Calcium-dependent Human Serum Homocysteine Thiolactone Hydrolase

A PROTECTIVE MECHANISM AGAINST PROTEIN N-HOMOCYSTEINYLATION*

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Hieronim Jakubowski‡

From the Department of Microbiology and Molecular Genetics, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, New Jersey 07103

Homocysteine thiolactone is formed in all cell types studied thus far as a result of editing reactions of some aminoacyl-tRNA synthetases. Because inadvertent reactions of thiolactone with proteins are potentially harmful, the ability to detoxify homocysteine thiolactone is essential for biological integrity. This work shows that a single specific enzyme, present in mammalian but not in avian sera, hydrolyzes thiolactone to homocysteine. Human serum thiolactonase, a 45-kDa protein component of high density lipoprotein, requires calcium for activity and stability and is inhibited by isoleucine and penicillamine. Substrate specificity studies suggest that homocysteine thiolactone is a likely natural substrate of this enzyme. However, thiolactonase also hydrolyzes nonnatural substrates, such as phenyl acetate, p-nitrophenoxy acetate, and the organophosphate paraoxon. N-terminal amino acid sequence of pure thiolactonase is identical with that of human paraoxonase. These and other data indicate that paraoxonase, an organophosphate-detoxifying enzyme whose natural substrate and function remained unknown up to now, is in fact homocysteine thiolactonase. By detoxifying homocysteine thiolactone, the thiolactonase/paraoxonase would protect proteins against homocysteinylolation, a potential contributing factor to atherosclerosis.

The non-protein amino acid homocysteine (Hcy),1 an obligatory precursor of methionine, poses an accuracy problem for the protein biosynthetic apparatus (1, 2). Several aminoacyl-tRNA synthetases (AARSs), such as MetRS, IleRS, LeuRS, ValRS, and LysRS, misactivate Hcy and form an AARS-bound homocysteinyl adenylate according to Reaction 1.

\[
\text{AARS + Hcy + ATP } \rightleftharpoons \text{AARS-Hcy \sim AMP + PPi} \quad \downarrow \quad \text{Hcy thiolactone}
\]

REACTION 1

However, misactivated Hcy is not transferred to tRNA by any of these enzymes (1–5). Instead, Hcy-AMP is efficiently edited (3), as indicated by the side reaction in Reaction 1. Editing of Hcy-AMP involves an intramolecular reaction in which the side chain thiolate of Hcy displaces the AMP group from the carbonylate of the activated Hcy, forming thiolactone as a product (Reaction 2).

\[
\begin{align*}
\text{NH}_2 & \quad \text{O} & \quad \text{SH} \\
\text{Hcy thiolactone} & \quad \text{AMP} & \quad \text{NH}_2 \\
\text{NH}_2 & \quad \text{O} & \quad \text{S} \\
\end{align*}
\]

REACTION 2

The energy of the anhydride bond of Hcy-AMP is conserved in an intramolecular thioester bond of Hcy thiolactone. Consequently, Hcy thiolactone easily acylates side chain amino groups of protein lysine residues (6–9).

Hcy thiolactone is synthesized by MetRS (1, 2, 9, 10) in all cell types tested thus far, including human vascular endothelial cells (7). Because of its mostly neutral character at physiological pH (pKₐ = 7.1; Ref. 11), thiolactone accumulates in culture media (6, 7, 9, 10). The synthesis of Hcy thiolactone increases with an increase in Hcy levels and decreases with an increase in Met levels. For example, folate-limited cultures of cystathionine β-synthase-deficient human fibroblasts (6), or normal human fibroblasts (6) and vascular endothelial cells (7), accumulate both Hcy and Hcy thiolactone. As much as 60% of the metabolized Hcy is converted to thiolactone in human vascular endothelial cells maintained on Hcy in Met-free, folate-limited media (7).

