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PROTECTIVE MECHANISMS AGAINST HOMOCYSTEINE TOXICITY: THE ROLE OF BLEOMYCIN HYDROLASE *

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Running Title: Bleomycin Hydrolase is a Homocysteine-thiolactone Hydrolase

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Homocysteine (Hcy) editing by methionyl-tRNA synthetase results in the formation of Hcy-thiolactone and initiates a pathway that has been implicated in human disease. In addition to being cleared from the circulation by urinary excretion, Hcy-thiolactone is detoxified by the serum Hcy-thiolactonase/paraoxonase carried on high density lipoprotein. Whether Hcy-thiolactone is detoxified inside cells was unknown. Here we show that Hcy-thiolactone is hydrolyzed by an intracellular enzyme, which we have purified to homogeneity from human placenta and identified by proteomic analyses as human bleomycin hydrolase (hBLH). We have also purified an Hcy-thiolactonase from the yeast *Saccharomyces cereviasiae* and identified it as yeast bleomycin hydrolase (yBLH). BLH belongs to a family of evolutionally conserved cysteine aminopeptidases and its only known biologically relevant function was deamidation of the anticancer drug bleomycin. Recombinant hBLH or yBLH, expressed in *E. coli*, exhibits Hcy-thiolactonase activity similar to that of the native enzymes. Active site mutations, C73A for hBLH and H369A for yBLH, inactivate Hcy-thiolactonase activities. Yeast *blh1* mutants are deficient in Hcy-thiolactonase activity *in vitro* and *in vivo*, produce more Hcy-thiolactone, and exhibit greater sensitivity to Hcy toxicity than wild type yeast cells. Our data suggest that BLH protects cells against Hcy toxicity by hydrolyzing intracellular Hcy-thiolactone.

Homocysteine (Hcy) is a sulfur-containing amino acid that is found as a normal metabolite in the three domains of life. In all organisms Hcy is metabolized to Hcy-thiolactone by methionyl-tRNA synthetase in an error-editing reaction in protein biosynthesis when Hcy becomes mistakenly selected in place of methionine (reviewed in refs.1-3). In each organism examined (bacteria, yeast, plant, mouse, and human) the Hcy-thiolactone pathway becomes predominant when re-methylation or trans-sulfuration reactions are impaired by genetic alterations of enzymes involved in Hcy metabolism, such as cystathionine β-synthase (4-6) and methionine synthase (4, 6), or by inadequate supply of folate (5, 7-9), vitamin B_{12} or vitamin B_6.

In recent years Hcy has become a focus of intense studies in the context of human pathophysiology. Elevated serum Hcy levels observed in genetic disorders of Hcy metabolism are associated with severe pathologies, which affect multiple organs and lead to premature death due to vascular complications (10). Although severe hyperhomocysteinemia is rare, mild hyperhomocysteinemia is quite prevalent in a general population and is associated with an increased risk of cardiovascular (11) and

neurodegenerative diseases such as Alzheimer's (12). The strongest evidence that Hcy plays a causal role in cardiovascular disease comes from studies of hyperhomocysteinemia in animal models (10) and small trials in humans (13). Although large clinical trials testing whether lowering Hcy can lead to better vascular outcomes have not been successful (13), an efficacy analysis shows that high risk stroke patients do benefit from lowering of plasma Hcy by vitamin supplementation (14).

Although Hcy is a normal metabolite, its excess can be extremely toxic to human (15-17), animal (18), yeast (4, 6), and bacterial cells (19). Why Hcy is toxic is not entirely clear and is a subject of intense studies (1, 3, 10). One hypothesis suggests that the conversion to Hcy-thiolactone contributes to Hcy toxicity and is linked to atherosclerosis in humans (1, 3, 5, 7). The formation of Hcy-thiolactone can be detrimental for two reasons. First, it requires ATP and thus causes non-productive consumption of cellular energy (4, 20). Second, Hcy-thiolactone is a reactive intermediate that causes protein *N*-homocysteinylation through the formation of amide bonds with ε-amino groups of protein lysine residues (5, 7, 21-22). Resulting protein damage necessitates removal of *N*-homocysteinylated proteins by proteolytic degradation, which would further deplete cellular energy and limit cell growth. Hcy-thiolactone appears to be more toxic to human cells than Hcy (17). Hcy-containing proteins are also toxic (24) and induce an auto-immune response, which is associated with atherosclerosis in humans (1, 3, 24-26).

To minimize Hcy-thiolactone toxicity, cells had to evolve mechanism of its disposal. Indeed, in all organisms the bulk of Hcy-thiolactone is eliminated by excretion from cells into the extracellular media. In mice and humans, Hcy-thiolactone (27) is cleared out from the circulation by urinary excretion in the kidney (3, 28). Hcy-thiolactone can also be disposed of by enzymatic hydrolysis by the serum Hcy-thiolactonase/paraoxonase (PON1) carried on high density lipoprotein (29-32).

As the serum PON1 is present extracellularly, it was unknown whether Hcy-thiolactone can be detoxified intracellularly. The present work describes intracellular Hcy-thiolactonase (HTLase) in humans and yeast, and provides evidence that the HTLase is identical with bleomycin hydrolase (BLH), whose natural substrate and function were unknown. Purified human, or yeast, HTLase/BLH exhibits catalytic efficiency of 10^3 $M^{-1}s^{-1}$ in the hydrolysis of Hcy-thiolactone, ~100-fold greater than the catalytic efficiency of human serum Hcy-thiolactonase. Our data suggest that BLH is a major intracellular Hcy-thiolactone-hydrolyzing enzyme that protects cells against Hcy toxicity. Preliminary accounts of this work were published in abstract forms (33, 34).

