with Met; full inhibition of Hcy editing is observed only after supplementation with excess Ile and Leu, in addition to Met. The C-terminal domain of bacterial MetRS is essential for editing of endogenous, but not exogenous, Hcy. These data suggest that amino acids are channelled from the Met biosynthetic pathway to protein synthesis (Jakubowski, 1995a).

PATHOPHYSIOLOGICAL CONSEQUENCES OF HOMOCYSTEINE EDITING

Hcy editing inhibits cell growth

Hcy is known to inhibit the growth of microbial cells. Experimental evidence suggests that growth inhibition is due to increased unproductive ATP consumption associated with Hcy editing by AARSs (Sikora & Jakubowski, 2009). In *E. coli* growing in a minimal medium one molecule of Hcy is edited by the conversion to Hcy-thiolactone per 109 molecules of Met incorporated into protein (Jakubowski, 1990). Because one mole of ATP is hydrolyzed per one mole of Hcy-thiolactone formed (reaction 1) (Jakubowski, 1997a) and Met activation consumes also one mole of ATP per each mole of Met-tRNA formed (Mulvey & Fersht, 1977), under these conditions unproductive ATP hydrolysis associated with Hcy editing is relatively low, equal to 1% of productive ATP consumption for Met activation for protein synthesis. In *E. coli* cultures supplemented with Hcy, the accumulation of Hcy-thiolactone and associated ATP dissipation increases 100-fold and the growth rate is inversely related to Hcy-thiolactone accumulation (Fig. 3). Thus, in the presence of Hcy one mole of ATP is unproductively dissipated for Hcy-thiolactone synthesis per each mole of ATP that is consumed for Met activation, i.e. unproductive ATP dissipation is equal to 100% of productive ATP consumption. Because Met constitutes 2.9% of the total amino acid content in bacterial protein, ATP energy dissipation for Hcy editing increases from 0.029% in minimal medium to 2.9% in Hcy-supplemented cultures, relative to total energy required for protein synthesis in *E. coli*. Since ATP production is a determinant of growth rate, the increased energy dissipation associated with editing explains the growth inhibition observed in Hcy-supplemented *E. coli* cultures. This conclusion is supported by findings that in Hcy-supplemented cultures, the inhibition of Hcy-thiolactone synthesis with Ile, Leu, or Met accelerates growth (Fig. 3). (Supplementation with Ile, Leu, or Met does not affect the growth rate in the absence of Hcy (Sikora & Jakubowski, 2009).) Similarly, a yeast strain in which unproductive energy dissipation for Hcy editing represents just 13% of the energy used for activation of Met, grows significantly slower than an isogenic strain which dissipates only 0.95% energy (Jakubowski, 1991).

The role of Hcy editing in human disease

Clinical studies have established that elevated Hcy is a risk factor for heart and brain diseases (Ebbing *et al*., 2010; Smith *et al*., 2010). A preponderance of biochemical and genetic data suggest that elevated Hcy promotes a proatherothrombotic phenotype in humans and experimental animals (Lentz, 2005; Manolescu *et al*., 2010). Normal Hcy homeostasis is maintained by regulation of the fluxes through the methyltransferase pathways that generate Hcy and the transsulfuration and remethylation pathways that remove Hcy (Brosnan *et al*., 2004). Deficiency in any of these fluxes leads to Hcy accumulation and disease.

Several hypotheses have been proposed to explain Hcy-toxicity (reviewed in: Glushchenko & Jacobsen, 2007; Lentz, 2005; Perla-Kajan *et al*., 2007b). The Hcy-thiolactone hypothesis (Jakubowski, 1997b) states that a pathway initiated by the conversion of Hcy to Hcy-thiolactone contributes to the pathophysiology of Hcy excess and is involved in atherothrombotic disease in humans (Fig. 4) (Jakubowski, 2007). Consistent with this hypothesis, plasma Hcy-thiolactone is elevated under conditions predisposing to atherothrombosis, such as hyper-homocysteinemia caused by mutations in *CBS* or *MTHFR* gene in humans (Table 4) or a high-Met diet in mice (Chwatko *et al*., 2007). A product of Hcy editing, Hcy-thiolactone is a reactive metabolite that causes protein N-homocysteinylation through the formation of amide