In addition to Hcy thiolactone, homocysteinylated proteins are also present in cultured human cells (6, 7). In vascular endothelial cells in which methionine synthase was inhibited by folate limitation, Hcy incorporation into protein represented 36% of the incorporation of Met (7). When endothelial cells were maintained on Hcy, its incorporation into intracellular and extracellular protein represented 15% and 65%, respectively, of Met incorporation (7). Cellular levels of Hcy bound to protein via amide bonds are greater than the levels of free Hcy (7). Hcy incorporation into protein is post-translational, reflecting facile homocysteinylolation of protein lysine residues by Hcy thiolactone (6–9). Translational incorporation of Hcy into protein is not believed to be possible because AARSs exhibit absolute selectivity against Hcy in the tRNA aminoacylation reaction (1–5).

The efficacy of protein damage by homocysteinylolation has been demonstrated in vitro and in vivo. Enzymes, such as MetRS and trypsin (7, 8), are irreversibly inactivated by homocysteinylolation. Lysine oxidase, an enzyme responsible for post-
translational modification essential for the biogenesis of connective tissue matrices, is also inactivated by Hcy thiolactone (12). Homocysteinylated proteins can be physiologically detrimental; for instance, they can elicit immune response, as shown recently in rabbits (13).

Because protein homocysteinylation can potentially lead to cell and tissue damage (6–9, 14), human beings most likely evolved mechanisms to detoxify Hcy thiolactone. The present work shows that a calcium-dependent Hcy thiolactone hydrolase (HTase) is tightly associated with HDL in human serum. In addition, it shows that HTase is identical with paraoxonase, an organophosphate-depotoxifying enzyme whose natural substrate and function are unknown at present.

**MATERIALS AND METHODS**

**Preparation of 1-[35S]Hcy Thiolactone—**[35S]Hcy thiolactone was prepared from [35S]Met according to Baernstein (15) and purified by two-dimensional TLC on 20 × 10-cm cellulose plates (Eastman Kodak Co.) (6). The overall yield of the procedure was 65%. The preparation of 1-[35S]Hcy thiolactone was at least 96% pure on analytical two-dimensional TLC. Maximum levels of contamination with Met, Hcy, and homocysteine were <1%, <0.8%, and <1%, respectively.

**Human Serum—**Human serum was obtained from healthy volunteers, as approved by the institutional human investigation review board. It was taken into vacutainer tubes and allowed to clot for 2 h. Serum was collected after centrifugation (15 min, 2,000 × g). Animal sera were purchased from Sigma.

**Reactions of Hcy Thiolactone in Human Serum—**Human serum was incubated with 1-[35S]Hcy thiolactone at 37 °C. Incorporation of Hcy into protein was assayed by precipitation with 5% trichloroacetic acid before and after treatment with 10 mM DTT. Protein-N-Hcy was determined as DTT-resistant thiolactone-precipitable material. Protein-S-S-Hcy was measured as DTT-sensitive fraction of trichloroacetic acid-precipitable material. Protein-S-Hcy was assayed by precipitation with 5% trichloroacetic acid from 12 μM [35S]Hcy thiolactone.

**Preparation of Lipoproteins—**LDL and HDL were prepared from fresh human serum by sequential precipitation with dextran-CaCl2, as described by Burstein et al. (16). Lipoproteins were also prepared by ultracentrifugation in KBr (density of 1.225 g/ml) and gel filtration (17) on a Sephacryl HR S-300 (Amersham Pharmacia Biotech) column in the presence of 1 mM CaCl2.

**Enzyme Assays—**Unless indicated otherwise, incubations were carried out at 37 °C in 0.1 M K-Hepes buffer (pH 7.4), 2 mM CaCl2. HTase activity was determined by following formation of [35S]Hcy from [35S]Hcy thiolactone. Hcy was separated from thiolactone by TLC (18–20) and quantitated by scintillation counting. Paraoxonase was assayed by following hydrolysis of phenyl acetate or paraoxon (22).

**Spectrophotometric assays** were used in substrate specificity studies with non-radiolabeled substrates (all from Sigma). Hydrolysis of Hcy thiolactones was determined from the decrease of their characteristic UV absorption at λ = 240 nm (ε = 3500–1 M⁻¹ cm⁻¹) (21). Hydrolysis of phenyl acetate and p-nitrophenylacetate was determined spectrophotometrically using ε = 13000 M⁻¹ cm⁻¹ at 270 nm for phenol and ε = 13000 M⁻¹ cm⁻¹ at 412 nm for p-nitrophenol, respectively. Hydrolysis of diethyl p-nitrophenyl phosphate (paraoxon) was measured spectrophotometrically using ε = 13000 M⁻¹ cm⁻¹ at 412 nm for p-nitrophenol (22).