EXPERIMENTAL PROCEDURES

Strains and Plasmids — *S. cerevisiae* strains used are listed in Table 1. Plasmids encoding hBLH and yBLH, and their enzymatically inactive variants C73A and H369A, respectively, were kindly provided by Leemor Joshua-Tor and Paul O'Farrell (35, 36). *E. coli* BL21 (DE3) was used as a host for plasmid maintenance and recovery.

[^{35}S]Hcy-thiolactone — Carrier-free *L*-[^{35}S]Met (Amersham Pharmacia Biotech) was supplemented with unlabeled *L*-methionine (Sigma-Aldrich) to specific activity of 40,000 Ci/mol, converted to [^{35}S]Hcy-thiolactone by a 4-h digestion with hydriodic acid at 128°C, and purified by 2D TLC as previously described (7, 23, 29).

Purification of Native Yeast Hcy-thiolactone Hydrolase (yHTLase) — All steps were carried out at 4°C. Fresh yeast cake (1kg) was taken up in 10 mM K-

phosphate buffer (pH 6.8), 0.5 mM mercaptoethanol, 5% glycerol (3 l) and disrupted in a high pressure homogenizer. The crude extract was clarified by centrifugation, mixed with DEAE-Sephacel (0.8 l), and poured into a 5 x 40 cm column. The column was washed with the pH 6.8 buffer (4 l) followed by a linear gradient of 0-0.5 M KCl in the pH 6.8 buffer (8 l). At pH 6.8 HTLase activity is not retained on DEAE-Sephacel and elutes in the break-through fractions. Protein precipitated from these fractions with 60% ammonium sulfate was collected by centrifugation, dissolved in 50 mM K-phosphate buffer (pH 6.8), and purified by Superdex 200 gel filtration. Active fractions were dialyzed against 50 mM Tris/HCl (pH 8.7), 0.5 mM mercaptoethanol, 5% glycerol, and applied on a DEAE-Sephacel column. The yHTLase was eluted with a linear 0-0.5 M KCl gradient in the pH 8.7 buffer (Fig. 1A,B). Pure yHTLase migrates on SDS-PAGE gels as a 48 kD protein (Fig. 1D).

Purification of Native Human Placenta Hcy-thiolactone Hydrolase — All steps were carried out at 4°C. Human placenta (100 g) was homogenized in 20 mM K-phosphate buffer (pH 6.8), 0.5 mM mercaptoethanol, 5% glycerol (0.2 l). The homogenate was clarified by centrifugation and a protein fraction precipitated between 50-70% ammonium sulfate saturation was collected. Hcy-thiolactone-hydrolyzing activity was further purified by ion exchange chromatography on DEAE-Sephacel, gel filtration on Superdex 200, and chromatography on a hydroxyapatite column. Purification to homogeneity was achieved by preparative electrophoresis on non-denaturing polyacrylamide gels.
Purified hHTLase migrated on SDS-PAGE gels as a 48 kD protein (Fig. 1D).

MALDI-TOF Mass Spectrometric Analysis of Tryptic Peptides — Samples of native hBLH and yBLH were digested overnight at 37°C with sequencing grade trypsin (Sigma-Aldrich) in 0.1 M ammonium bicarbonate. Peptide mass analysis was performed at the Autoflex mass spectrophotometer (Bruckner Daltonics, Leipzig, Germany) at the proteomics facility of the Institute of Biochemistry and Biophysics, Warsaw, Poland). Proteins were identified by the use of the MASCOT Server 1.9 based on mass searches within human and yeast sequences.

Purification of Recombinant Human and Yeast Bleomycin Hydrolases — Plasmid encoding His-tagged hBLH or yBLH (35, 36) was transformed into *E. coli* strain BL21 (DE3) for protein expression. The cells were grown in LB medium (0.6 l) to mid-log phase at 37°C, the culture was shifted to 25°C and the BLH expression was induced with 0.5 mM IPTG for 16 h. The cells were harvested, resuspended in 50 mM K-phosphate pH 8.5, 300 mM NaCl, 7 mM 2-mercaptoethanol and frozen at -80°C. For BLH purification the cells were thawed and disrupted by sonication on ice. Crude extracts were clarified by centrifugation at 4°C, and BLH was purified by an affinity chromatography on a 1 ml Ni-Agarose (Amersham Biotech) column. Pure BLH, eluted with 0.2 M imidazole, was dialyzed against 50 mM K-phosphate buffer pH 7.4, 7 mM 2-mercaptoethanol, 10% glycerol and stored at -20°C.

Preparation of Yeast Cell Extracts — Yeast cells from 10 ml cultures at 10^7cells/ml were collected by centrifugation at 2°C and disrupted by vortexing with glass beads (100-400 µm, Sigma-Aldrich) in 50 µl ice-cold buffer (50 mM potassium phosphate, pH 7.5, 1 mM EDTA, 1 mM DTT; 3 x 0.5 min with 1 min cooling on ice intervals). Crude cells extracts were clarified by centrifugation using a JA25.50 rotor in a Beckman-Coulter J2 centrifuge (30,000 x g, 15 min, 2°C) and assayed for Hcy-thiolactonase activity.

Enzyme Assays — Unless indicated otherwise, incubations were carried out at 25°C in 50 mM potassium-phosphate buffer (pH 7.4), 1 mM DTT, 1 mM EDTA. Hcy-thiolactonase activity was determined by following the formation of [^{35}S]Hcy from [^{35}S]Hcy-thiolactone as previously described (29). Hcy-thiolactonase activity was also assayed with unlabeled *L*-Hcy-thiolactone, or its analogue, by monitoring changes in UV absorption at A_{240} using a Varian Cary 50 UV-Vis spectrophotometer (29).