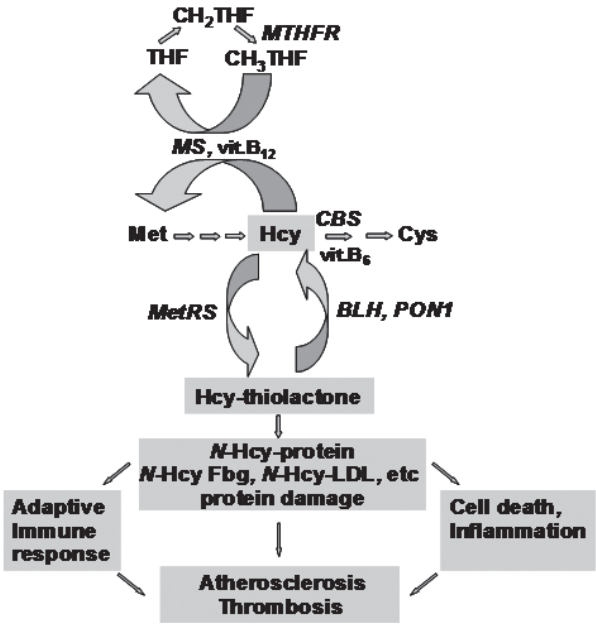

Figure 4. The Hcy-thiolactone hypothesis of vascular disease
In humans and animals Hcy is formed from dietary protein Met as a result of cellular methylation reactions. In this pathway Met is first activated by ATP to yield S-adenosylmethionine (AdoMet). As a result of the transfer of its methyl group to an acceptor, AdoMet is converted to S-adenosylhomocysteine (AdoHcy). *Enzymatic hydrolysis of AdoHcy is the only known source of Hcy in the human body*. Levels of Hcy are regulated by remethylation to Met, catalyzed by Met synthase (MS), and transsulfuration to cysteine, the first step of which is catalyzed by cystathionine β-synthase (CBS). The remethylation requires vitamin B_{12} and 5,10-methyl-tetrahydrofolate (CH_3-THF), generated by 5,10-methylene-THF reductase (MTHFR). The transsulfuration requires vitamin B_6. Hcy is also metabolized to the thioester Hcy-thiolactone by methionyl-tRNA synthetase (MetRS) in an error-editing reaction in protein biosynthesis when Hcy is mistakenly selected in place of Met. The flow through the Hcy-thiolactone pathway increases when re-methylation or trans-sulfuration reaction is impaired by genetic alterations of enzymes, such as CBS, MS, and MTHFR, or by inadequate supply of CH_3-THF. Hcy-thiolactone is a reactive metabolite that modifies protein lysine residues, which causes protein damage leading to an autoimmune response and thrombosis. *N*-Hcy-Fbg and *N*-Hcy-LDL, *N*-homocysteinylated forms of fibrinogen and low density lipoprotein, respectively. (Adapted with permission from Chwatko *et al.*, 2007)

bonds with protein lysine residues (Fig. 5), which impairs or alters the protein's function (Jakubowski, 1999b). *N*-linked protein Hcy (*N*-Hcy-protein), first discovered in cultured human cells (Jakubowski, 1997b; Jakubowski *et al.*, 2000), occurs in the human body, is elevated in hyperhomocysteinemic CBS or MTHFR deficient patients (Table 4) (Jakubowski *et al.*, 2008), and accumulates in atherosclerotic lesions in mice (Perla-Kajan *et al.*, 2008). In humans, *N*-Hcy-protein accumulation has been linked to an autoimmune response and atherothrombosis (Jakubowski, 2007). Recent human clinical studies show that plasma *N*-Hcy-protein levels are associated with a risk of coronary heart disease (Yang *et al.*, 2006), whereas plasma Hcy-thiolactone levels are associated with the development vascular damage in diabetic patients (Gu *et al.*, 2008).

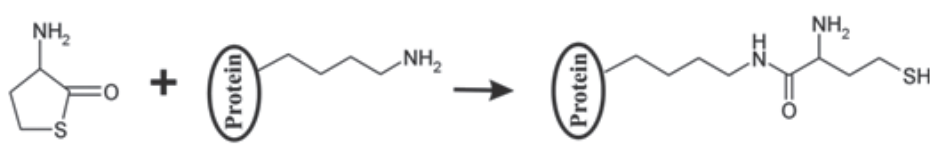

Figure 5. Schematic illustration of chemical modification of a protein lysine residue by Hcy-thiolactone
Adapted from (Jakubowski, 1997b)