In experiments in which utilization of other (thio)esters (10 mM) by HTase was tested, potential substrates and products were separated by TLC and visualized by staining with ninhydrin (3, 5) or under UV. With all potential substrate-product pairs, complete separation was achieved on cellulose plates (Kodak) using butanol:acetic acid:water (4:1:1, v/v) as solvent. Complete separation of acetyl-S-coenzyme A from coenzyme A-SH was achieved on polyethyleneimine-cellulose plates (Sigma) using 1.2 M LiCl as solvent.

**Preparation of Protein-S-Hcy—**All steps were carried out at 4 °C. Unless otherwise indicated, a buffer containing 20 mM Tris-HCl (pH 8.0), 0.5 mM CaCl2 was used. When necessary, fractions containing HTase activity were concentrated by ultrafiltration on Centricon 30 cartridges (Amicon).

Human serum (20 ml) was brought to 35% saturation by the addition of 4.4 g of solid ammonium sulfate, and the pellet was discarded. Proteins precipitated by subsequent addition of 4.8 g of ammonium sulfate (to 70% saturation) were collected, dissolved in 5 ml of buffer, dialyzed, and concentrated to 6 ml. The 35–70% ammonium sulfate fraction was chromatographed on a 1.6 × 85-mm Sephacryl S300HR column (Amersham Pharmacia Biotech). Active fractions (20 ml), eluting between LDL and serum albumin fractions, were applied onto a 1 × 10-cm Mono Q Sephacryl (Amersham Pharmacia Biotech). Bound proteins were eluted with 0–0.4 M NaCl gradient (100–100 ml) in the standard Tris-Ca buffer. Active fractions (30 ml) eluting at 0.25–0.30 M NaCl, were concentrated to 4 ml and applied onto a second Sephacryl S300HR column (1.6 × 85 cm). Active fractions from the second Sephacryl S300HR (20 ml) column were applied onto a 1 ml Hitrap Blue column (Amersham Pharmacia Biotech). The Blue column was washed successively with 5 ml of 1, 2, or 3 M NaCl in the buffer, and the buffer alone. HTase was eluted with 5 ml 0.1% sodium deoxycholate in standard buffer. Fractions containing the enzyme (1.5 ml) were diluted with 4 ml of buffer, supplemented with 0.1% Triton X-100, and applied onto Mono Q Sephacryl column (1.6 × 85 cm) equilibrated with the Tris-Ca-Triton buffer. Bound proteins were eluted with 0–0.4 M NaCl gradient (50 × 50 ml) in the Tris-Ca-Triton buffer. Fractions containing HTase activity (9.5 ml) were concentrated to 0.1 ml.

**N-terminal Sequencing—**N-terminal sequence determination was carried out by Dr. R. Donnelly on Applied Biosystems model 491 Procise™ sequencer at institutional Molecular Core Facility.

**RESULTS**

**Reactions of Hcy Thiolactone in Human Serum—**Incubation of exogenous [35S]Hcy thiolactone with human serum resulted in progressive incorporation of the [35S]radiolabel into protein. At 3 h, most of the thiolactone became trichloroacetic acid-precipitable (Fig. 1A). Addition of DTT to [35S]Hcy thiolactone-modified serum proteins resulted in a release of ~30% of the incorporated 35S as free [35S]Hcy. DTT treatment of protein-S-[35S]Hcy adducts, prepared by a 2-h incubation of free [35S]Hcy with serum, rendered all radioactivity trichloroacetic acid-soluble (Fig. 1B). Native polyacrylamide gel electrophoresis demonstrated that albumin-S-[35S]Hcy represented most (>95%) of protein-S-[35S]Hcy in human serum (data not shown). As described elsewhere (6–8), [35S]Hcy in the DTT-resistant fraction of 35S-protein adducts is bound to side chain amino groups of protein lysine residues. Thus, Hcy thiolactone undergoes two major reactions in serum: 1) protein homocysteinylation, forming protein-N-Hcy (Fig. 2A); and 2) enzymatic hydrolysis to give Hcy, which then attaches to protein cysteine residues, forming protein-S-Hcy disulfides (Fig. 2B).