During enzyme purification, the HTLase activity was monitored by a TLC-based assay. Reaction mixtures (25 µl) contained 50 mM potassium phosphate (pH 7.5), 10 mM *L*-Hcy-thiolactone, 1 mM dithiothreitol, and a protein fraction (5 µl). Incubation was carried out at 37°C. At appropriate time, the reaction was stopped by transferring 3 µl aliquots onto the origin of a TLC plate (aluminium precoated with silica gel containing fluorescent indicator, Merck). The plate was developed with ethyl acetate:isopropanol:ammonia:water (27:23:5:3, by volume) for 15-20 min, dried and visualized under UV light. In assays containing HTLase activity, dark spots of Hcy-thiolactone (R_f = 0.4) diminished or disappeared completely (Fig. 1B).

Aminopeptidase activity was assayed with 0.1 mM R-AMC. After TLC separation, AMC, a highly fluorescent product of R-AMC hydrolysis, was visualized under UV light (Fig. 1C).

In experiments in which utilization of other compounds (10 mM) was tested, potential substrates and products were separated by TLC and visualized by staining with ninhydrin or under UV. With all potential substrate-product pairs, complete separation was achieved on cellulose plates (Analtech) using 1-butanol:acetic acid:water (4:1:1, by volume) as a solvent (5, 29).

Determination of Hcy-thiolactone in Yeast Cultures — Yeast cultures were maintained on minimal media for 24 h at 30°C. Aliquots of the cultures (50 µl) were clarified by microcentrifugation (14,000 x g, 2 min, 25°C). Cell-free media (2 µl) were diluted 20-fold with water and a half of the sample was applied onto a cation exchange HPLC column.

HPLC Analyses — HPLC was carried out using a cation-exchange polysulfoethyl aspartamide column (1.0 x 150 mm, 5 µ, 300 Å) from PolyLC, Inc. and System Gold Nouveau HPLC instrumentation from Beckman-Coulter as previously described (27, 28). After sample (20 µl) application the column was eluted isocratically with 10 mM sodium phosphate buffer, pH 6.6, 25 mM NaCl at a flow rate 0.15 ml/min. A post-column derivatization and fluorescence detection was used for the quantification of Hcy-thiolactone (eluting at 8 min) as previously described (27, 28). The HPLC column effluent was mixed in a three-way tee with 2.5 mM *o*-phthaldiladehyde, 0.25 M NaOH, delivered at a flow rate 0.07 ml/min. The mixture passed through Teflon tubing reaction coil (0.3 mm I.D. x 3 m) and then was monitored with a Jasco 1520 fluorescence detector using excitation at 370 nm and fluorescence emission at 480 nm.

RESULTS

Human and Yeast Hcy-thiolactonases are Identical with Corresponding Bleomycin Hydrolases — In order to answer a question whether Hcy-thiolactone can be detoxified inside cells, we carried out systematic search for an intracellular HTLase. We have examined HTLase activity levels in several tissues and found that human placenta is a better source of this activity than porcine liver. We have purified Hcy-thiolactone-hydrolyzing activity to homogeneity from human placenta. We have also purified to homogeneity an Hcy-thiolactone-hydrolyzing enzyme from the yeast *Saccharomyces cerevisiae* (Fig. 1).

To determine their identity, the purified human and yeast HTLases were digested with trypsin and the resulting peptides were subjected to MALDI-TOF mass spectrometric analysis. The identified peptides from each enzyme were then used to search the NCBI database. Mascot Server 1.9 (Bruckner Daltonics) searches revealed that the best match for human HTLase was human bleomycin hydrolase (hBLH, accession number gi|1321858). Sequence coverage was 72% and the score was 1659. The score is the negative logarithm of the probability that the observed match is a random set. A similar analysis of the yeast HTLase revealed that the best match for the yeast enzyme was yeast bleomycin hydrolase (yBLH, accession number gi|3891714) encoded by the *BLH1* gene, also known as *GAL6* or *LAP3*. Sequence coverage was 54% and the score was 2536.

To confirm the identity of the human and yeast HTLases, we examined recombinant wild type and mutant clones, C73S of hBLH and H369A of yBLH (35, 36), for the ability to hydrolyze Hcy-thiolactone. Plasmids encoding BLH proteins under the control of β-galctosidase promoter were transformed into *E. coli* strain BL21(pLysS) for protein expression. Cells were grown in LB medium to mid-log phase and induced with isopropyl-β-thiogalactosyl pyranoside (IPTG). To assay HTLase activity, 10 mM Hcy-thiolactone was added to 0.1 ml aliquots of the cultures. Only cells expressing active variants of yBLH or hBLH completely hydrolyzed Hcy-thiolactone in 2 h at 30°C. No hydrolysis of Hcy-thiolactone was observed by un-induced cells (in the absence of IPTG) or induced cells expressing inactive H369A or C73S BLH variants. We have confirmed by SDS-PAGE of bacterial cell extracts that both active and inactive BLH variants were expressed at similar levels after IPTG induction. *E. coli* cells alone do not possess any significant ability to hydrolyze Hcy-thiolactone.

Next, we purified recombinant hBLH and yBLH from the overproducing *E. coli* strains and found that the recombinant BLH proteins have the ability to hydrolyze HTL *in vitro*. We then re-assayed DEAE-Sephacel fractions from our native HTLase purification procedures using arginine aminomethyl-coumaryl-amide (R-AMC), an artificial substrate for BLH. We found that the yBLH aminopeptidase activity (Fig. 1C) correlated with yHTLase activity (Fig. 1B). Similar correlation between HTLase and BLH aminopeptidase activities was observed during the purification of hHTLase (not shown). We also found that BLH inhibitors, such as *trans*-epoxysuccinyl-*L*-leucilamido-(4-guanidino)butane (E-64), iodoacetate, or zinc (37) also inhibited HTLase activities of hBLH and yBLH (Table 2). Dipeptides were also found to be inhibitors of the yHTLase activity (Table 2). Taken together, these results exclude a possibility that the HTLase activity may be due to a contaminating enzyme and show that the ability to hydrolyze HTL is an intrinsic property of the human as well as the yeast BLH.