Toxicity of Hcy-thiolactone

Consistent with the Hcy-thiolactone hypothesis, chronic treatments of animals with Hcy-thiolactone cause pathophysiological changes similar to those observed in human genetic hyperhomocysteinemia (Jakubowski, 2007). For example, Hcy-thiolactone infusions or Hcy-thiolactone-supplemented diet produce atherosclerosis in baboons or rats, whereas treatment with Hcy-thiolactone causes developmental abnormalities in chick embryos, including optic lens dislocation (Maestro de las Casas *et al.*, 2003), characteristic of the CBS-deficient human patients (Mudd *et al.*, 2001). Furthermore, Hcy-thiolactone is more toxic to cultured human cells than Hcy itself, and induces apoptotic death in vascular endothelial cells (Kerkeni *et al.*, 2006) and endoplasmic reticulum stress and unfolded protein response in retinal epithelial cells (Roybal *et al.*, 2004).

Hcy-thiolactone hydrolyzing enzymes

Because of its toxicity, Hcy-thiolactone clearance is essential for homeostasis in an organism (Chwatko & Jakubowski, 2005; Chwatko *et al.*, 2007). Two enzymes are known that have the ability to hydrolyze Hcy-thiolactone: extracellular (serum) paraoxonase 1 (PON1) (Jakubowski, 2000b) and intracellular bleomycin hydrolase (Blmh) (Zimny *et al.*, 2006).

PON1, named for its ability to hydrolyze the organophosphate paraoxon, is synthesized exclusively in the liver and carried on high-density lipoprotein (HDL) in the circulation, although recent studies suggest a wider expression pattern (Marsillach *et al.*, 2008). Pon1 protects against high-fat diet-induced atherosclerosis in mice (Shih *et al.*, 1998) and humans (Bhattacharyya *et al.*, 2008). Pon1-deficient mice are more susceptible to high-fat diet-induced atherosclerosis than wild type littermates, but do not develop atherosclerosis on normal chow diet (Shih *et al.*, 1998). Pon1-deficient mice are also more susceptible to neurotoxicity induced by intraperitoneal injections of Hcy-thiolactone than wild type animals (Borowczyk *et al.*, 2011) *In vitro*, HDL and the purified PON1 protein have the ability to hydrolyze Hcy-thiolactone (Jakubowski, 2000b) and to protect against protein N-homocysteinylation in serum (Jakubowski *et al.*, 2001) and cultured human endothelial cells (Jakubowski *et al.*, 2000). *In vivo*, PON1 protects proteins against N-homocysteinylation in humans (Perla-Kajan & Jakubowski, 2009). Furthermore, Hcy-thiolactonase activity of PON1 predicts cardiovascular disease (Domagała *et al.*, 2006). Serum Hcy-thiolactonase activity is absent in Pon1-knockout mice (Jakubowski, 2000b; Jakubowski, 2001a).

Blmh, named for its ability to hydrolyze the anticancer drug bleomycin, is ubiquitously expressed in various mammalian tissues (Bromme *et al.*, 1996; Kamata *et al.*, 2007) and is studied in the context of cancer therapy (Bromme *et al.*,

1996), Alzheimer's disease (Kajiya *et al.*, 2006b, Papassotiropoulos *et al.*, 2000, Lefterov *et al.*, 2001; Kajiya *et al.*, 2006a), Hcy toxicity (Zimny *et al.*, 2006; Suszynska *et al.*, 2010), and protein breakdown (Kamata *et al.*, 2009). Its physiological function was unknown until 2006 when it was demonstrated that Blmh is a major Hcy-thiolactonase in humans and yeast, and protects against Hcy toxicity in yeast (Zimny *et al.*, 2006). A recent finding that Hcy-thiolactonase activity of Blmh is significantly reduced in brains from Alzheimer's disease patients compared with unaffected brains (Suszynska *et al.*, 2010) suggests that diminished functional Blmh activity could contribute to the pathology of the disease. *Blmh*$^{-/-}$ mice are more sensitive to bleomycin toxicity than wild type animals and prone to tail dermatitis (Schwartz *et al.*, 1999). Blmh-deficient mice are also more susceptible to Hcy-thiolactone neurotoxicity than wild type animals (Borowczyk *et al.*, 2011).

