The non-protein amino acid homocysteine (Hcy), owing to its structural similarity to the protein amino acids methionine, isoleucine, and leucine, enters first steps of protein synthesis and is activated by methionyl-, isoleucyl-, and leucyl-tRNA synthetases in vivo. However, translational incorporation of Hcy into protein is prevented by editing mechanisms of these synthetases, which convert misactivated Hcy into thiolactone. The lack of efficient interactions of the side chain of Hcy with the specificity subsite of the synthetic/editing active site is a prerequisite for editing of Hcy. Thus, if the side chain thiol of Hcy were reversibly modified with a small molecule that would enhance its binding to the specificity subsite and prevent editing, such modified Hcy is predicted to be transferred to tRNA and incorporated translationally into protein. Here I show that S-nitroso-Hcy is in fact transferred to tRNA by methionyl-tRNA synthetase and incorporated into protein by the bacterium Escherichia coli. S-Nitroso-Hcy-tRNA also supports translation of mRNAs in a rabbit reticulocyte system. Removal of the nitroso group yields Hcy-tRNA and protein containing Hcy in peptide bonds. S-Nitrosylation-mediated translational incorporation of Hcy into protein may occur under natural conditions in cells and contribute to Hcy-induced pathogenesis in atherosclerosis.

Aminoacyl-tRNA synthetases (AARSs) establish the genetic code relationships in translation by matching amino acids with their cognate tRNAs. The non-protein amino acid homocysteine (Hcy), an obligatory precursor of methionine in all cell types, presents a major problem for translation, because it is misactivated by MetRS, IleRS, LeuRS, and LysRS. However, the misactivated Hcy is never transferred to tRNA. Instead, specific editing mechanisms prevent its editing and favor the transfer to tRNA. A possible way to achieve this is by utilizing S-nitrosothiol chemistry. Here I show that MetRS uses S-nitroso-Hcy as a substrate in the tRNA aminoacylation reaction, forming S-nitroso-Hcy-tRNA, and that S-nitroso-Hcy-tRNA transfers S-nitroso-Hcy into protein.
ampoule. The ampoule was then placed on a heating block, and the digestion was allowed to proceed for 4.5 h at 128 °C. After digestion, the resulting crude preparation of L-[S-35S]Hcy thiolactone was lyophilized, dissolved in 20 µl of water, and purified by 2D TLC on cellulose plates (20 × 10 cm) using butanol-acetic acid/water (4:1:1, v/v) as the first-dimension solvent and 2-propanol/ethyl acetate/water (5:5:1, v/v) as the second-dimension solvent (7, 8). Thiolactone spot, localized under UV light and by autoradiography, was scraped off the plate and eluted with 2 mM HCl. The overall yield of the procedure was 65%. The preparation of L-[35S]thiolactone was at least 96% pure on analytical 2D TLC. Maximum levels of contamination with Met, Hcy, and homocysteine were <1%, <0.8%, and <1%, respectively.



![Figure 1. Editing of homocysteine by MetRS.](image)

**Figure 1. Editing of homocysteine by MetRS.** After formation of Hcy–AMP in the synthetic/editing active site of MetRS, the side chain of Hcy moves from the specificity subsite to the thiol subsite, which facilitates the formation of Hcy thiolactone (3, 13, 14).

**TABLE I**

| Amino acid | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
|------------|----------|------|---------------|
| S-Nitroso-Hcy | 11.2 | 0.37 | 2.9 × 10⁶ |
| Hcy | 23 | 5.3 | 4.3 × 10⁶ |
| Met | 76 | 0.02 | 3.8 × 10⁵ |

**Activation of S-nitroso-Hcy and Other S-nitrosothiols.**

Activation of S-nitroso-Hcy was measured at pH 7.4, 37 °C in the ATP/PPi exchange reaction as described under “Materials and Methods.” For comparison, data for activation of Hcy and methionine from Ref. 13 are shown.

**RESULTS AND DISCUSSION**

**S-Nitroso-Hcy Is a Substrate for MetRS in the Adenylate Formation and tRNA Aminoacylation Reactions—S-Nitroso-Hcy was a substrate for MetRS in the aminoacyl adenylate formation reaction (Table I).** Catalytic efficiency for S-nitroso-Hcy was intermediate between those for methionine and Hcy. The $k_{cat}$ value for S-nitroso-Hcy (11.2 s⁻¹) was 7- and 2-fold lower than the $k_{cat}$ values for methionine and Hcy, respectively. The $K_m$ value for S-nitroso-Hcy (0.37 mM) was 18.5-fold higher and 14.3-fold lower than the $K_m$ values for methionine and Hcy, respectively. The enzyme-bound S-nitroso-Hcy—AMP intermediate was relatively stable and, in contrast to the Hcy—AMP intermediate (6), was not edited, as determined by the lack of S-nitroso-Hcy-dependent ATP hydrolysis (Fig. 2B).