These reactions of Hcy thiolactone were observed in 10 different human serum samples, as well as in bovine, horse, rabbit, and mouse sera, and in human sera from different donors, rates of thiolactone hydrolysis to Hcy ranged from about equal to 2–3-fold slower than the rate of protein-N-homocysteinylation. The hydrolysis of the thiolactone to Hcy in rabbit and mouse sera was 5–10 times faster than protein-N-homocysteinylation. There was no enzymatic hydrolysis of thiolactone in chicken serum (data not shown).

**HTase Is Associated with HDL—**When a sample of human

![Fig. 1. Reactions of Hcy thiolactone in human serum. A. formation of protein-N-Hcy (filled circles) and protein-S-S-Hcy (filled squares) from 12 μM [35S]Hcy thiolactone. B. Formation of protein-S-S-Hcy (filled squares) from 12 μM [35S]Hcy. At the point indicated by the arrow, 10 mM DTT was added.](image-url)
serum was subjected to gel filtration, Hcy thiolactone hydrolyzing activity eluted in a single peak at a position characteristic of HDL, between LDL and serum albumin peaks (Fig. 3). The HTase activity co-migrated with paraoxonase, a known component of HDL (23). To determine if HTase is in fact associated with HDL, human serum lipoprotein fractions were prepared by sequential precipitation with dextran-CaCl₂ (16) and assayed. HTase activity, like paraoxonase activity, was present in HDL and absent in the LDL fraction (Table I), indicating that HTase is tightly associated with HDL in human serum. Similar association of HTase with HDL was observed in rabbit serum (not shown).

**Purification of Human Serum HTase**—At all steps of purification, a single peak of HTase activity was obtained, suggesting that a single enzyme is responsible for Hcy thiolactone hydrolysis in human serum (Table II). Due to its tight association with HDL, pure HTase could only be obtained by including the non-ionic detergent Triton X-100 at the final step of purification. For example, HTase preparation obtained after first Mono Q column chromatographed as a high molecular mass complex of ~300 kDa on a gel filtration column and contained protein components (major of mass 28 kDa and minor of mass 45 kDa) identical to those of authentic HDL (17) as revealed by SDS-polyacrylamide gel electrophoresis (not shown). Re-chromatography on Mono Q in the presence of Triton X-100 resulted in separation of HTase (eluting at 0.25 M NaCl) from apolipoprotein A-I (eluting at 0.1 M NaCl). Pure HTase migrated as a 45-kDa protein on 10% SDS-polyacrylamide gel electrophoresis. A 1300-fold purification with a yield of 10% was achieved (Table II), suggesting that HTase is present in human serum at a concentration ~50 μg/ml or ~1 μM. Similar purification of HTase was achieved when HDL (prepared by ultracentrifugation in KBr and gel filtration; Ref. 17) was subjected to chromatography on a Mono Q column in the presence of Triton X-100.

**N-terminal Sequence Analysis**—Twelve cycles of automatic Edman degradation of the purified HTase yielded the following N-terminal amino acid sequence: AKLIALTLGMGMG. Search of data banks revealed a 100% match of this sequence with N-terminus of human serum paraoxonase (24).

**Calcium Requirement of Human Serum HTase**—HTase could not be purified in the absence of CaCl₂ because the enzyme quickly lost its activity. The inactivated enzyme could not be re-activated by addition of any divalent metal. Effects of divalent cations and EDTA on HTase activity is shown in Table III. Partial activity, observed in the absence of exogenous divalent metal ions, was due to 0.02 mM CaCl₂ carried over from HTase preparation. The activity was inhibited by EDTA and stimulated by CaCl₂, NiCl₂ (2 mM) stimulated HTase activity but was ineffective at lower concentrations. Other divalent metal ions, tested as dichloride salts, did not affect HTase activity. Taken together, these data indicate that calcium is required for stability and activity of the enzyme.