BIOLOGICAL CONSEQUENCES OF PROTEIN N-HOMOCYSTEINYLATION

The reaction of Hcy-thiolactone with proteins — protein *N*-homocysteinylation — results in the substitution of the ε-amino group of a protein Lys residue with an Hcy residue containing a free thiol group (Fig. 5). This reaction impairs or alters the protein's structure and function (Jakubowski, 2007; Jakubowski, 2004; Jakubowski, 2008; Jakubowski, 2005b). For instance, *N*-Hcy-proteins are prone to oxidative damage (Glowacki & Jakubowski, 2004; Sibrian-Vazquez *et al.*, 2010), aggregation (Perla-Kajan *et al.*, 2007a; Glowacki & Jakubowski, 2004; Paoli *et al.*, 2010; Jakubowski, 1999b), and induce an autoimmune response (Undas *et al.*, 2004) and cell death (Ferretti *et al.*, 2004; Paoli *et al.*, 2010). Although studied with many proteins, the structural, functional, and biological consequences of *N*-homocysteinylation are best understood for albumin (Glowacki & Jakubowski, 2004; Paoli *et al.*, 2010) and fibrinogen (Sauls *et al.*, 2006), known targets for the modification by Hcy-thiolactone *in vivo* (Jakubowski *et al.*, 2008; Jakubowski, 2002b).

N-Hcy-albumin

Albumin is the major target for N-homocysteinylation in the human blood. Its Lys-525 residue is a predominant site of N-homocysteinylation *in vitro* and *in vivo* (Glowacki & Jakubowski, 2004). *N*-homocysteinylation affects the susceptibility of albumin to oxidation and proteolysis. A disulfide at the conserved Cys^{34} of albumin promotes conversion of *N*-(Hcy-SH)-albumin-Cys^{34}-SH to a proteolytically sensitive form *N*-(Hcy-S-S-Cys)-albumin-*Cys34*-SH, which would facilitate clearance of the N-homocysteinylated form of mercaptoalbumin (Glowacki & Jakubowski, 2004). Recent studies identify Lys-4, Lys-12, Lys-137, Lys-159, Lys-205, and Lys-212 of human albumin as targets for N-homocysteinylation by Hcy-thiolactone *in vitro* and provide evidence that two of those residues, Lys-137 and Lys-212, in addition to Lys-525, are N-homocysteinylated *in vivo* in human plasma (Marczak *et al.*, 2011; Sikora *et al.*, 2010).

Low levels of protein N-homocysteinylation can induce mild conformational changes leading to the formation of native-like aggregates, which evolve over time to amyloid-like structures and are toxic to cells (Paoli *et al.*, 2010). N-Homocysteinylation initiates albumin aggregation process under physiological-like conditions generating large protein complexes formed by native albumin molecules. The aggregation does not involve disulfide bond formation, but hydrophobic interactions facilitated by local protein unfolding. The early aggregates are cytotoxic and induce apoptosis in mammalian cell cultures. Furthermore, early aggregates of *N*-Hcy-albumin can act as a seed, stimulating the conversion of native albumin to molecular forms with greater propensity to aggregate. Over time, aggregated *N*-Hcy-albumin undergoes structural reorganization, generating curly protofibrils and circular structures similar to amyloid pores. Importantly, the levels of protein N-homocysteinylation that induce aggregation and toxicity (Paoli *et al.*, 2010) are close to those observed in albumin from patients with hyperhomocysteinemia (Jakubowski *et al.*, 2008). Taken together, these findings identify a mechanism that may explain the role of Hcy in neurological abnormalities observed in CBS- (Mudd *et al.*, 1985) or MTHFR-deficient patients (Strauss *et al.*, 2007), as well as in cognitive impairment and Alzheimer's disease in the general population (Smith *et al.*, 2010).

