Essential Binding and Functional Domains of Human Bleomycin Hydrolase†

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ABSTRACT: Bleomycin hydrolase (BH) is unusual among cysteine proteinases because it appears to form multihomomeric structures, inactivates the antitumor glycopeptide bleomycin, and contains a unique C-terminal amino acid sequence. We now demonstrate intrinsic endopeptidase activity associated with human BH (hBH) using artificial substrates and intracellular dimerization of hBH using a yeast two-hybrid assay. To determine domains important for homomeric interactions and catalysis, we constructed N- and C-terminal deletion mutants and identified an N-terminal region (hBH_{1-82}) that interacted with two nonoverlaping hBH domains: one near the N-terminus (hBH_{14-103}) and another neighboring the C-terminus ($hBH_{358-455}$). In vitro hBH aggregated with a molecular mass of 235 kD corresponding to a homotetramer and the C-terminus was critical for this oligomerization since no tetramers were found when the last 40 amino acids were deleted. The penultimate 8 amino acids, which constitute a unique and highly conserved *b*leomycin *hy*drolase-like *d*omain (BHYD), were essential for BH and aminopeptidase activity but not for endopeptidase activity or oligomer formation. Thus, the C-terminus of hBH has two independent roles controlling both the catalytic activity and oligomerization of hBH.

Bleomycin hydrolase (BH)[1] is an intracellular neutral cysteine proteinase originally identified by its ability to enzymatically inactivate bleomycin (*1*). The bleomycins are a family of DNA-cleaving glycopeptides isolated from *Streptomyces verticillus* and widely used in the treatment of human cancers. Unfortunately, tumor resistance and the development of fatal pulmonary fibrosis limit the therapeutic efficacy of bleomycin (*2*). The susceptibility of both normal and malignant tissues to the toxic actions of bleomycin has been linked to low levels of BH, which deamides a β-aminoalanine moiety in bleomycin (*3*). It seems likely that a comprehensive understanding of the structure and biochemical properties of BH could lead to a more rational use of the anticancer drug bleomycin.

The primary amino acid sequence of BH was first deduced using a rabbit liver cDNA library and revealed the 10-residue signature sequence of the cysteine active site found in the papain superfamily (*3*, *4*). Subsequent studies indicate homologous enzymes are expressed in bacteria, yeast, birds, reptiles, and mammals (*5*−*10*). The ubiquitous distribution and evolutionary conservation of BH implies important physiological roles, which have not been defined. Brömme et al. (*6*) and Ferrando et al. (*7*) recently independently cloned human bleomycin hydrolase (hBH), a 455 amino acid protein that has the highly conserved, putative, active site amino acid sequences surrounding Cys^{73}, His^{372}, and Asn^{396}, typical for cysteine proteases of the papain superfamily (Figure 1A). The corresponding Cys and His in the yeast BH have been identified as essential for catalysis of bleomycin (*11*). Montoya et al. (*12*) have mapped hBH to 17q11.1−11.2 and have shown that it has the genomic structure typical for cysteine proteases. Interestingly, we have noticed the C-terminus is highly conserved in BH-like enzymes and is not present in other proteases found in the current database (Figure 1B). Our previous results with a neutralizing, epitope-specific, antibody to a C-terminal region of BH (*13*) support the hypothesis that the C-terminus could have a role in controlling enzyme activity. Joshua-Tor et al. (*14*) noted that the structure of the C-terminal region was structurally reminiscent of known inhibitors of the papain superfamily, such as E-64, leupeptin, and stefin, and postulated a potential regulatory function for that region.

Unlike other members of the papain superfamily, BH is thought to form homo-oligomeric structures; this is based on in vitro biochemical studies (*6*, *9*). Both bacterial and yeast BH (yBH) form tetramers in vitro (*9*, *15*), although the recently described crystal structure of yeast bleomycin hydrolase (yBH) supports a homohexameric structure formed by the association of three dimers (*14*). It has been suggested that oligomerization is not required for enzyme activity of

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[1] Abbreviations: BH, bleomycin hydrolase; yBH, yeast bleomycin hydrolase; hBH, human bleomycin hydrolase; dA2, deamidobleomycin A2; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; PBS, phosphate buffer saline; IPTG, isopropyl β-thiogalactoside; GST, glutathione *S*-transferase; 3-AT, 3-aminotriazole; BD, binding domain; AD, activating domain; BD-hBH, GAL4BD-hBH fusion protein; AD-hBH, GAL4AD-hBH fusion protein; BHYD, bleomycin hydrolase-like domain; AMC, amino acid methyl coumarylamide; Z, benzyloxycarbonylphenylalanine; H, amino; Cit, citrulline.

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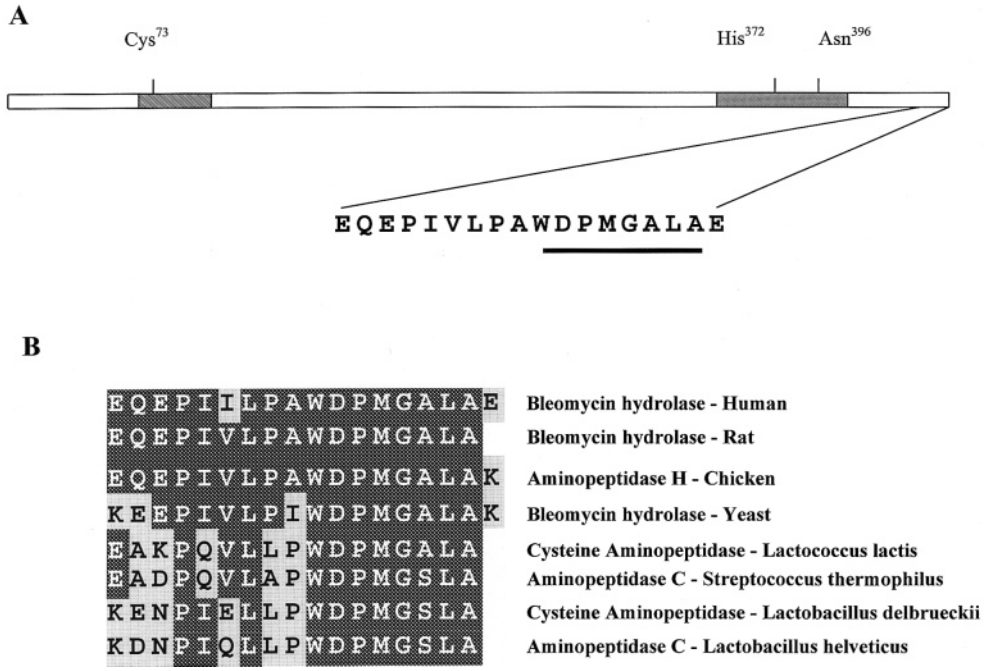

FIGURE 1: Schematic of hBH and the C-terminus. (A) Block diagram of hBH with the active site amino acids and the consensus adjacent regions noted. The highly conserved C-terminal sequence is also outlined and the BHYD is underlined. (B) Amino acid sequence of the C-terminus of several BH-related proteins. The darkened box indicates the conserved amino acids.