**Effects of Thiol Reagents on HTase Activity**—HTase was resistant to iodoacetate or iodoacetamide (data not shown), suggesting that thiol groups are not required for activity. Up to 0.01 M cysteine, Hcy, or DTT did not affect HTase activity. However, HTase was 60% inhibited by 0.1 M DTT, most likely due to reduction of intramolecular -S-S- disulfide bridges of the enzyme. Although S-nitroso-N-acetyl-D-penicillamine, a donor of NO groups, inhibited HTase (Table V), another NO donors, S-nitroso-glutathione and sodium nitroprusside, did not affect HTase activity. Taken together, these data indicate that calcium is required for activity of the enzyme.

**Substrate Specificity of HTase**—Several thioesters and esters were tested as potential substrates for HTase (Table IV). D-Hcy thiolactone and L-Hcy thiolactone were hydrolyzed 4.2- and 2.3-fold slower, respectively, than L-Hcy thiolactone by HTase. L-Homoserine lactone (25) was also a substrate, but, in contrast to L-Hcy thiolactone, it is not known to occur in human cells. Lactams of lysine and ornithine (5) were not substrates (data not shown). Other related thioesters, such as N-acetyl-D,L-Hcy thiolactone and acetyl-S-coenzyme A, or esters, such as O-
reaction mixtures.

Incubations were carried out for up to 1 h at 37 °C in 10-μl reaction mixtures containing indicated additions, 0.1 M K-Hepes buffer, pH 7.4, 10 mM [35S]Hcy thiolactone, and 2 μg of HTase. Because HTase can be purified only in the presence of calcium, assays contained 0.02 mM CaCl2 carried over from the enzyme preparation. Unless stated otherwise, divalent metals were tested as dichloride salts at 2 mM.

**TABLE II**

| Step       | Volume (ml) | Protein (μg) | HTase activity (μmol/mg/h) | Paraoxonase activity (μmol/mg/min) | Ratio HTase/paraoxonase (× 10³) | Purification (fold) |
|------------|-------------|--------------|----------------------------|----------------------------------|---------------------------------|-------------------|
| Serum      | 20.0        | 1200         | 0.057                      | 2.8                              | 0.34                            | 3.8               |
| Ammonium   | 6.2         | 220          | 0.22                       | 11.4                             | 0.31                            | 3.8               |
| Sephaeryl  | 19.8        | 44           | 0.73                       | 38.6                             | 0.31                            | 12.8              |
| S300HR     | 30.0        | 8.4          | 2.6                        | 121.4                            | 0.36                            | 45.7              |
| Sephaeryl  | 19.6        | 6.4          | 2.9                        | 162.5                            | 0.29                            | 50.4              |
| HTrap Blue | 1.5         | 2.6          | 3.0                        | 217                               | 0.23                            | 52.6              |
| Mono Q     | 9.5         | 0.1          | 76.0                       | 3619                             | 0.35                            | 1333              |

**TABLE III**

**HTase requires calcium for activity**

| Additions   | HTase activity (%) |
|-------------|--------------------|
| None        | 41 ± 1             |
| EDTA (2 mM) | 8 ± 0.4            |
| Ca 0.007 mM | 58 ± 1             |
| 0.03 mM     | 75 ± 1             |
| 0.12–2 mM   | 100 ± 1            |
| Mg 4 ± 0.2  | 12 ± 0.5           |
| Mn 8 ± 0.4  | 24 ± 1             |
| Zn 4 ± 0.2  | 30 ± 0.6           |
| Cd 12 ± 0.5 |                  |
| Co 6 ± 1    |                  |
| Fe 0.12 mM  | 12 ± 1             |
| Ni 0.5 mM   | 29 ± 1             |
| 2 mM        | 67 ± 1             |
| Cu 0.007 mM | 25 ± 1             |
| Pb 0.03 mM  | 20 ± 1             |

**TABLE IV**

**Substrate specificity of human serum HTase**

| Compound          | Relative activity (Km) |
|-------------------|------------------------|
| l-Hcy thiolactone | 100 ± 6 (Km = 23 mM)   |
| d-Hcy thiolactone | 24 ± 1                 |
| dl-Hcy thiolactone| 43 ± 6                 |
| N-Acetyl-l,l-Hcy thiolactone | 0 |
| Acetyl-S-CoA      | 0                      |
| O-Acetylserine    | 0                      |
| Homoserine lactone| +                     |
| Methionine methyl ester | 0 |
| Phenylalanine ethyl ester | 0 |
| Phenyl acetatea   | 280,000 ± 3,000 (Km = 0.6mM) |
| p-Nitrophenyl acetateb | 4,000 ± 800 (Km = 2.5mM) |
| Paraoxonaseb      | 330 ± 30 (Km = 0.5mM)  |

- a Hydrolysis of aryl esters by HTase (0.01 μg) was carried out in 0.5-mL reaction mixtures.
- b Hydrolysis of paraoxon by HTase (0.05 μg) was carried out in 0.5-mL reaction mixtures.