N-Hcy-fibrinogen

Fibrinogen (Fbg) is known to undergo facile N-homocysteinylation by Hcy-thiolactone *in vitro* (Jakubowski, 1999b) and *in vivo* in humans (Jakubowski *et al.*, 2008; Jakubowski, 2002b). Sauls and coworkers showed that clots formed from Hcy-thiolactone-treated normal human plasma or Fbg lyse slower than clots from untreated controls (Sauls *et al.*, 2006). Some of the lysine residues susceptible to N-homocysteinylation are close to tissue plasminogen activator and plasminogen binding, or plasmin cleavage, sites, which can explain abnormal characteristics of clots formed from *N*-Hcy-Fbg (Sauls *et al.*, 2006). Although *N*-Hcy-Fbg forms disulfide-linked complexes with albumin, this does not seem to increase its thrombotic propensity (Sauls *et al.*, 2011). The detrimental effects of elevated plasma Hcy on clot permeability and resistance to lysis in humans are consistent with a mechanism involving Fbg modification by Hcy-thiolactone (Undas *et al.*, 2006a). CBS-deficient patients, who suffer from atherothrombosis have significantly elevated plasma levels of prothrombotic *N*-Hcy-Fbg (Jakubowski *et al.*, 2008). Furthermore, Fbg purified from hyperhomocysteinemic patients (with plasma *N*-Hcy-Fbg = 2.38 μM) produces fibrin clots with a 1.2 fold longer lysis time and a denser clot structure compared with Fbg isolated from control patients (with plasma *N*-Hcy-Fbg = 0.34 μM) (Cilia La Corte *et al.*, 2011). These findings suggest that *N*-homocysteinylation of fibrinogen leads to abnormal resistance of fibrin clots to lysis and contributes to increased risk of thrombosis in humans (Fig. 4).

Auto-immunogenic properties of *N*-Hcy-protein

Similar to other post-translationally modified proteins, *N*-Hcy-proteins elicit an auto-immune response in humans, manifested by the induction of IgG auto-antibodies directed against *N*ε-Hcy-Lys epitopes. This response is enhanced in stroke (Undas *et al.*, 2004) and coronary artery disease (CAD) patients (Undas *et al.*, 2005), suggesting that it is a general feature of atherosclerosis (Jakubowski, 2005b). Elevated levels of anti-*N*-Hcy-protein IgG auto-antibodies are a consequence of elevated levels of Hcy-thiolactone (Gu *et al.*, 2008) and *N*-Hcy-protein (Yang *et al.*, 2006) observed in CAD patients. Anti-*N*-Hcy-protein IgG auto-antibodies vary considerably among individuals and are strongly correlated with plasma total Hcy, but not with Cys or Met (Undas *et al.*,

2004). Such correlation is explained by direct mechanistic links between Hcy-related species, predicted by the Hcy-thiolactone hypothesis (Fig. 4): elevation in Hcy leads to inadvertent elevation in Hcy-thiolactone which mediates the formation of neo-self antigens, *N*-Hcy-protein (Fig. 5). Raising levels of these antigens trigger an auto-immune response (Fig. 4). If the neo-self *N*ε-Hcy-Lys epitopes were present on endothelial cell membrane proteins, anti-*N*-Hcy-protein autoantibody would form antigen-antibody complexes on the surface of the vascular vessel. Endothelial cells coated with anti-*N*-Hcy-protein auto-antibodies would be taken up by the macrophage *via* the Fc receptor, resulting in injury to the vascular surface. If the *N*-Hcy-proteins were present chronically, repeating attempts to repair the damaged vascular wall would lead to an atherosclerotic lesion (Perla-Kajan *et al.*, 2008).

The involvement of an auto-immune response against *N*-Hcy-protein in CAD is supported by the findings that lowering plasma Hcy by folic acid supplementation lowers anti-*N*-Hcy-protein auto-antibodies levels in control subjects but not in CAD patients (Undas *et al.*, 2006b). These findings suggest that once accumulated, the *N*-Hcy protein antigens causing the auto-antibody response persist, and that chronic protein damage caused by Hcy-thiolactone cannot be easily reversed in CAD patients. Furthermore, these findings also suggest that while primary Hcy-lowering intervention by B-vitamin supplementation is beneficial, secondary intervention may be ineffective, and may explain at least in part the failure of B-vitamin therapy to lower cardiovascular events in myocardial infarction patients (Bonaa *et al.*, 2006; Ebbing *et al.*, 2010).

Turnover of *N*-Hcy-protein

The discovery of anti-*N*-Hcy-protein IgG auto-antibodies, which specifically recognize *N*ε-Hcy-Lys epitopes (Jakubowski, 2005b), suggests that proteolytic turnover of *N*-Hcy-protein occurs in the human body. The formation of these auto-antibodies is most likely initiated by proteolytic degradation of *N*-Hcy-protein to antigenic peptides, including those containing *N*ε-Hcy-Lys epitopes, which are then displayed on the cell surface. Because turnover of post-translationally modified proteins yields modified amino acids (e.g., methylated arginine and methylated lysine residues, ref. (Wang *et al.*, 2009)) one can predict that *N*-Hcy-protein would yield *N*-Hcy-Lys isopeptide among products of its hydrolysis. Indeed, recent data show that the *N*ε-Hcy-Lys isopeptide is present in mouse and human plasma, and that its levels are elevated in genetic (CBS deficiency in humans and mice, MTHFR deficiency in mice) or dietary (high Met diet in mice) deficiencies in Hcy metabolism. *N*ε-Hcy-Lys is generated by proteolytic degradation of *N*-Hcy-protein in mouse liver extracts. Taken together, these data indicate that free *N*ε-Hcy-Lys is an important pathology-related component of Hcy metabolism in humans and mice.