BH (*11*). Oligomerization, catalytic, and helical domains within the yeast protein have been proposed based on the hexameric crystal structure (*14*). Because there is only 40% amino acid identity between yeast BH and hBH and even less in the predicted key hook domains, it is not certain that the proposed functional domains exist in hBH. Chicken aminopeptidase H, a member of the BH family, forms an octamer (*16*) consistent with a fundamental dimeric structural unit. hBH displays a monomeric molecular mass of 52 kD by SDS PAGE and has been reported to assume a hexameric structure in vitro based on gel filtration elution at 310 kD (*6*).

In this paper we demonstrated for the first time that hBH also forms homotetramers and has endopeptidase activity. Moreover, we defined two independent sites of homomeric interactions by deductive domain analysis including one in the putative cysteine catalytic region. We also tested the significance of the carboxyl terminus in oligomerization and catalytic activity of the hBH and found that the penultimate, 8 C-terminal amino acids, which we have termed the *b*leomycin *hy*drolase-like *d*omain (BHYD), were essential for bleomycin deamidation and aminopeptidase activity but not endopeptidase activity. The highly conserved C-terminus was also essential for hBH oligomerization, but the reduced catalytic activity was not linked to loss of oligomerization.

MATERIALS AND METHODS

Plasmids and Plasmid Constructs. Commercially available and previously described vectors, namely, pSP72 (Promega, Madison, WI), pLitmus29 (NEB, Beverly, MA), and pMTL22 (*17*), were used to subclone full-length hBH cDNA (kindly provided by D. Brömme, Arris Pharmaceuticals, South San Francisco, CA), which had GGA as the second codon instead of AGC and 15 base pairs 5′ to the ATG codon that facilitated subcloning and primer design. We used pT7Blue-2 and pCite4 (Novagen, Madison, WI), which carry the T7 promoter upstream of 5′ untranslated enhancer, as cloning vectors for optimal expression of cloned inserts by in vitro transcription and translation. We used prokaryotic expression vectors pTrcHis (Invitrogen, Carlsbad, CA), pET15b+, and pET30c+ (Novagen), designed for expression of fusion proteins with 6 histidine residues, which enabled further purification using Ni−NTA agarose (Qiagen, Santa Clarita, CA). We employed the following yeast shuttle expression vectors: pAS2−1 [$GAL4_{(1-147)}$ DNA-BD, *TRP1*, amp^r, *CYH*^{S}*2*]; pACT2 [$GAL4_{(768-881)}$ AD, *Leu2*, amp^r, HA epitope tag]; pTD1, pACT2 encoding a GAL4-AD-SV40 large T-antigen fusion protein; pVA3, pAS2−1 encoding a GAL-BD-p53 fusion protein; and pLAM5′. Yeast vectors were purchased from Clontech (Palo Alto, CA) as a part of Matchmaker 2. The HeLa Matchmaker cDNA library prepared in the pGADgh vector was from Clontech. Methods for yeast manipulations and standard molecular biology techniques were according to the Matchmaker 2 protocol (Clontech) or as described previously (*18*−*21*).

Full-length hBH as a BamHI−BglII fragment was inserted into the BamHI site of the binding domain vector pAS2−1 and used as bait (pAS2−1hBH) and as a BamHI−XhoI fragment into activating domain vector pACT2 (pACT2hBH). The following forward (F) and reverse (R) primers were designed for generation of truncated forms and deletion mutants of hBH used in yeast two-hybrid experiments, for in vitro binding studies, and for overexpression of fusion proteins in *Escherichia coli* cells. The in-frame position of all cDNA inserts was confirmed by dye terminator labeling and sequencing using a ABI Prism 373 DNA sequencer (University of Pittsburgh Research Facility).

Primers used for subcloning into the yeast two-hybrid system vectors were F-5′-CACGAG*CCATGG*AAGGACAG-CAGATGAGC-3′ and R-5′-GCT*AGATCT*GCTACTGG-AACACATGCTG-3′ to generate pAS2−1hBH$_{1-54}$ (the restriction enzyme recognition sites within the primer are

italicized); F-5′-CATGAG*CATATG*ACTGGACAGCAGATGAGC-3′ and R-5′-GCGC*GGATCC*TTCTGGTTGCCTCTGTTGTATAA-3′ to generate pAS2−1hBH$_{1-175}$; F-5′-GCGCGGA*CATATG*GACAAGGTTGAACGCTG-3′ and R-5′-GCGCGC*GGATCC*TGCTTAAGTATTCCACTG-3′ to generate pACT2hBH$_{106-357}$; F-5′CATGAG*CATATG*AACCTGGTACACAGTGGAGC-3′ and R-5′-GCGC*CTGCAG*TTCATGTTCTTCAAGGAG-3′ to generate pAS2−1hBH$_{194-357}$; and F-ACT-AD5 and R-5′-GCGAGCGA*CTCGAG*AAGTATTCCACTGTGTAA-3′ to generate pACT2-hBH$_{14-289}$. The ACT-AD5 primer (Clontech) has the sequence 5′-CTATTCGATGATGAAGATACCCCACCAAAC-3′ positioned ∼100 bp upstream from the multiple cloning site of the AD domain vectors so that the 5′- BamHI recognition site remained available for the subsequent restriction digestion and subcloning steps. The reverse primers were designed to contain a XhoI recognition site as a 5′ extension. We used F-ACT-AD5 and R-5′-GCGAGCGA*CTCGAG*AACAGGTAAGATTGTCTA-3′ to generate pACT2hBH$_{14-103}$; F-ACT-AD5 and R-5′-GCGAGCGA*CTCGAG*CATAACATTCAGACAAGA-3′ to generate pACT2-hBH$_{14-82}$; and F-ACT-AD5 and R-5′-GCGAGCGA*CTCGAG*CAGAATATCATTCATCCT-3′ to generate pACT2-hBH$_{14-181}$.

The following primers were used for subcloning into pCite4 vectors (Novagen) for in vitro transcription and translation: F-5′-CATGAG*CATATG*ACTGGACAGCAGATGAGC-3′ and R-5′-GCGC*GGATCC*AGTATCACTCACTCAGCCAA-3′ to generate pCite4hBH; F-ACT-AD5 and R-5′- GCGC*CTGCAG*TTCATGTTCTTCAAGGAG-3′ to generate pCite4hBH$_{14-357}$; F-5′-GAGAC*GGATCC*CAACAGAGGCAACCAGA-3′ and R-5′-GCGA*CTCGAG*ATCACTCACTCAGCCAA-3′ to generate pCitehBH$_{170-455}$; and F-ACT-AD5 and R-5′-GCGAGCGA*CTCGAG*GAACCACTCATCTGTCAT-3′ to generate pCite4hBH$_{14-415}$. pCite4-hBH$_{14-82}$, pCite4hBH$_{14-103}$, and pCite4hBH$_{14-181}$ were generated by subcloning restriction enzyme fragments from corresponding pACT2 recombinant vectors (see the description of yeast two-hybrid system vectors) into pCite4 vectors. We applied the same approach to generate recombinant vectors for overexpression of corresponding GST-fusion proteins (Figure 3).

