Protective Mechanisms against Homocysteine Toxicity

THE ROLE OF BLEOMYCIN 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/para-oxonase 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 cerevisiae and identified it as yeast bleomycin hydrolase (yBLH). BLH belongs to a family of evolutionarily conserved cysteine aminopeptidases, and its only known biologically relevant function was deamidation of evolutionarily conserved cysteine aminopeptidases, and its only known biologically relevant function was deamidation of the anticancer drug bleomycin. Recombinant hBLH or yBLH, expressed in Escherichia 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 blhl 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 remethylation 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 B12, or vitamin B6.

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 disease (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, 19), and bacterial cells (20). 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 nonproductive consumption of cellular energy (4, 19). Second, Hcy-thiolactone is a reactive intermediate that causes protein N-homocysteinylatation through the formation of amide bonds with e-amino groups of protein lysine residues (5, 7, 21, 22). Resulting protein damage necessitates the removal of N-homocysteylnated 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 (3, 24) and induce an autoimmune response, which is associated with atherosclerosis in humans (1, 3, 24–26).
Bleomycin Hydrolase Is a Homocysteine-thiolactone Hydrolase

To minimize Hcy-thiolactone toxicity, cells had to evolve the 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 detoxified by enzymatic hydrolysis by the serum Hcy-thiolactonase/paraoxonase (PON1) carried on high density lipoprotein (29–32).

Since the serum PON1 is present extracellularly, it was unknown whether Hcy-thiolactone can be detoxified intracellularly. The present work describes intracellular Hcy-thiolactone-hydrolyzing enzyme that protects cells against Hcy-thiolactone toxicity.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—Saccharomyces 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). Escherichia coli BL21 (DE3) was used as a host for plasmid maintenance and recovery.

[^35]S[Hcy-thiolactone]—Carrier-free l[^35]S-Met (Amersham Biosciences) was supplemented with unlabelled l-methionine (Sigma) to a specific activity of 40,000 Ci/mol, converted to[^35]S-Hcy-thiolactone by a 4-h digestion with hydroxid acid at 128 °C, and purified by two-dimensional 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 (1 kg) was taken up in 10 mM potassium phosphate buffer (pH 6.8), 0.5 mM mercaptoethanol, 5% glycerol (3 liters) and disrupted in a high pressure homogenizer. The crude extract was clarified by centrifugation, mixed with DEAE-Sephacel (0.8 liters), and poured into a 5 × 40-cm column. The column was washed with the pH 6.8 buffer (4 liters) followed by a linear gradient of 0–0.5 M KCl in the pH 6.8 buffer (8 liters). At pH 6.8, HTLase activity is not retained on DEAE-Sephacel and elutes in the breakthrough fractions. Protein precipitated from these fractions with 60% ammonium sulfate was collected by centrifugation, dissolved in 50 mM potassium 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. 1, A and B). Pure yHTLase migrates on SDS-polyacrylamide gels as a 48-kDa 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 potassium phosphate buffer (pH 6.8), 0.5 mM mercaptoethanol, 5% glycerol (0.2 liters). The homogenate was clarified by centrifugation, and a protein fraction precipitated between 50 and 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 nondenaturing polyacrylamide gels. Purified hHTLase migrated on SDS-polyacrylamide gels as a 48-kDa 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) 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 liters) to midlog phase at 37 °C, the culture was shifted to 25 °C, and the BLH expression was induced with 0.5 mM isopropyl-β-D-thioglactopyranoside for 16 h. The cells were harvested; resuspended in 50 mM potassium phosphate, pH 8.5, 300 mM NaCl, 7 mM 2-mercaptoethanol; and frozen and stored at −80 °C. For BLH purification, the cells were thawed and disrupted by sonication on ice. Crude extracts were purified by centrifugation at 4 °C, and BLH was purified by affinity chromatography on a 1-ml Ni²⁺-agarose (Amersham Biosciences) column. Pure BLH, eluted with 0.2 M imidazole, was dialyzed against 50 mM potassium phosphate buffer, pH 7.4, 7 mM 2-mercaptoethanol, 10% glycerol and stored at −20 °C.