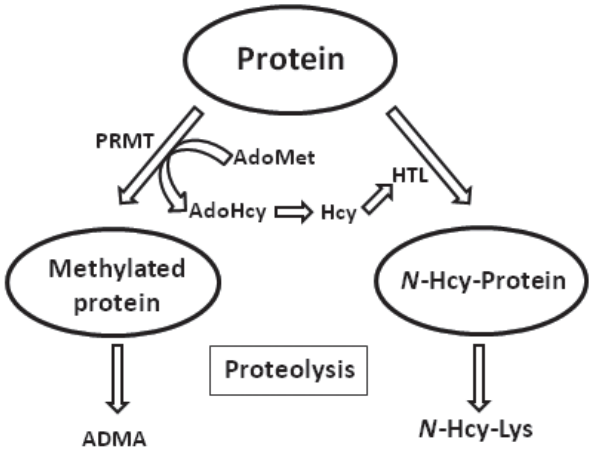

Figure 6. *N*-Hcy-Lys and asymmetric dimethylarginine (ADMA) are derived from the proteolysis of modified proteins
Arginine residues in proteins are methylated by protein arginine methyltransferase (PRMT), which uses S-adenosylmethionine (AdoMet) as a methyl donor and produces S-adenosylhomocysteine (AdoHcy). Hcy derived from the enzymatic hydrolysis of AdoHcy is converted by methionyl-tRNA synthetase to Hcy-thiolactone (HTL), which modifies protein lysine residues, affording *N*-Hcy-protein. Subsequent proteolytic degradation of *N*-Hcy-protein affords the isopeptide *N*-Hcy-Lys. (Reprinted with permission from Zabczyk *et al.,* 2011).

Recent data show that *N*ε-Hcy-Lys is significantly increased in acute myocardial infarction patients compared with controls. The plasma levels of *N*ε-Hcy-Lys are positively correlated with the nitric oxide synthase inhibitor asymmetric dimethylarginine, which is consistent with their common origin as products of protein degradation (Fig. 6) (Zabczyk *et al.*, 2011). Further studies are required to assess a prognostic value of *N*ε-Hcy-Lys in myocardial infarction.

CONCLUSIONS

Most of the effort over the last dozen years has been devoted to dissecting molecular details of the post-transfer editing mechanism, which are now known in considerable detail. However, except for the classical studies with ValRS and PheRS, transient accumulation of misacylated tRNA, a crucial intermediate in the proposed misacylation-deacylation pathway, remains to be demonstrated for most native AARSs. New studies within last few years, aided by high-resolution AARS structures, have revived interest in the pre-transfer hydrolytic editing mechanism and began to examine the role of the alternative editing pathways first described in 1978–1981. Experiments following the fate of the AA-AMP and AA-tRNA intermediates, preferably by fast kinetic approaches should provide insights into editing mechanisms. Roles of aminoacyl-tRNA deacylases in correcting tRNA mischarging errors have been documented both *in vitro* and *in vivo*. The loss of editing reduces the growth and viability in stressed cells, but not in cells under normal growth conditions. Editing and non-editing forms of the same AARS can exist in different organelles or organisms. Pre-transfer Hcy editing by MetRS yields Hcy-thiolactone in all organisms examined, from bacteria to man. This shows that continuous editing is part of the process of charging a tRNA with its amino acid *in vivo*. Pathophysiological consequences of excessive Hcy editing in bacteria and mammals have been identified and, in humans, linked to heart and brain diseases. Roles of other products of editing such as Hse-lactone and Orn-lactam in health and disease remain to be examined. The pathological phenotype of a mouse AlaRS mutant defective in the post-transfer editing, which affects brain function, underscores the importance of editing in maintaining normal physi-

ological function. Ongoing research is expected to clarify the role of the amino acid selectivity of AARSs in heart and brain diseases.

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