The following primers were used for subcloning into pTrcHis vectors: F-5′-TATGA*CTCGAG*AGGACAGCAGATGAGC-3′ and R-5′-GCGC*GGTACC*TAACACAGCTAGCACCT-3′ to generate pTrcHishBH$_{1-437t}$. Because the reverse primer for this amplification did not contain a stop codon, the overexpressed recombinant protein consisted of hBH amino acids 1−437 and the amino acid sequence GTIWEFEAWLFWRMRED-FQPDTD, N-terminally fused to hBH$_{1-437}$. We used F-5′-TATGA*CTCGAG*AGGACAGCAGATGAGC-3′ and R-5′-GCGC*GGTACC*CATGCTGGCAGGATAAT-3′ to generate pTrcHishBH$_{1-447t}$. Similar to the former construct, the reverse primer for this amplification did not contain a stop codon so the overexpressed recombinant protein consisted of hBH−amino acids 1−447 and the amino acid sequence VPYCNSKLGCFCG, N-terminally fused to hBH$_{1-447}$.

The following primers were used for subcloning into pET vectors: F-5′-GCGAGGACAG*CATATG*GG-3′ and R-5′-CGC*GGATCC*TCATAACACAGCTAGCACCT-3′ to generate pET15bhBH$_{1-437}$; F-5′-CCG*AAGCTT*GACCATGGCCAGTATGAC-3′ and R-5′- CCG*CTCGAG*TTATGCTGGCAGGATAATG-3′ to generate pET30chBH$_{1-446}$.

Yeast Two-Hybrid Screening and Analysis. Yeast two-hybrid analysis was performed using the Matchmaker 2 protocol provided by Clontech with the Y190 yeast strain (MATa, ura3−52, his3−200, lys2−801, ade2−101, trp1−901, leu2−3, 112, gal4Δ, gal80Δ, cyh^{r}2, LYS2::GAL1$_{UAS}$-HIS3TATA-HIS3, URA3::GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ). Briefly, Y190 were transformed with the appropriate pAS2−1 and pACT2 derivatives (100 ng of DNA of each plasmid) or for screening the library with pAS2−1hBH (500 μg of DNA) and pGADgh containing HeLa library (250 μg of DNA) using lithium acetate. Cotransformed Y190 were plated on Petri dishes lacking leucine, tryptophan, and histidine and containing 35 mM 3-aminotriazole (3-AT). Y190 cotransformed with pVA3 and pTD served as positive controls. We confirmed the expression of the hBH fusion proteins by Western immunoblotting with GAL4AD and GAL4BD monoclonal antibodies (Clontech). We performed the β-galactosidase assay after transferring the yeast colonies on Whatman filters, lysing the submerged filters in liquid nitrogen, and thawing them at room temperature. Filters were placed on filter paper presoaked in a buffer containing 60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, β-mercaptoethanol, and 0.8 mM 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal). In general, β-galactosidase activity was apparent within 1 h, but the filters were allowed to incubate for 8 h.

Generation of hBH Transcription/Translation Products. Clones containing either hBH or hBH deletion mutants were cloned into pCite4 vectors (Novagen). The in vitro hBH products were synthesized according to the manufacture's directions for the TnT Rabbit Reticulocyte Lysate system (Promega). The resulting proteins were examined by 12% SDS PAGE.

In Vitro Binding Assays. We use the methods of Kho et al. (*22*) to express and purify glutathione *S*-transferase (GST) fused to hBH, hBH$_{14-82}$, or hBH$_{170-455}$. Fresh overnight cultures of DH5α *E. coli* transformed with pGEX4T were diluted 1:10 in LB medium containing ampicillin and incubated for 3−4 h at 37 °C until A_{600} reached 0.7−0.9. Isopropyl β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.6 mM, and incubation was allowed to continue for another 3 h. Bacterial cultures were pelleted and resuspended in PBS with 1 mM (phenylmethyl)-sulfonyl fluoride and 1% aprotinin. The bacteria were then lysed on ice by mild sonication, mixed with Triton X-100 to a final concentration of 1%, and centrifuged at 14000*g* for 5 min at 4 °C. Aliquots of bacterial supernatant fractions (1 mL) were rocked for 30 min at 4 °C with 25 μL of glutathione-Sepharose 4B (Pharmacia). After four washings with PBS containing 1% Nonidet P-40, ^{35}S-labeled proteins (3 μL) were incubated with the beads (25 μL) in 50 mM NaCl and bovine serum albumin (1 mg/mL) at 4 °C for 1 h. The beads were washed four times with 0.1% Nonidet P-40 in PBS, boiled, and loaded on the SDS PAGE. The gels were soaked in fluorographic reagent (Amplify, Amersham, Arlington Heights, IL), dried, and exposed to Kodak X-ray film.

Generation of His-Tagged Fusion Proteins. The full-length hBH (derived from the recombinant pLitmus29hBH), hBH$_{1-447t}$, and hBH$_{1-437t}$ were subcloned in-frame into