Substrate Specificity — Substrate specificity studies, summarized in Table 3, indicate that the hHTLase and yHTLase exhibit a high specificity for the *L*-stereoisomer of Hcy-thiolactone; *D*-Hcy-thiolactone was not hydrolyzed by any of the enzymes. For both enzymes, non-saturating kinetics were observed for up to 20 mM *L*-Hcy-thiolactone, which precluded determinations of individual k_{cat} and K_m values. Catalytic efficiency values, k_{cat}/K_m, were obtained from the slopes of linear plots of initial *L*-Hcy-thiolactone hydrolysis rates divided by enzyme concentration *vs*. *L*-Hcy-thiolactone concentrations. The hHTLase and yHTLase had similar catalytic efficiencies of 10^3 $M^{-1}s^{-1}$ in the hydrolysis of

L-Hcy-thiolactone, ~100-fold greater than the catalytic efficiency of human serum Hcy-thiolactonase (29). For comparison, the catalytic efficiencies of hBLH and yBLH for the degradation of bleomycin A2 are 5000 $M^{-1}s^{-1}$ (37) and 4.6 $M^{-1}s^{-1}$ (calculated from the data of ref. 38). *L*-Hse-lactone was not hydrolyzed. γ-Thiobutyrolactone or *N*-acetyl-*D,L*-Hcy-thiolactone also were not hydrolyzed, which shows that the alpha amino group is essential for hydrolysis of *L*-Hcy-thiolactone. The hHTLase and yHTLase hydrolyzed also methyl esters of *L*-Cys and *L*-Met; however, methyl esters of α-*L*-Ala, β-*L*-Ala, *L*-Lys, and *L*-Trp were not hydrolyzed (Table 3).

BLH Is a Major Hcy-thiolactonase in Yeast — To determine the contribution of BLH to total intracellular Hcy-thiolactone-hydrolyzing activity, we examined the HTLase activity in cell extracts from *blh1* mutant and *BLH1* wild type strains using [^{35}S]Hcy-thiolactone as a substrate. We found that *blh1* mutants exhibit significantly diminished HTLase activity relative to the *BLH1* strains (Table 4). The thiol reagent iodoacetamide, an inhibitor of BLH (37), inhibited the HTLase activity in cell extracts from *BLH1* strains. The low activity in extracts from *blh1* strains was not affected by iodoacetamide (not shown). These observations suggest that at least 70% of Hcy-thiolactone hydrolyzing activity in wild type yeast cells is due to BLH.

HTLase Activity Can be Increased by Overexpression of BLH in Yeast — To test if HTLase activity can be increased *in vivo*, BLH-overexpressing and control yeast strains (39) were utilized. The BLH-overexpressing strains harbor the pYES2-BLH1 plasmid encoding yeast BLH under the control of the galactose-inducible *GAL1* promoter. Control isogenic strains harbor empty pYES2 vector. The strains were grown in raffinose medium (which prevents expression from the *GAL1* promoter) followed by the addition of 0.5% galactose for 12 h to induce the expression of BLH1. Total cell extracts were prepared from those strains and analyzed for HTLase activity. As shown in Table 4, BLH over-expression in yeast cells results in 3-4-fold higher HTLase activity, relative to controls.

BLH Controls the Levels of Hcy-thiolactone in Yeast — In order to determine whether BLH participates in Hcy-thiolactone hydrolysis *in vivo*, effects of *BLH1* deletion on the accumulation of Hcy-thiolactone were examined using strains YS18, YS18-ΔBLH1, ABJ6-9 (*met6*), and ABJ6-28 (*met6blh1*). A *met6* mutant accumulates Hcy and Hcy-thiolactone due to a mutation in the methionine synthase gene responsible for the conversion of Hcy into Met in the last step of the methionine biosynthetic pathway (4). Yeast cells were grown to saturation on SD minimal medium plus auxotrophic requirements, diluted 2-fold into fresh medium without methionine and incubated at 30°C for 24 h. As shown in Table 4, Hcy-thiolactone levels were elevated 2-fold in a culture of the *blh1* strain YS18-ΔBLH1, relative to the *BLH1* strain YS18. As expected (4), the *met6* strain ABJ6-9 accumulated 7.6-fold more Hcy-thiolactone than the *MET6* strain YS18. However, the *met6 blh1*strain ABJ6-28 accumulated 3-fold more Hcy-thiolactone that the *met6 BLH1* strain ABJ6-9 (Table 4).

In another experiment effects of manipulations of BLH expression on Hcy-thiolactone synthesis from exogenous Hcy were examined by using HWY22, HWY23, and HWY24 strains. These strains exhibit different levels of HTLase activity due to different levels of BLH expression (Table 4). For these experiments, the strains were maintained on raffinose/galactose medium plus auxotrophic requirements (Leu, His) and 0.5-4 mM Hcy for 24 h. For each strain tested, Hcy-thiolactone accumulation increased with increasing Hcy concentration

(Fig. 2). For each Hcy concentration, the highest levels of Hcy-thiolactone were observed in cultures of the *blh1* strain HWY24. Hcy-thiolactone levels were lower in the cultures of *BLH1* strain (HWY22) than in the cultures the *blh1* strain (HWY24). The lowest levels of Hcy-thiolactone were observed in the BLH-overexpressing strain HWY23 (Fig. 2). The levels of Hcy-thiolactone (Fig. 2) correlate negatively with the levels of HTLase activity (Table 4) in those strains. Taken together, these findings suggest that BLH controls the size of the Hcy-thiolactone pool formed from exogenous Hcy.