\[\text{TABLE 1} \]

\begin{tabular}{|c|c|c|}
\hline
Strain & Genotype & Source \\
\hline
HWY22 & MATa his3-1 leu2-0 met15-0 ura3-0/pYES2-BLH & D. Ramotar \\
HWY23 & MATa his3-1 leu2-0 met15-0 ura3-0/pYES2 & D. Ramotar \\
HWY24 & MATa his3-1 leu2-0 met15-0 ura3-0 blh1::KanMX/pYES2 & D. Ramotar \\
W303-1ΔBLH1 & MATa ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 blh1Δ::URA3 & Yeast Genetic Stock Center \\
XJ93-1B & MATa met6 gal2 & This laboratory \\
ABf6-9 & MATa met6 leu2-3,112 trp1-1 blh1Δ::URA3 & This laboratory \\
ABf6-28 & MATa met6 leu2-3,112 trp1-1 & This laboratory \\
YS18 & MATa ura3-1 his3-11,15 leu2-3,112 CANv & D. H. Wolf \\
YS18-ΔBLH1 & MATa ura3-1 his3-11,15 leu2-3,112 CANv::blh1Δ::KanMX & D. Ramotar \\
\hline
\end{tabular}
Preparation of Yeast Cell Extracts—Yeast cells from 10 ml cultures at 10^7 cells/ml were collected by centrifugation at 2 °C and disrupted by vortexing with glass beads (100–400 μm; Sigma) in 50 μl of ice-cold buffer (50 mM potassium phosphate, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol; 3 × 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 J2 centrifuge (30,000 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 dithiothreitol, 1 mM EDTA. Hcy-thiolactonase activity was determined by following the formation of [35S]Hcy from [35S]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 A240 using a Varian Cary 50 UV-visible 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 the appropriate time, the reaction was stopped by transferring 3-μl aliquots onto the origin of a TLC plate (aluminum precoated with silica gel containing fluorescent indicator; Merck). The plate was developed with ethyl acetate/isopropyl alcohol/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 arginine aminomethylcoumarylamide (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/acetate 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 × g, 2 min, 25 °C). Cell-free media (2 μl) were diluted 20-fold with water, and 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 × 150 mm, 5 μm, 300 Å) from PolyLC, Inc. and System Gold Nouveau HPLC instrumentation from Beckman as previously described (27, 28). After sample (20 μl) application the column was eluted isocratically with 1 mM sodium phosphate buffer, pH 6.6, 25 mM NaCl at a flow rate 0.15 ml/min. A postcolumn 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-phthalaldehyde, 0.25 M NaOH, delivered at a flow rate of 0.07 ml/min. The mixture was 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 a 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 S. 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 data base. Mascot Server 1.9 (Bruckner Daltons) searches revealed that the best match for human HTLase was human bleomycin hydrolase (hBLH, accession number gi|3321858). 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|3391714) 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 the β-galactosidase promoter were transformed into E. coli strain BL21(pLysS) for protein expression. Cells were grown in LB medium to midlog phase and induced with isopro- pyl-β-d-thiogalactopyranoside to midlog phase and induced with isopro- pyl-β-d-thiogalactopyranoside. Only cells expressing active variants of yBLH or hBLH exhibited a high specificity for the L-stereoisomer of Hcy-thiolactone; D-Hcy-thiolactone was not hydrolyzed by any of the enzymes. For both enzymes, nonsaturating 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 versus 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-Homoserine-lactone was not hydrolyzed. The α-amino group is essential for hydrolysis of L-Hcy-thiolactone. The hHTLase and yHTLase

### TABLE 2

| Tested compound          | Inhibition |
|--------------------------|------------|
|                          | yHTLase    | hHTLase |
|                          | %          | %       |
| ZnCl$_2$, 2 mM           | 69.8       | ND      |
| CdCl$_2$, 2 mM           | 5.2        | ND      |
| CuCl$_2$, 2 mM           | 91.1       | ND      |
| Iodoacetamide, 2 mM      | 94.0       | 99.9    |
| H$_2$O$_2$, 2 mM         | 41.3       | ND      |
| E-64, 12.5 μM            | 99.5       | 14.6    |
| E-64, 62.5 μM            | ND         | 94.4    |
| E-64, 125 μM             | 98.7       | 97.1    |
| Arg-Ala, 10 mM           | 86.0       | ND      |
| Lys-Ala, 10 mM           | 77.4       | ND      |
| Lys-Leu, 10 mM           | 26.4       | ND      |
| Leu-Ala, 10 mM           | 50.0       | ND      |