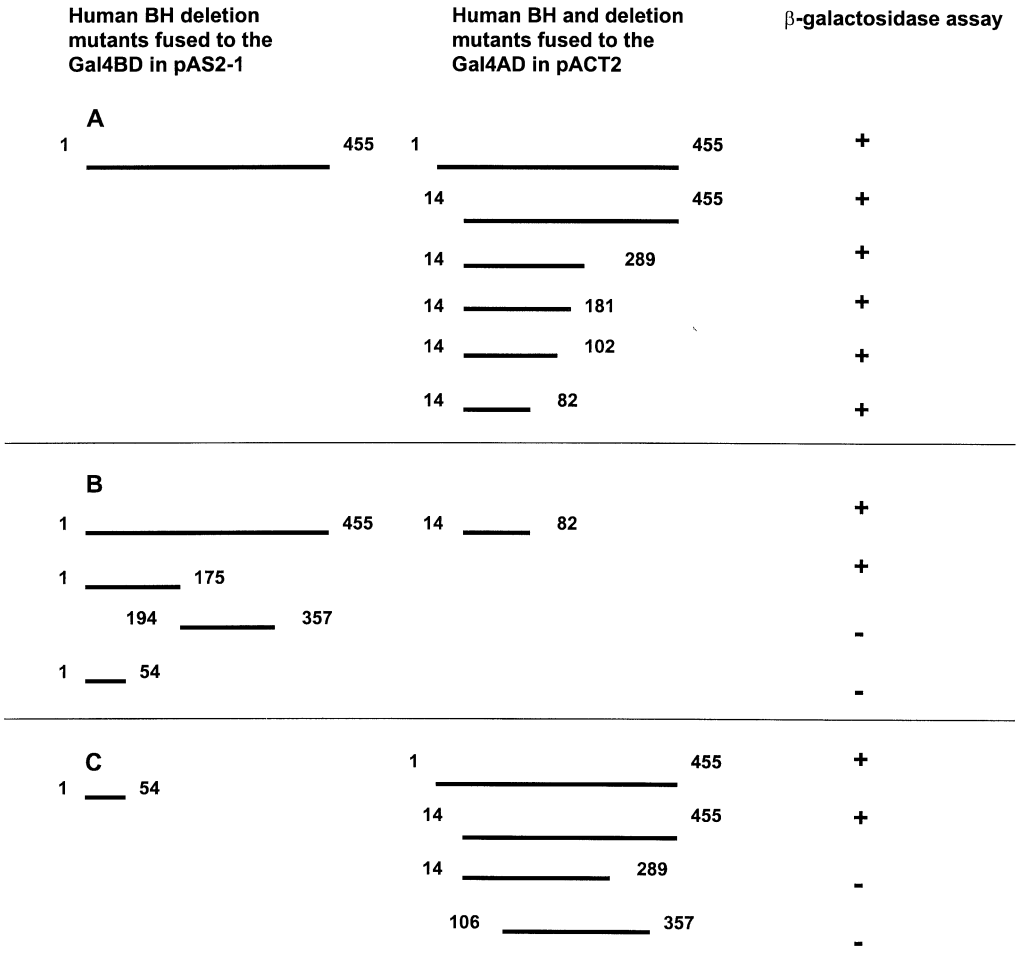

FIGURE 2: hBH domain interactions measured by a two-hybrid β-galactosidase expression. Yeast 190 strain cells were transformed with different combinations of BD (binding domain) and AD (activation domain) plasmids. Amino acids delimiting the N- and C-termini are indicated. β-Galactosidase activity was monitored by using the qualitative filter assay as described under the Materials and Methods section and scored + if blue color developed and − if no color appeared.

pTrcHisC or pTrcHisB vectors using XhoI−KpnI recognition sites and expressed as a His(6)-tagged fusion protein in DH5α *E. coli* cells. hBH_{1-446} and hBH_{1-437} were subcloned into pET30c and pET15b, respectively, and expressed as His-(6)-tagged fusion proteins in BL21(DE3) cells. Expression of His-tagged proteins was induced with IPTG at a final concentration of 0.6 mM. IPTG was added to the liquid bacterial culture ($A_{600} = 0.5-0.7$), and after 3 h of induction at 37 °C the bacterial cells were harvested and resuspended in lysis buffer (20 mM Tris-HCl (pH 8.0), 10 mM β-mercaptoethanol, 1 mM (phenylmethyl)sulfonyl fluoride). The His-tagged proteins were then purified by mildly sonicating and treating with Triton X-100 at a final concentration of 1% for 1 h to solubilize the fusion proteins. The supernatant fractions were then collected and added to a 50% slurry of Ni−NTA resin equilibrated with a buffer containing 20 mM Tris-HCl (pH 8.0), 10 mM β-mercaptoethanol, 20 mM imidazole, 100 mM KCl, 10% glycerol, and 0.1% Nonidet P-40. The His-tagged proteins were eluted by the addition of a buffer containing 20 mM Tris-HCl (pH 8.0), 10 mM β-mercaptoethanol, 200 mM imidazole, 100 mM KCl, 10% glycerol, and 0.1% Nonidet P-40.

Gel Filtration. The molecular mass of ^{35}S-labeled hBH, ΔBH, hBH_{14-415}, and hBH_{14-357} was determined by gel filtration on a Superose 12 HR 10/30 column (Pharmacia). The column was equilibrated and eluted with Tris-buffered saline (1 mL/min). The molecular mass standards (Pharmacia) used were thyroglobulin (669 kD), ferritin (440 kD), catalase (232 kD), aldolase (158 kD), albumin (67 kD), ovalbumin (43 kD), ribonuclease A (14 kD), and chymotrypsinogen (25 kD). Fractions (0.5 mL) were collected, and radioactivity was determined using a Beckman LS 1801 scintillation counter. Nonradioactive protein was determined by absorbance at 280 nm.

SDS PAGE and Western Blotting. His-tagged hBH and deletion mutants were separated on 10% SDS PAGE with or without preboiling of samples. In some experiments proteins were stained with Coomassie Blue after electrophoresis for detection. We also used Western immunoblotting with AntiXpress antibody (Invitrogen) (diluted 1:2000), followed by horseradish peroxidase-conjugated polyclonal goat anti-mouse IgG (diluted 1:5000). Signals were detected using a chemiluminiscent detection assay and a 20 s exposure with X-ray film.

Bleomycin Hydrolase Assay. The metabolism of bleomycin was determined by our previously described HPLC method that separates bleomycin A_2 from its inactive metabolite deamidobleomycin A_2 (dA_2) (*3*). Briefly, His-hBH or deletion mutants (2 μg) were incubated with 70 μM bleomycin A_2 (Bristol Myers Squibb, Wallingford, CT, and Nippon Kayaku Co. Ltd., Tokyo, Japan) in a 50 μL reaction buffer (20 mM Tris, pH 7.5) at 37 °C for 0−2 h. The