A blh1 Mutant is More Sensitive to Hcy Toxicity Than a Wild Type — To determine whether BLH protects against Hcy toxicity, we examined growth of HWY24 (*blh1Δ::KanMX met15*) and HWY22 (*BLH1met15*) strains (39) in the presence of Hcy. The HWY strains require Met (or Hcy) for growth due to a mutation in the *MET15* gene. The strains were grown on SD medium plus auxotrophic requirements to a density of 10^7 cells/mL, washed, diluted into fresh SD medium containing 0 - 8 mM Hcy, and grown on a shaker at 30°C. As shown in Fig. 3, Hcy inhibited the growth of HWY22 in a concentration-dependent manner. More severe growth inhibition was observed with the HWY24 strain, which grew much slower on 0.5 mM, 1 mM, or 2 mM Hcy than the HWY22 strain. In contrast to HWY22 strain, the HWY24 strain did not grow on 4 mM or 8 mM Hcy. Consistent with the expected *met15* phenotype, both strains did not grow in the absence of Hcy (Fig. 3).

We also examined effect of Hcy on cell viability in the cultures of yeast strains HWY22 (*BLH1*) and HWY 24 (*blh1*). Exposure to 10 mM Hcy for 60 h diminished cell viabilty in *blh1* cultures to 13.0±3.1% (on raffinose/galactose medium) or 65±4% (on SD minimal medium). The viablity of *BLH1* cells was not affected by 10 mM Hcy (not shown).

DISCUSSION

This work identifies novel intracellular Hcy-thiolactone-hydrolyzing enzymes in humans and yeast. An unexpected outcome of the present work is that each of these enzymes is identical with BLH, a bleomycin-detoxifying enzyme whose natural function and substrate were unknown. Our findings suggest that HTLase/BLH plays an important role in Hcy metabolism in humans and yeast. We also show that BLH protects yeast cells against Hcy toxicity.

Human and yeast BLH have almost identical high molecular structure, similar to the 20S proteasome and belong to a family of self-compartmentalizing intracellular cysteine proteases (35, 36). The evolutionary conservation and wide distribution of BLH suggests that the enzyme has a conserved cellular function. However, this function has not yet been elucidated. The natural substrates of BLH were unknown and the only biologically relevant function was deamidation of the anticancer drug bleomycin due to an aminopeptidase activity (37-39). Although yBLH and hBLH have been shown to interact with several cellular proteins, such as yeast cAMP binding ectoprotein (40), the human homologue of ubiquitin-conjugating enzyme 9 (41), human ribosomal proteins (42), and amyloid precursor protein (43, 44), the physiological role of those interactions is unclear. BLH-knockout mice are healthy and fertile, but more sensitive to bleomycin toxicity and prone to tail dermatitis, compared with wild type littermates (45). Yeast BLH, in contrast to human BLH (35), is a DNA-binding protein that acts as a negative regulator of *GAL* gene expression (46). Our data demonstrate that Hcy-thiolactone is a natural substrate of BLH and

provide evidence that BLH controls levels of Hcy-thiolactone in yeast.

Our conclusion that HTLase is identical with BLH, and that BLH has an intrinsic ability to hydrolyze Hcy-thiolactone, is based on several lines of evidence. First, proteomic analyses of purified native hHTLase and yHTLase, reveal that they are identical with hBLH and yBLH, respectively. Second, purified native hHTLase and yHTLase hydrolyze arginine methylcoumarylamide, a fluorogenic substrate of BLH. Third, recombinant variants of hBLH and yBLH purified from *E. coli* exhibit HTLase activity similar to that of corresponding native enzymes. Fourth, recombinant BLH mutants devoid of aminopeptidase activity are also devoid of HTLase activity. Fifth, bleomycin hydrolase-deficient *blh1* yeast mutants are also deficient in HTLase activity. Sixth, BLH overproduction results in elevated HTLase activity in yeast strains.

Our data also suggest that the hydrolysis of Hcy-thiolactone occurs at the aminopeptidase active site of the BLH. First, the HTLase activity of the human or yeast enzyme is inhibited by known BLH inhibitors, such as E-64, iodoacetamide, or zinc. Second, mutations of the active site residues, important for the aminopeptidase activity of the human (C73S) or yeast (H369A) enzyme (35), result in the inactivation of the HTLase activity.

Intracellular Hcy-thiolactonase has not been reported before in humans or yeast. As shown in the present communication, the human and yeast HTLases are different from the two other known Hcy-thiolactonases: human serum Hcy-thiolactanase (29) and plant Hcy-thiolactonase (9). Human serum Hcy-thiolactonase, encoded by the *PON1* gene, is a calcium-dependent extracellular enzyme carried on HDL in the blood. As shown in Table 3, the human serum Hcy-thiolactonase has broad specificity of a (thio)lactonase (29, 47). The plant Hcy-thiolactonase has a broad substrate specificity of an α-aminoacyl-(thio)ester hydrolase (9) (Table 3). In contrast, substrate specificity of the human and yeast HTLases is restricted to *L*-α-aminoacyl thioesters, esters of thio-amino acids (Table 3), and amino acid amides (37).

Our results demonstrate that the physiological function of BLH involves control of Hcy-thiolactone accumulation in yeast. We have also demonstrated that the disruption of the *BLH1* gene results in hypersensitivity to Hcy toxicity (Fig. 3). The *blh1* cells loose viability in the presence of 10 mM Hcy and fail to grow in the presence of Hcy-concentrations that permit growth of *BLH1* cells. Taken together, these data show that BLH contributes to the resistance against Hcy toxicity in yeast, most likely due to its ability to hydrolyze Hcy-thiolactone. This conclusion is supported by the observation that a yeast *cys2cys4* strain, which overproduces Hcy-thiolactone due to a mutation in the cystathionine β-synthase gene (4), is also extremely sensitive to Hcy: its viability is 22, 8, and 0.1% after a 24 h exposure to 0.1, 1, and 10 mM mM Hcy, respectively (6). Thus, cellular overproduction of Hcy-thiolactone caused by mutations in two different metabolic pathways leads to similar Hcy-sensitive phenotypes.