*Data from Ref. 29.
*b Recalculated from Ref. 47.
*c Recalculated from Ref. 38.
*d Recalculated from Ref. 37.
also hydrolyzed 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 [35S]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 overexpression 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 5, 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 than the met6 BLH1 strain ABJ6-9 (Table 5).

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 and His) and 0.5–4 mM Hcy. Hcy-thiolactone was assayed by HPLC as described under “Experimental Procedures.” Hcy-thiolactone levels, in pmol/10,000 cells/24 h, are plotted as a function of Hcy concentration for the following yeast strains: HWY22 (BLH1met15), HWY23 (BLH1met15/pYES2-BLH1), and HWY24 (blh1Δ::KanMX met15).

**Ablh1 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

### TABLE 4

| Yeast strain (relevant genotype) | HTLase activity |
|-------------------------------|-----------------|
|                               | 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                      | ND*             | 0.18 ± 0.02 |
| met6BLH1                      | ND              | 0.67 ± 0.04 |

* ND, not determined.

### TABLE 5

| Strain (relevant genotype) | Hcy-thiolactone |
|----------------------------|-----------------|
|                            | nmol/10^6 cells/24 h |
| YS18 (MET6 BLH1)           | 1.25 ± 0.01     |
| YS18-ΔBLH1 (MET6 blh1)     | 2.32 ± 0.20     |
| ABJ6-28 (met6 BLH1)        | 9.5 ± 2.1       |
| ABJ6-9 (met6 blh1)         | 29.6 ± 4.7      |

**FIGURE 2.** Hcy-thiolactone accumulation depends on the levels of BLH expression in yeast. Yeast strains expressing different levels of BLH were maintained on raffinose/galactose minimal medium containing auxotrophic requirements (His and Leu) and 0.5–4 mM Hcy. Hcy-thiolactone was assayed by HPLC as described under “Experimental Procedures.” Hcy-thiolactone levels, in pmol/10,000 cells/24 h, are plotted as a function of Hcy concentration for the following yeast strains: HWY22 (BLH1met15), HWY23 (BLH1met15/pYES2-BLH1), and HWY24 (blh1Δ::KanMX met15).
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FIGURE 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) (top) and HWY24 (blh1Δ:KanMX met15) (bottom) 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 the indicated time intervals using a Klett densitometer.

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 20 S proteasome, and belong to a family of self-compartmentalizing intracellular cysteine proteases (35, 36). The evolutionary conservation and wide distribution of BLH suggest 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 deamination 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 knock-out 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 zine. 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 work, the human and yeast HTLases are different from the two other known Hcy-thiolactonases: human serum Hcy-thiolactonase (29) and plant Hcy-thiolactonase (9). Human serum Hcy-thiolactonase, encoded by the PON1 gene, is a calcium-dependent extracellular enzyme carried on high density lipoprotein in the blood. As shown in Table 3, the human serum Hcy-thiolactonase has broad specificity of a (thio)lac-
tonase (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 α-aminoacyl thioesters, esters of thioamino 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 lose 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 cyss2cyss4 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 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, and 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 I443V polymorphism of hBLH and an increased risk for Alzheimer disease have been observed (44). Other studies have found associations between elevated plasma Hcy and Alzheimer disease (12). However, whether the genetic hBLH polymorphism influences the association between Hcy and Alzheimer disease has not been examined. The structure of hBLH (35) shows that the I443V polymorphic site is located in the C-terminal domain important for the aminopeptidase activity of the enzyme (48). This site can also affect HTLase activity of hBLH either directly or indirectly via interactions with another protein, which in turn can modulate the HTLase activity. Effects of the I443V polymorphic site on the HTLase activity of hBLH would provide a possible explanation for the involvement of Hcy in Alzheimer disease.

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