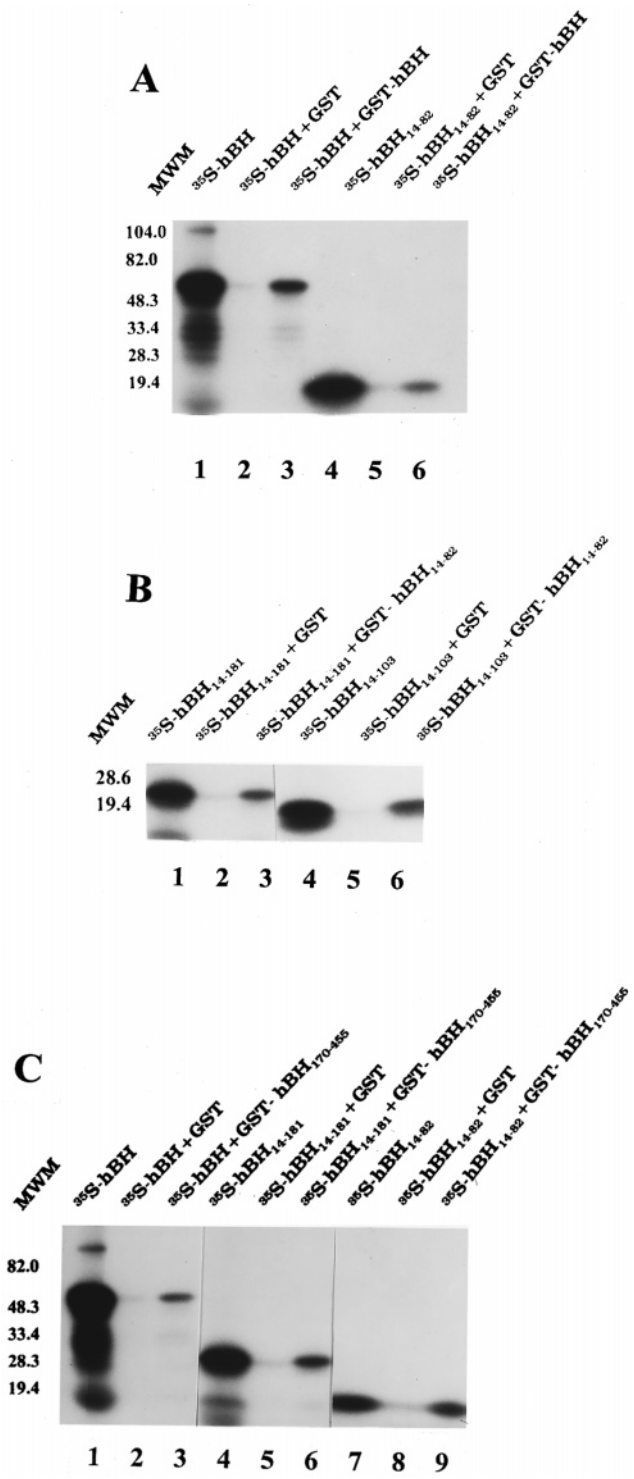

FIGURE 3: *In vitro* dimerization of ^{35}S-labeled hBH or mutants with GST-hBH immobilized on glutathione-agarose. (A) In vitro binding of ^{35}S-labeled full-length hBH or hBH_{14-82} to GST-hBH linked to glutathione-agarose. ^{35}S-labeled full-length hBH or hBH_{14-82} was transcribed and translated, and the resulting products were analyzed by 12% SDS PAGE (lanes 1 and 4). The radioactive mixtures were incubated with glutathione-agarose that either contained GST (lanes 2 and 5) or GST-hBH (lanes 3 and 6). (B) In vitro binding of ^{35}S-labeled hBH_{14-181} or hBH_{14-103} to GST-hBH_{14-82} linked to glutathione-agarose. (C) In vitro binding of ^{35}S-labeled full-length hBH, hBH_{14-181}, or hBH_{14-82} to GST-$hBH_{170-455}$.

reaction was stopped by adding 40 μL of methanol and 10 μL of 7.5 mM $CuSO_4$, and the resulting supernatant fractions were injected onto a C_8 reverse-phase HPLC column (3.9 mm $\times$ 150 mm; 5 μm particle size, Waters Chromatography, Milford, MA). Bleomycin A_2 and dA_2 were eluted at 1 mL/min with 17% methanol, 7.2% acetonitrile, 0.8% acetic acid, 2 mM heptanesulfonic acid, and 25 mM triethylamine (pH 5.5) and were detected by absorbance at 292 nm. BH activity was defined as the amount (μmoles) of bleomycin A_2 converted into dA_2 per minute.

Assay for Cysteine Proteinase Activities. The assay was described previously (*11*). The His-tagged hBH and deletion mutants were used at a concentration of 1 μg/mL. The proteins were diluted to 500 μL with 0.1% Brij 35 solution (Sigma) and added to 250 μL of buffer (352 mM KH_2PO_4, 48 mM Na_2HPO_4, 10 mM dithiothreitol). The assays were started by adding 250 μL of a 25 μM stock solution of the amino acid methyl coumarylamide (AMC) substrate (Bachem Bioscience Inc., King of Prussia, PA) in 0.1% Brij 35, and the mixtures were incubated for 15 min at 37 °C. We determined aminopeptidase activity using amino-methyl-citrulline-AMC (H-Cit-AMC) and amino-methyl-methionine-AMC (H-Met-AMC) as substrates and endopeptidase activity using the N-protected AMC benzyloxycarbonylphenylalanine-arginine-arginine-AMC (Z-Arg-Arg-AMC), benzyloxycarbonylphenylalanine-arginine-glutamic acid-lysine-arginine-AMC (Z-Arg-Glu-Lys-Arg-AMC), and benzyloxycarbonylphenylalanine-glycine-proline-arginine-AMC (Z-Gly-Pro-Arg-AMC) (*6*). The reactions were stopped with 500 μL of 100 mM sodium monochloroacetate in 100 mM sodium acetate (pH 4.3). The fluorescence of the liberated aminomethylcoumarin was measured in a Perkin-Elmer fluorimeter at an excitation wavelength of 370 nm and an emission wavelength of 460 nm. Product formation was calculated from a standard curve of 7-amino-4-methylcoumarin fluorescence. All reactions were linear with time. Some reactions were preincubated for 15 min with 50 or 250 μM E-64, a known inhibitor of BH (*3*, *6*) and aminopeptidase or endopeptidase activity determined as above. Cysteine proteinase activity was defined as the amount (pmoles) of aminomethylcoumarin released from the substrate per minute. For kinetic measurements we used substrate concentrations between 50 nM and 1 mM.

RESULTS

Yeast Two-Hybrid System. hBH is a 455 amino acid protein with three predicted catalytic amino acids: Cys^{73}, His^{372}, and Asn^{392} (Figure 1A). Previous studies suggest BH forms homomeric structures in vitro, with dimers being a key unit (*14*). To examine for possible hBH self-association within cells, we cloned a cDNA sequence, encoding the 455 amino acid full-length hBH into pAS2−1 (pAS2−1hBH) and pACT2 (pACT2hBH) expression vectors, fusing this protein to the DNA-binding (BD), and activating domains (AD) of GAL4, respectively. Yeast Y190 strain was cotransformed with pAS2−1hBH and pACT2hBH, and His+ colonies were examined for expression of the *lacZ* reporter gene. When two hBH fusion proteins were expressed in yeast, β-galactosidase activity was readily detected using a quantitative filter assay. This interaction was specific because the production of any of the two fusion proteins in combination with an irrelevant protein, such as p53, or the binding domain of GAL4 was unable to induce β-galactosidase activity (data not shown). We also found that hBH did not bind to the GAL4 upstream activating sequence in the yeast two-hybrid system, consistent with previous reports that hBH, unlike

yBH, did not bind to DNA (*6*). These results permitted us to exploit the yeast two-hybrid system for further analysis of hBH interactions.