In humans, BLH is expressed in a tissue-dependent manner. For example, BLH mRNA is expressed at low to moderate levels in most human organs tested (spleen, thymus, prostate, ovary, small intestine, heart, brain, placenta, lung) (37). Elevated expression levels of BLH mRNA are observed in testis, skeletal muscle, and pancreas. Very low expression levels are seen in liver, kidney, colon, and peripheral blood leukocytes (37). The present work, using a protein purification approach combined with HTLase and aminopeptidase

assays, demonstrates that active BLH is expressed in human placenta.

Human BLH is also expressed in a cell-dependent manner. For example, high levels of BLH mRNA expression are observed in human cancer cell lines, such as HeLa, HL-60, or K-562 (37). Western blot analysis and aminopeptidase activity assays show that active BLH protein is expressed in HeLa cells (35). We have previously observed elevated HTLase activity in human breast cancer cells (5). We have also shown that HTLase activity is present in human fibroblasts (5) but absent in human umbilical vein endothelial cells (7). The lack of HTLase/BLH in endothelial cells may contribute to their susceptibility to Hcy toxicity (14-16).

In some but not all studies, genetic associations between the Ile443Val polymorphism of hBLH and an increased risk for Alzheimer's disease have been observed (44). Other studies have found associations between elevated plasma Hcy and Alzheimer's disease (12). However, whether the genetic hBLH polymorphism influences the association between Hcy and Alzheimer's disease has not been examined. The structure of hBLH (35) shows that the Ile443Val polymorphic site is located in the C-terminal domain important for the aminopeptidase activity of the enzyme (48). This site can also affect HTase activity of hBLH either directly or indirectly *via* interactions with another protein, which in turn can modulate the HTLase activity. Effects of the Ile443Val polymorphic site on the HTLase activity of hBLH would provide a possible explanation for the involvement of Hcy in Alzheimer's disease.

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FOOTNOTES

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[1] The abbreviations used are: AMC, aminomethylcoumarine; R-AMC, arginine aminomethyl coumarylamide; BLH, bleomycin hydrolase; DTT, dithiothreitol; E-64, *trans*-epoxysuccinyl-*L*-leucilamido-(4-guanidino)butane; Hcy, homocysteine; HPLC, high performance liquid chromatography; HTLase, homocysteine-thiolactonase; IPTG, isopropyl-β-D-thiogalactopyranoside; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; TLC, thin layer chromatography.

FIGURE LEGENDS

Fig. 1. **A final step of the purification of yeast BLH/HTLase: ion exchange chromatography on DEAE-Sephacell, pH 8.7.** *A*, Protein elution profile. *B*, HTLase activity monitored by TLC. Hcy-thiolactone was visualized under short-wave UV light. *C*, Aminopeptidase activity monitored by TLC using R-AMC as a substrate. AMC, a highly fluorescent product of R-AMC hydrolysis was visualized under long-wave UV light. *D*, SDS-PAGE gel electrophoresis of purified native hHTLase (*lane 1*) and yHTLase (*lane 2*). Molecular weight markers are shown on the right.

Fig. 2. **Hcy-thiolactone accumulation depends on the levels of BLH expression in yeast.** Yeast strains expressing different level of BLH were maintained on raffinose/galactose minimal medium containing auxotrophic requirements (His, Leu) and 0.5-4 mM Hcy. Hcy-thiolactone was assayed by HPLC as described in Materials and Methods. Hcy-thiolactone levels, in pmoles/10,000 cells/ 24 h, are plotted as a function of Hcy concentration for the following yeast strains: HWY22 (*BLH1met15*) (empty circles, ○), HWY23 (*BLH1met15*/pYES2-BLH1) (filled circles, ●), and HWY24 (*blh1Δ::KanMX met15*) (triangles, Δ).

Fig. 3. **A *blh1* yeast mutant is more sensitive to growth inhibitory effects of Hcy than *BLH1* wild type yeast strain.** Overnight cultures of yeast strains HWY22 (*BLH1met15*)(*upper panel*) and HWY24 (*blh1Δ::KanMX met15*) (*lower panel*) were diluted to a cell density of about 3 Klett units into fresh SD minimal medium supplemented with 0-8 mM Hcy. The cultures were incubated at 30°C in a rotary shaker and the increase in cell density was monitored at indicated time intervals using a Klett densitometer.