**SDS-PAGE Analyses—Products of in vitro and in vivo translation were analyzed on 13% denaturing polyacrylamide gels (21).**

**TABLE I**

| Amino acid | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
|------------|----------|------|---------------|
| S-Nitroso-Hcy | 11.2 | 0.37 | 2.9 × 10⁶ |
| Hcy | 23 | 5.3 | 4.3 × 10⁶ |
| Met | 76 | 0.02 | 3.8 × 10⁵ |
Met catalyzed by MetRS was assayed as described under "Materials and Methods." Nonsaturating kinetics were observed with 2–160 μM MetRS. ([35S]Met-tRNA<sup>Met</sup>) editing activity was assayed by following the hydrolysis of [α-32P]ATP (2 mM, 50 Ci/mol) to [32P]AMP in the presence of 1 mM amino acid and 0.2 μM MetRS.

**TABLE II**

Aminoacylation activities of methionyl-tRNA synthetases

| MetRS        | Rate of Aminoacylation with [S-NO-Hcy] | Rate of Aminoacylation with Met |
|--------------|--------------------------------------|--------------------------------|
| Wild type    | 100                                  | 100                            |
| (k<sub>cat</sub>/K<sub>m</sub> = 2 × 10<sup>-2</sup> s<sup>-1</sup> M<sup>-1</sup>) | (k<sub>cat</sub>/K<sub>m</sub> = 1.6 × 10<sup>6</sup> s<sup>-1</sup> M<sup>-1</sup>) |
| W305A        | 7.2                                  | 46                             |
| Y15A         | 10.4                                 | 19                             |
| Y15F         | 9.4                                  | 1.2                            |
| R233Q        | 0.16                                 | 0.3                            |
| D52A         | 0.22                                 | 0.07                           |

Fig. 2. S-Nitroso-Hcy is a substrate for aminoacylation of tRNA<sup>Met</sup>, but not for editing, catalyzed by MetRS. A, incorporation of [S-nitroso-[35S]Hcy] (●), [35S]Hcy (■), and [35S]Met (●) (30 μM, 20,000 Ci/mmol) into tRNA<sup>Met</sup> catalyzed by 2 μM MetRS. B, S-nitroso-Hcy- (●) and Hcy-dependent (■) editing activity was assayed by following the hydrolysis of (α-[32P]ATP (2 mM, 50 Ci/mol) to [32P]AMP in the presence of 1 mM amino acid and 0.2 μM MetRS.

**TABLE III**

Table III shows the results of the deacetylation assay. The aminoacylation activities of [35S]Aminoacyl-tRNAs (2–5 μM) were deacylated at pH 7.4, 37 °C in the absence and presence of 1 μM MetRS. ([35S]Hcy-tRNA<sup>Met</sup> was prepared in situ by treatment of [S-nitroso-[35S]Hcy-tRNA<sup>Met</sup> with 1–4 mM Hcy. TLC analyses revealed that Hcy thiolactone and S-nitroso-Hcy were products of deacetylations of Hcy-tRNA and S-nitroso-tRNA, respectively.

**Fig. 4.** SDS-PAGE analysis of [S-nitroso-[35S]Hcy incorporation into proteins in *E. coli*. Cultures of *E. coli* strain CAG1849 (metE::Tn10/pREP4/pQE15) were grown at 37 °C in M9 medium containing 0.1 mM methionine, 0.1 mg/ml ampicillin, and 0.03 mg/ml kanamycin. Bacterial cells were labeled with [35S]amino acids (10 μM, 20,000 Ci/mmol) in M9 medium without methionine and antibiotics in the absence and presence of 0.5 mM IPTG. After labeling, bacterial proteins were subjected to SDS-PAGE on 13% polyacrylamide gels. A, proteins form *E. coli* cells labeled with [S-nitroso-[35S]Hcy for 10 min (lanes 1 and 3), 1 h (lanes 2 and 6), 2 h (lanes 3 and 7), and 4 h (lanes 4 and 8) in the absence (lanes 1–4) and presence (lanes 5–8) of 0.5 mM IPTG. B, in control experiments *E. coli* cells were labeled with [35S]Hcy (lanes 1 and 2) or [35S]Met (lanes 3 and 4) in the absence (lanes 1 and 3) and presence (lanes 2 and 4) of IPTG for 1 h. In experiments with [S-nitroso-[35S]Hcy and [35S]Hcy, 10-μl aliquots of labeled cultures were analyzed. In experiments with [35S]Met, 1-μl aliquots of labeled cultures were analyzed. Position of IPTG-inducible mouse DHFR protein encoded by the plasmid pQE15 is indicated.