To identify hBH protein partners, we cotransformed yeast Y190 strain with pAS2−1hBH as a target and HeLa cDNA library cloned into the activating domain vector pGADgh. Among the >15 true positives that we will describe elsewhere, one was a truncated version of hBH without the first 13 amino acids: pGADghΔBH. Previous structural studies by others suggested the 50 amino acids in the N-terminus of yeast BH comprise an oligomerization or hook domain (*14*). Nonetheless, there is only 10% identity in the N-terminal region of hBH and yeast BH. Consequently, predicted domains deduced from the yeast crystal structure may not necessarily apply to hBH. To define further the amino acid sequences important for homodimerization of hBH, we constructed various C-terminal truncations of ΔBH (Figure 2A). The smallest C-terminal deletion of ΔBH that dimerized with hBH was hBH_{14-82}. We therefore examined the interactions of hBH_{14-82} with other domains of hBH (Figure 2B). hBH_{14-82} associated with hBH_{1-175} but not with $hBH_{195-357}$ or hBH_{1-54} (Figure 2B), localizing the probable region for binding between amino acids 55 and 175. In addition to the N-terminal interaction with the amino terminal end, we also observed an interaction with the C-terminus. hBH_{1-54} interacted with full-length hBH and hBH_{14-455} but not with hBH_{14-289} and $hBH_{106-357}$ (Figure 2C) as well as a number of other fragments spanning the internal portions of residues 14−357 (data not shown). We also performed quantitative analysis using liquid β-galactosidase assays (data not shown) that fully confirmed the qualitative data. These results documented an interaction between residues 1−54 and 358−455. Interestingly, this region, namely, amino acids 382−410, is highly conserved among members of the cysteine proteinase family and contains two of the active residues: His^{372} and Asn^{392}. Therefore, the N-terminus (hBH_{1-82}) bound to two separate nonoverlapping domains on hBH: one located on the N-terminus (hBH_{55-175}) and the second on the C-terminus of the molecule ($hBH_{358-455}$).

hBH Interactions in Vitro. The interactions between hBH and the deletion mutants were examined further using in vitro binding assays and GST fusion proteins. Deletion mutants were made based on yeast two-hybrid results, optimal primer designs, and predicted catalytic regions. The in vitro translated ^{35}S-labeled hBH used in our studies migrated on PAGE at the predicted 52 kD range, although we also noted a band at approximately twice the mass that we suspect is a dimer and a number of lower molecular weight protein products that have not been defined (Figure 3A, lane 1). When ^{35}S-hBH was incubated with GST-hBH immobilized on glutathione-Sepharose beads, washed, and then eluted, we found only a single radioactive band on PAGE that comigrated with authentic hBH (Figure 3A, lane 3). No radioactive hBH binding was observed with glutathione-Sepharose beads containing only GST (Figure 3A, lane 2). The ^{35}S-hBH_{14-82} deletion mutant migrated at the expected 10 kD range and bound specifically to GST-hBH beads (Figure 3A, lanes 4−6). We next synthesized ^{35}S-hBH_{14-181} and ^{35}S-hBH_{14-103}, which migrated on PAGE at approximately 18 and 23 kD, respectively (Figure 3B, lanes 1 and 4). Both ^{35}S-hBH_{14-181} and ^{35}S-hBH_{14-103} bound to GST-hBH_{14-82} immobilized on glutathione-Sepharose beads (Figure 3B, lanes 3 and 6), which supported and extended the yeast two-hybrid results (Figure 2B), suggesting hBH_{14-82} interacted with at least one region between amino acids 14 and 103. hBH_{14-82} did not interact with GST-hBH_{14-82} (data not shown). In addition to the interactions between the N-terminal regions, we also confirmed in vitro interactions between the N-terminus and domains closer to the C-terminus, namely, $hBH_{170-455}$. Thus, we found $hBH_{170-455}$ bound specifically with not only hBH but also hBH_{14-181} and hBH_{14-82} (Figure 3C, lanes 3, 6, and 9). Because of nonspecific binding of ^{35}S-$hBH_{170-455}$ to GST, we were unable to test if it interacted with itself. Collectively, therefore, two-hybrid and in vitro binding assays identified interactions between the N-terminal region of hBH, namely, hBH_{1-82}, with two nonoverlapping parts of hBH: one located close to the N-terminus (hBH_{14-103}) and the second on the C-terminus of hBH ($hBH_{358-455}$). Obviously, we cannot exclude multiple sites within these regions. Nonetheless, hBH_{14-82} contains the catalytic cysteine (Cys^{73}) and sequences between amino acids 60 and 80, which are highly conserved among all enzymes that belong to the papain superfamily. BH is unusual among cysteine proteases because of its propensity to form higher order homomeric structures. We do not know if the binding domains we have defined here are important for intra- or intermolecular interactions.

Gel Filtration. To address whether the N- or C-terminus regions were important for oligomerization of hBH, we determined the molecular mass of ^{35}S-hBH and three deletion mutants, ^{35}S-ΔBH, ^{35}S-hBH_{14-415}, and ^{35}S-hBH_{14-357}, by gel filtration on a Superose 12 column. As shown in Figure 4, ^{35}S-hBH and ^{35}S-ΔBH eluted as a single peak of molecular mass 235 kD corresponding to tetramer. Similar results were seen with PAGE (data not shown). These results suggest that first 13 amino acids are not essential for the higher order structure formation of hBH. Deletion of either 98 or 40 amino acids from the C-terminus resulted in the loss of tetramer formation, with only the monomer being seen (Figure 4D,E). To test the significance of the C-terminus for catalytic function of hBH, we made four C-terminal mutants. hBH_{1-446} and hBH_{1-437} are deletion mutants without the last 9 and 18 amino acids, respectively. In hBH_{1-447t} and hBH_{1-437t} the last 8 and 18 amino acids, respectively, were replaced with unrelated amino acids that are not specifically basic or acidic. The recombinant proteins were expressed as His-tagged proteins in bacteria and affinity purified, and the protein concentration was estimated on Coomassie blue stained gels using a known concentration of bovine serum albumin (Sigma) as the standard. The expression of the proteins was also established with Western blotting using AntiXpress and T7 antibodies and suggested the hBH was the primary protein expressed. We determined the molecular mass of the deletion mutants and replacement mutants by gel filtration on a Superose 12 column and found that they retained the hBH oligomer structure (Figure 5). Similar results were seen by PAGE (data not shown). These results also support the notion that the addition of tagging amino acids on the N-terminus does not disrupt the ability of hBH to oligomerize.

Bleomycin Hydrolase and Cysteine Proteinase Activities of hBH and Deletion Mutants. hBH and deletion mutants were tested for their ability to metabolize bleomycin A_2 to