Figure 1

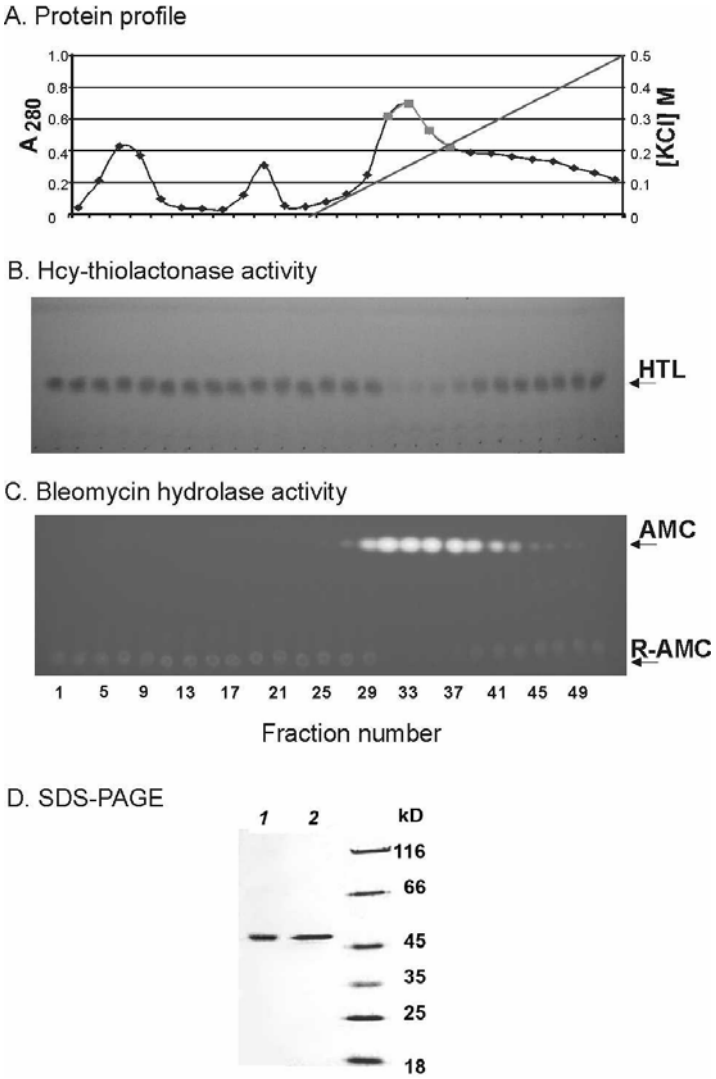


Figure 2

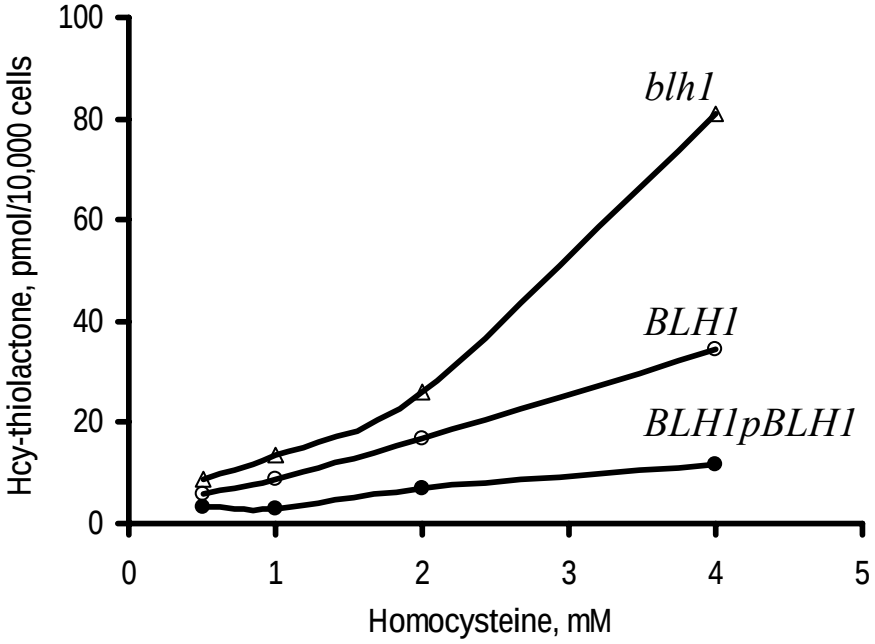
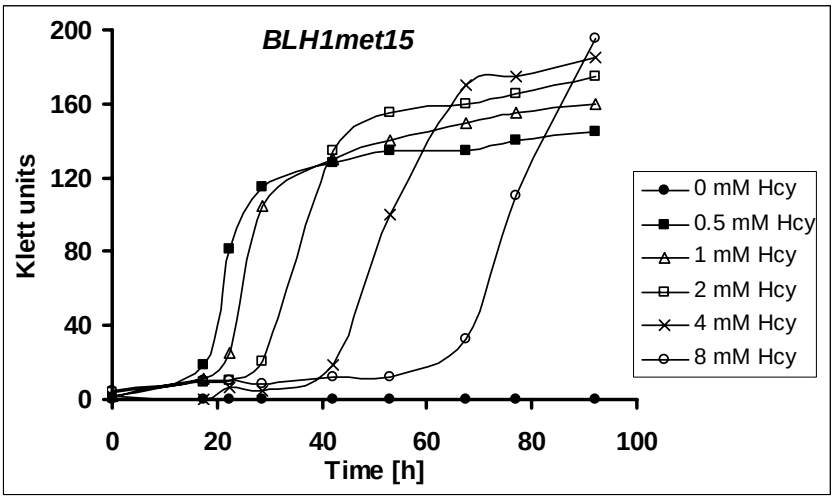