Acetyl-serine, methyl and ethyl esters of methionine, phenylalanine (Table IV), tryptophan, alanine, β-alanine, and cysteine also were not substrates (data not shown). However, HTase hydrolyzed non-natural aryl esters, such as phenyl acetate and p-nitrophenyl acetate, as well as the organophosphate paraoxon (Table IV). Kinetic constants for HTase with aryl esters and paraoxon as substrates were similar to the corresponding constants measured before for paraoxonase (22). For example, turnover numbers for HTase (Table IV) and paraoxonase (22) are 846- and 833-fold higher, respectively, with phenyl acetate than with paraoxon.

The substrate specificity studies indicate that HTase exhibits a certain degree of selectivity toward l-Hcy thiolactone. This suggests that Hcy thiolactone is a likely natural substrate of HTase. Although Kₘ for Hcy thiolactone is relatively high (23 mM, Table IV), other enzymes also have high Kₘ values for physiologically important substrates. For example, Kₘ for bicarbonate in the bicarbonate dehydration reaction catalyzed by human carbonic anhydrase is 32 mM (26); Kₘ for Hcy in the cystathionine synthesis reaction catalyzed by human cystathionine β-synthase is 25 mM (27). The amount of HTase present in human serum is sufficient to hydrolyze Hcy thiolactone within a few hours (Fig. 1A).

**Inhibitors of HTase**—Because HTase was inhibited by S-nitroso-N-acetyl-penicillamine, but not by other NO donors, it was concluded that penicillamine or a related amino acid may be inhibitory. Indeed, isoleucine and penicillamine were found to be noncompetitive inhibitors of the enzyme, with the D-forms being more inhibitory than the L-forms (Table V). Among natural amino acids tested, isoleucine was the most effective inhibitor (I₅₀ = 2 mM). Leucine, valine, and cysteine (Table V), as well as phenylalanine, tryptophan, tyrosine, and methionine, did not inhibit HTase (data not shown). Phenyl acetate and paraoxon were noncompetitive inhibitors of HTase (Table V). Phenol and p-nitrophenol did not inhibit HTase (data not shown). Phenyl acetate is a noncompetitive inhibitor of paraoxon hydrolysis by paraoxonase (22). Taken together, these data indicate that Hcy thiolactone, phenyl acetate, and paraoxon are hydrolyzed by the same enzyme, but probably at different sites. It is possible that HTase possesses multiple binding sites for phenyl acetate or paraoxon.

**DISCUSSION**

This work identifies a calcium-dependent HTase, a Hcy thiolactone-hydrolyzing enzyme in human serum. HTase is a 45-kDa protein component of HDL. An unanticipated outcome of the present work was that HTase is identical with serum paraoxonase, an organophosphate-detoxifying enzyme whose natural substrate and function were unknown (22–24).

Two different thiolactone-hydrolyzing activities have been reported before, one in human serum and another in human endothelial cells (14). However, we were not able to detect enzymatic hydrolysis of thiolactone by endothelial cells or cell extracts. In fact, enzymatic hydrolysis of Hcy thiolactone to Hcy in human endothelial cell cultures was due exclusively to
human serum contains small amounts of protein-Hcy bound via amide bonds, most likely as protein-N-Hcy (7, 9).