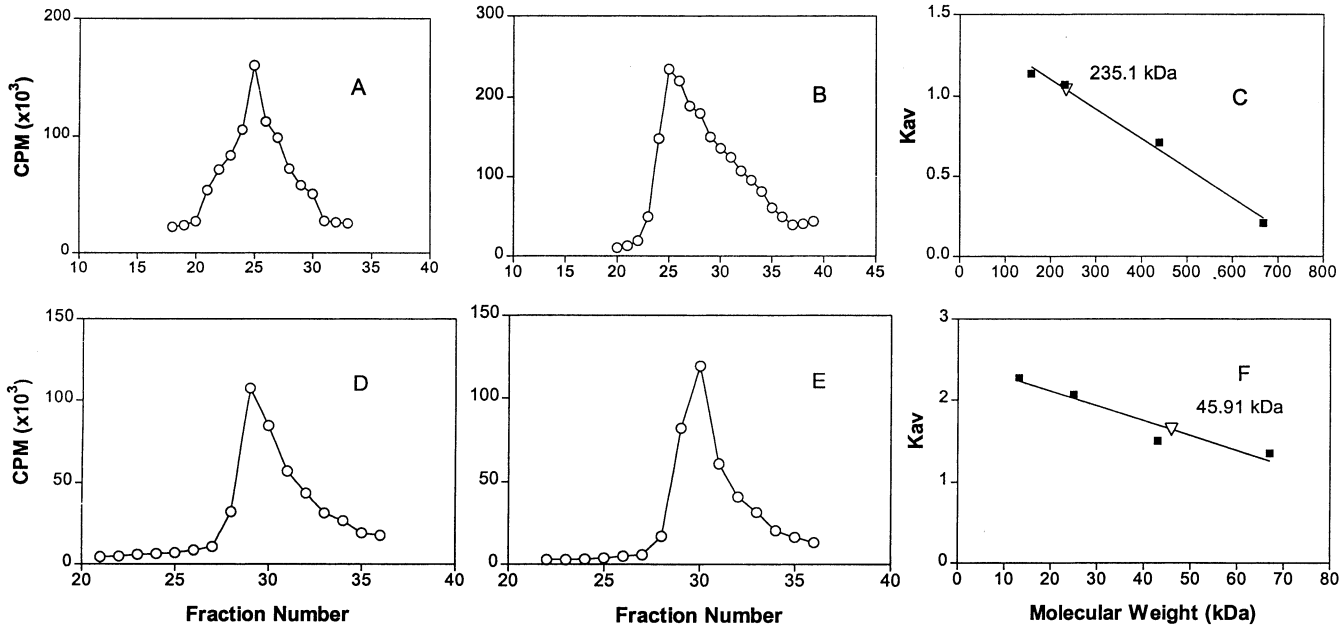

FIGURE 4: Molecular mass determination of full-length and truncated hBH mutants by gel filtration. ^{35}S-labeled hBH, ΔBH, hBH_{14-415}, and hBH_{14-357} were analyzed by a Superose 12 HR 10/30 column as described under the Materials and Methods section. The elution profile of (A) ^{35}S-labeled hBH and (B) ^{35}S-labeled ΔhBH. Determination of (C) ^{35}S-labeled hBH mass. The elution profile of (D) ^{35}S-labeled hBH_{14-357} and (E) ^{35}S-labeled hBH_{14-415}. Determination of (F) ^{35}S-labeled hBH_{14-415} mass. The molecular mass markers are noted. $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume for the protein, V_0 is the column void volume, and V_t is the total bed volume.

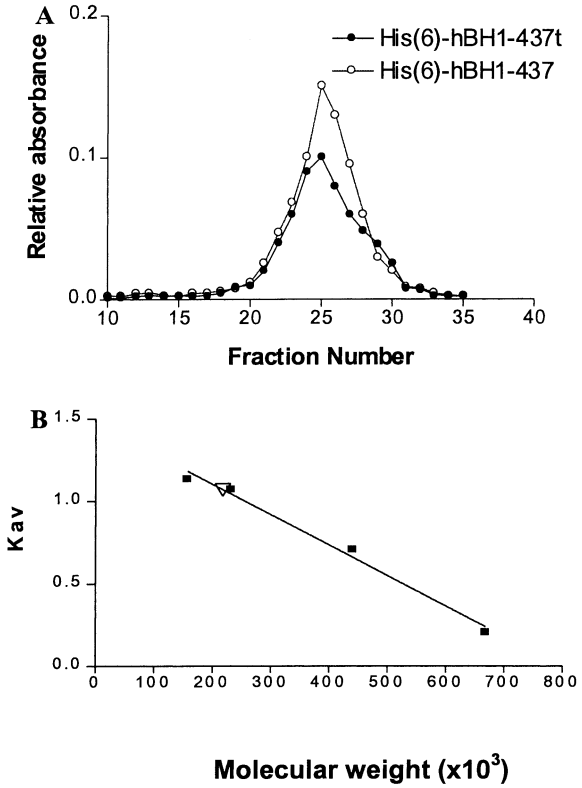

FIGURE 5: Molecular mass determination of replacement and deletion hBH mutant by gel filtration. His(6)-tagged fusion proteins were analyzed by a Superose 12 HR 10/30 column as described in the Materials and Methods section. The elution profiles of (A) His-(6)-hBH_{1-437t} (●) and His(6)-hBH_{1-437} (○). (B) Determination of molecular mass (▽). The mass was calculated as in Figure 4.

its inactive metabolite bleomycin dA_2 by HPLC (Figure 6). Bleomycin dA_2 eluted approximately 2 min earlier than the parent compound (Figure 6A,B). hBH metabolized more than 90% of bleomycin A_2 to dA_2 after a 120 min incubation. Incubation for much shorter periods allowed us to calculate the enzymatic activity during the linear phase of metabolism, and it was 0.52 μmol/min. The deletion of the last 8 or 18 amino acids on the C-terminus completely abolished BH activity whether the incubation was for 2 h (Figure 6C,D) or 10 h (data not shown). We also saw no metabolism of bleomycin A_2 using the hBH_{1-447t} or hBH_{1-437t} mutants (data not shown).

We next examined whether the amino or carboxyl termini were critical for cysteine proteinase activity of the enzyme. Previous studies (*6*) suggested hBH had aminopeptidase but not endopeptidase activity. We found significant hydrolysis of H-Cit-AMC [$K_{cat} = 101.7 \pm 5.3$ s^{-1} and $K_m = 14.3 \pm 3.1$ μM) and H-Met-AMC ($K_{cat} = 70.7 \pm 3.7$ s^{-1} and $K_m = 15.2 \pm 3.7$ μM) by hBH (Table 1). hBH also had low but detectable endopeptidase activity against N-blocked substrates that varied when different substrates were used. The highest endopeptidase activity was found against Z-Arg-Arg-AMC ($K_{cat} = 41.2 \pm 5.1$ s^{-1} and $K_m = 89.4 \pm 8.1$ μM) corresponding to catalytic efficiency 10-fold more than against Z-Arg-Glu-Lys-Arg-AMC ($K_{cat} = 11.7 \pm 1.7$ s^{-1} and $K_m = 250 \pm 20$ μM). hBH_{1-446} and hBH_{1-437}, which lacked the last 8 or 18 amino acids, had remarkably diminished aminopeptidase activity but retained endopeptidase activity against the Z-Arg-Arg-AMC and Z-Arg-Glu-Lys-Arg-AMC substrates (Table 1). The replacement of the lost amino acids with irrelevant amino acids did not restore catalytic activity. Preincubation with 50 μM of the cysteine protease inhibitor E-64 resulted in >50% inhibition of both aminopeptidase and endopeptidase activities, and >90% of both activities were inhibited with 250 μM.