Figure 3



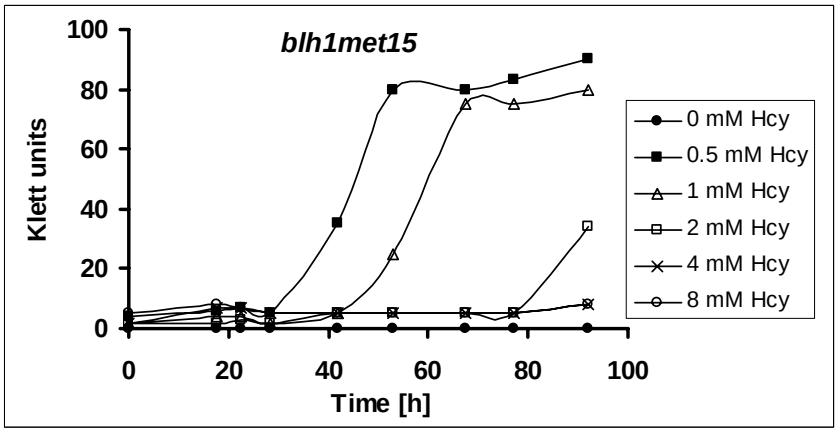


TABLE 1

S. cerevisiae strains used in this study

Strain	Genotype	Source
HWY22	*MATa his3-1 leu2-0 met15-0 ura3-0*/pYES2	D. Ramotar
HWY23	*MATa his3-1 leu2-0 met15-0 ura3-0*/pYES2-BLH1	D. Ramotar
HWY24	*MATa his3-1 leu2-0 met15-0 ura3-0 blh1Δ::KanMX*/pYES2)	D. Ramotar
W303-1AΔBLH1	*Mata ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 blh1Δ::URA3*	D. Ramotar
XJB3-1B	*MATα met6 gal2*	Yeast Genetic Stock Center
ABJ6-9	*MATα met6 leu2-3,112 trp1-1 blh1Δ::URA3*	This laboratory
ABJ6-28	*MATα met6 leu2-3,112 trp1-1*	This laboratory
YS18	*MATα ura3-1 his3-11,15 leu2-3,112 CAN^r*	D. H. Wolf
YS18-ΔBLH1	*MATα ura3-1 his3-11,15 leu2-3,112 CAN^r blh1Δ::KanMX*	D. Ramotar

TABLE 2

Inhibitors of the yeast and human HTLases

Assays were carried out at 25°C in 50 µl reaction mixtures containing 2.5 mM *L*-Hcy-thiolactone, 50 mM potassium-phosphate buffer, pH 7.4, 1 mM DTT, 1 mM EDTA, and 10 µM recombinant yeast or human BLH/HTLase. The hydrolysis of *L*-Hcy-thiolactone was monitored by changes in UV absorption at A_{240}.100% HTLase activity corresponds to 2.5 mM *L*-Hcy-thiolactone hydrolyzed per min.

Tested compound	% inhibition	
	yHLTase	hHTLase
$ZnCl_2$, 2 mM	69.8	n.d.[a]
$CdCl_2$, 2 mM	5.2	n.d.
$CuCl_2$, 2 mM	9.1	n.d.
Iodoactamide, 2 mM	94.0	99.9
H_2O_2, 2 mM	41.3	n.d.
E-64, 12.5 uM	99.5	14.6
E-64, 62.5 uM	n.d.	94.4
E-64, 125 uM	98.7	97.1
ArgAla, 10 mM	86.0	n.d.
LysAla, 10 mM	77.4	n.d.
LysLeu, 10 mM	26.4	n.d.
LeuAla, 10 mM	50.0	n.d.

[a] n.d., not determined.

TABLE 3

Substrate specificity of yeast, human, and plant Hcy-thiolactonases

Assays were carried out for 1 h at 25°C in 50 µl reaction mixtures containing 10 mM indicated compound, 50 mM potassium-phosphate buffer, pH 7.4, 1 mM DTT, 1 mM EDTA, and 10 µM recombinant yeast or human BLH/HTLase. Reaction products were analyzed by TLC on cellulose plates. Progress of the reactions involving thiolactones was monitored by changes in UV absorption at A_{240}. 100% yHTLase or hHTLase activity corresponds to 2.5 mM *L*-Hcy-thiolactone hydrolyzed per min. Symbols + or – indicate that a compound is hydrolyzed or is not hydrolyzed, respectively.

Substrate	Relative activity of			
	yHTLase	hHTLase	human serum HTLase[a]	plant HTLase[b]
L-Hcy-thiolactone	% 100	% 100	% 100	% 100
D-Hcy-thiolactone	<1	<1	24	6
γ-Thiobutyrolactone	<1	<1	545 [c]	–
N-Acetyl-*D,L*-HTL	<1	<1	–	n.d.
L-Hse-lactone	–[d]	–	++++	+
L-Cys methyl ester	++	++	–	++
L-Met methyl ester	+	++	–	++
D-Met methyl ester	–	+	–	+
α-*L*-Ala methyl ester	–	–	–	++
β-*L*-Ala methyl ester	–	–	–	–
L-Lys methyl ester	–	–	n.d.	++
L-Trp methyl ester	–	–	–	++
Phenyl acetate	–	–	280,000	–
p-Nitrophenyl acetate	–	–	4,000	–
Paraoxon	–	–	330	–
Bleomycin	0.46 [e]	500 [f]	n.d.[g]	n.d.

[a] Data from Ref. 28.

[b] Data from Ref. 9.

[c] Recalculated from Ref. 46.

[d] Symbols + or – indicate that a compound is hydrolyzed or is not hydrolyzed, respectively.

[e] Recalculated from Ref. 37.

[f] Recalculated from Ref. 46.

[g] n.d., not determined.

TABLE 4

The *BLH1* gene is a major determinant of HTLase activity in yeast

Yeast strain (relevant genotype)	HTLase activity, nmol/10^6 cells/1 h	
	Raffinose galactose medium	Glucose medium
HWY22 (*BLH1*)	1.18±0.11	0.31±0.02
HWY23 (*BLH1 pBLH1*)	4.02±0.43	0.38±0.04
HWY24 (*blh1*)	0.40±0.02	0.18±0.01
HWY25 (*blh1 pBLH1*)	1.42±0.05	0.24±0.02
YS18-ΔBLH1 (*blh1*)	<0.01	0.24±0.03
W303-1A-ΔBLH1 (*blh1*)	0.12±0.02	0.19±0.02
met6blh1	n.d.[a]	0.18±0.02
met6BLH1	n.d.	0.67±0.04

[a] n.d., not determined.

TABLE 5

Effects of *blh1* and *met6* mutations on Hcy-thiolactone levels in yeast cultured on SD minimal medium

Strain (relevant genotype)	Hcy-thiolactone nmol/10^7 cells/24 h
YS18 (*BLH1*)	1.25±0.01
YS18-ΔBLH1 (*blh1*)	2.32±0.20
ABJ6-28 (*met6 BLH1*)	9.5±2.1
ABJ6-9 (*met6 blh1*)	29.6±4.7