**Fig. 3.** Aminoacylation of tRNA with S-nitroso-Hcy catalyzed by MetRS. After formation of S-nitroso-Hcy–AMP, the side chain of S-nitroso-Hcy does not move to the thiol substrate but remains in the specificity subsite of the synthetic/editing active site of MetRS. This allows the transfer of S-nitroso-Hcy from the adenylate to tRNA.

Translational Incorporation of S-Nitroso-Hcy into Protein—

Once an amino acid is attached to tRNA, it is destined to become incorporated translationally into protein (23). Thus, the facile aminocacylation of tRNA with S-nitroso-Hcy catalyzed by MetRS suggested that S-nitroso-Hcy could be incorporated into protein. To test this, *E. coli* strain CAG1849/pREP4/pQE15, which produces mouse DHFR protein upon induction with IPTG was employed. The strain is a methionine auxotroph, unable to metabolize Hcy to methionine because of the insertion of the transposon Tn10 into the metE gene. As shown in Fig. 4A, *E. coli* utilized S-nitroso-Hcy for incorporation into proteins and, after induction with IPTG, into recombinant mouse DHFR. S-Nitroso-Hcy–DHFR co-migrated with the Met-DHFR on denaturing polyacrylamide gels (Fig. 4B). When [S-nitroso-[35S]Hcy-labeled proteins were subjected to hydrolysis by hydrochloric acid (11), [35S]Hcy was released (not shown).

TLC analyses confirmed that, like other metE strains (7, 10), the CAG1849 strain metabolized Hcy to thiolactone. Whereas [S-nitroso-[35S]Hcy was stable in M9 medium, it was converted into [35S]homocysteine and [35S]thiolactone in cultures of *E. coli* strain CAG1849; no other [35S]metabolites were observed (not shown). The conversion of S-nitroso-Hcy to homocysteine in *E. coli* was most likely because of the presence of enzymes denitrosylating S-nitrosothiols (24, 25). After reduction to Hcy, homocysteine is subsequently converted to thiolactone by MetRS, IleRS, and LeuRS in *E. coli* (7, 10). Because post-translational reaction of thiolactone with protein lysine residues may also lead to incorporation of Hcy into protein (26), control experiments in which *E. coli* cultures were incubated with Hcy or thiolactone were carried out. There was no incorporation of Hcy (Fig. 4B) or thiolactone (not shown) into bacterial proteins under these conditions. Taken together, these data indicate that S-nitroso-Hcy is incorporated translationally by denitrosylation and S-nitrosylation (24, 25).

H. Jakubowski, unpublished data.
Protein Synthesis with S-Nitrosohomocysteine

FIG. 5. [S-Nitroso-35S]Hcy-tRNA supports translation of luciferase and globin mRNAs in rabbit reticulocyte system. SDS-PAGE analyses of translation mixtures containing 10 μM [35S]S-nitroso-Hcy-tRNA in a rabbit reticulocyte system (Promega) in the absence (lane 3) and presence of globin mRNA (lane 1) or luciferase (Luc) mRNA (lane 2) are shown. Analyses of control reactions containing 10 μM [35S]Met-tRNA in a rabbit reticulocyte system in the absence (lane 6) and presence of globin mRNA (lane 4) or luciferase mRNA (lane 5) are also shown. Aminocyl-tRNAs used were 1:1 mixtures of charged initiator and elongator methionine tRNAs.

As shown in Fig. 5, S-nitroso-Hcy-tRNA supported translation of globin mRNA and luciferase mRNA in a methionine-free rabbit reticulocyte translation system. Globin and luciferase proteins labeled with [S-nitroso-35S]Hcy were indistinguishable from the corresponding [35S]Met-labeled proteins on polyacrylamide gels. The data thus indicate that S-nitrosylation provides a mechanism for the translational incorporation of Hcy into protein.

Conclusions—In cultured human cells, Hcy can be incorporated into proteins post-translationally in a two-step mechanism (11, 26–28). Because translational incorporation of Hcy into protein provides a plausible mechanism whereby Hcy can affect physiological function.

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