Evidence presented in this work strongly suggests that HTase is identical with serum paraoxonase, an organophosphate-detoxifying enzyme whose natural substrate and function heretofore remained unknown (22–24). 1) HTase and paraoxonase (23) exhibit identical species distribution; both activities are present in mammalian sera but absent in chicken sera. Rabbit and mouse sera contain 5–10-fold more HTase and paraoxonase (23, 24) than human serum. 2) HTase and paraoxonase activities co-purify at all chromatographic steps of the purification procedure (Table II). 3) Kinetic constants for phenyl acetate, p-nitrophenyl acetate, and paraoxon measured with HTase (Table IV) are similar to the corresponding constants with paraoxonase (22). 4) Paraoxon and phenyl acetate inhibit Hcy thiolactone hydrolysis by HTase, indicating that the three compounds are hydrolyzed by the same enzyme. 5) HTase, like paraoxonase (22–24), is a calcium-dependent, 45-kDa component of HDL (23). 6) N-terminal sequence of purified HTase is identical with that of paraoxonase (24). Thus, paraoxonase is HTase, and its likely natural function is to detoxify Hcy thiolactone and, therefore, to minimize protein-N-homocysteinylated. Vascular endothelial cell cultures supplemented with HTase contain lower amounts of Hcy thiolactone and Hcy-protein than unsupplemented cultures.2

The toxicity of Hcy thiolactone, as well as a protective role of HTase in vivo, has been demonstrated in some studies, or can be inferred from others. For example, chronic infusions of ba-boons with thiolactone or Hcy cause atherosclerosis (29). Similar infusions of rabbits, which have higher serum levels of HTase than primates (this work), failed to produce atherosclerosis (28). Hcy thiolactone, which cannot be hydrolyzed to Hcy in chicken due to lack of HTase (this work), is toxic to chicken embryos (36). L-Hcy, but not the D-form, is also toxic to rat embryos (32). However, both L- and D-forms of Hcy thiolactone are toxic (32). The stereospecific embryotoxicity of L-Hcy is consistent with the stereospecificity of MetRS, which converts L-Hcy, but not D-Hcy to thiolactone (3). On the other hand, embryotoxicity of both L- and D-forms of thiolactone is consistent with identical chemical reactivities of the two stereoisomers of thiolactone toward proteins.

Human genetic epidemiological studies indicate that reduced paraoxonase (PON1) activity may support the development of atherosclerosis (33). To examine a role of PON1 in vivo, PON1-deficient mice were created (34). Compared with their wild-type littermates, PON1-deficient mice were more susceptible to atherosclerosis (34). Results of a recent clinical trial (35), showing that low activity of paraoxonase is associated with the extent of coronary artery disease in human subjects homozygous for the methylenetetrahydrofolate reductase C677T genotype (i.e., having elevated Hcy levels) is consistent with the proposed Hcy thiolactone-detecting role of HTase/paraoxonase. These observations underline the importance of examining variability of HTase/paraoxonase in future studies of associations between Hcy and vascular diseases.

Note Added in Proof—Sera from PON1-deficient mice, kindly supplied by Drs. Diane M. Shih and Aldons J. Luis (UCLA), were also found to be deficient in HTase activity, which provides further evidence that paraoxonase is HTase.

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**Calcium-dependent Human Serum Hcy Thiolactone Hydrolase**

**TABLE V**

*Effects of amino acids and other compounds on HTase activity*

| Compound                  | HTase activity |
|---------------------------|----------------|
| None                      | 100 ± 10       |
| L-Isoleucine              | 2m M           |
| L-Isoleucine methyl ester | 2m M           |
| L-Isoleucine amide        | 2m M           |
| D-Isoleucine              | 2m M           |
| L-Leucine                 | 2m M           |
| L-Valine                  | 2m M           |
| D-Valine                  | 2m M           |
| D-Cysteine                | 2m M           |
| L-Penicillamine           | 2m M           |
| D-Penicillamine           | 2m M           |
| D-Penicillamine disulfide | 2m M           |
| S-Nitroso-N-acyethyl-d-penicillamine | 2m M |
| Phenyl acetate            | 2m M           |
| Paraoxon                  | 2m M           |

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*Noncompetitive inhibition. Enzymatic hydrolysis of [35S]Hcy thiolactone (1–20 mU) to [35S]Hcy was measured in the absence and presence of indicated compounds (0.05–10 mM). I₅₀ values show indicate inhibitor concentrations at which 50% inhibition was observed.*

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2 H. Jakubowski, unpublished data.
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