DISCUSSION

Phylogenetically BH-like enzymes are ubiquitous. Moreover, BHs are unique among cysteine proteases because they form homomeric structures. Studies of the bacterial and

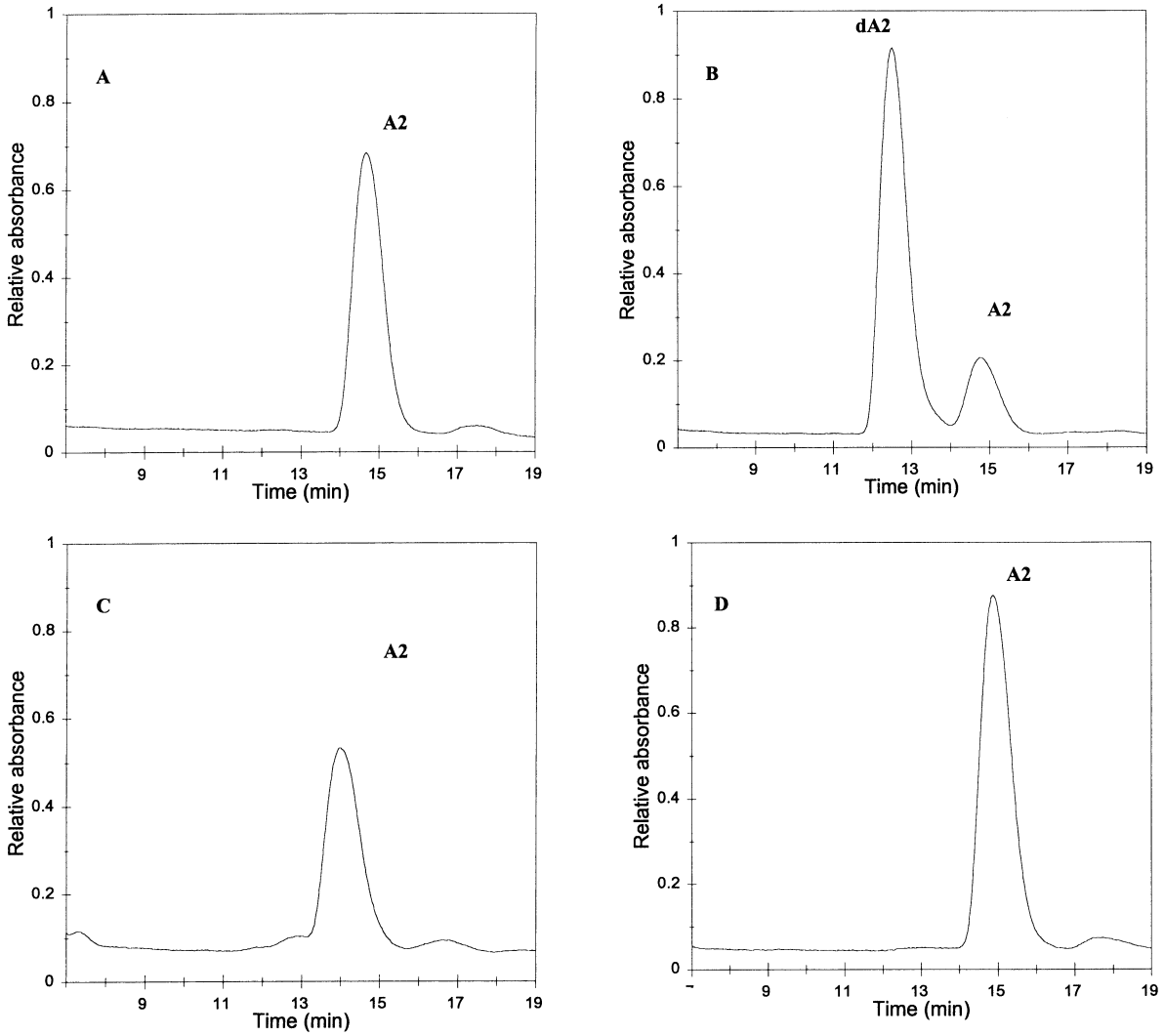

FIGURE 6: Bleomycin hydrolase activity. His-tagged hBH proteins were purified and incubated with bleomycin A_2 for 2 h. The amount of bleomycin A_2 metabolized to bleomycin dA_2 was determined by HPLC as described under the Materials and Methods section. (A) No enzyme. (B) hBH. (C) hBH_{1-446}. (D) hBH_{1-437}.

Table 1: Comparison of Aminopeptidase and Endopeptidase Activities of HBH and Deletion Mutants

	cysteine proteinase activity[b]				
proteins[a]	H-Cit-AMC	H-Met-AMC	Z-Arg-Arg-AMC	Z-Arg-Glu-Lys-Arg-AMC	Z-Gly-Pro-Arg-AMC
HBH	189.75 ± 1.9	94.5 ± 2.0	8.4 ± 0.2	1.9 ± 0.0	7.2 ± 0.3
hBH_{1-447t}	5.5 ± 0.5	5.7 ± 0.03	4.9 ± 0.4	0.9 ± 0.0	2.7 ± 0.1
hBH_{1-446}	3.7 ± 0.1	4.9 ± 0.4	7.09 ± 0.4	1.8 ± 0.0	4.5 ± 0.1
hBH_{1-437t}	4.7 ± 0.1	4.2 ± 0.1	3.6 ± 0.3	0.7 ± 0.0	2.5 ± 0.3
hBH_{1-437}	2.3 ± 0.0	3.0 ± 0.0	6.0 ± 0.4	1.1 ± 0.0	3.0 ± 0.1

[a] His-tagged hBH, hBH_{1-446}, hBH_{1-447t}, hBH_{1-437}, and hBH_{1-437} were purified, and their ability to hydrolyze H-Cit-AMC, H-Met-AMC, Z-Arg-Arg-AMC, Z-Arg-Glu-AMC, and Z-Gly-Pro-Arg-AMC was determined as described under the Materials and Methods section. [b] Cysteine proteinase activity is defined as the amount (pmol) of aminomethylcoumarin released from the substrate per minute. Data represent the mean ± standard error of three experiments.

yeast BH suggest it can assume both a tetrameric and a hexameric form (*14*). Chicken aminopeptidase H, which has >75% identity with hBH, has been reported to be an octamer (*16*). Brömme et al. (*6*) found hBH assumed a hexameric form, while we, using the same cDNA, have now observed a tetrameric form of hBH in vitro. Brömme et al. (*6*), however, used recombinant protein purified from S*f* 9 cells, while we estimated the protein mass of protein prepared by in vitro transcription and translation with rabbit reticulocyte lysate. We do not yet know what factors determine whether BH adopts one particular form or which is preferred within cells, but clearly this issue is worthy of further study. Nevertheless, these results collectively suggest the fundamental unit of BH is a dimer. In support of this hypothesis Joshua-Tor et al. (*14*) proposed extensive dimer interactions from the crystal structure of yBH and argued the observed hexamer comprises a trimer of dimers. Our results with the yeast two-hybrid system also are consistent with a dimer forming in vivo. In contrast to yBH, however, we did not observe any interactions between hBH and the Gal4 regulatory system. This is in agreement with the result of Brömme et al. (*6*), who did not observe DNA binding with hBH.

Because of the extensive differences in the primary amino acid sequence between yeast BH and hBH as well as the possible oligomer state, we examined the hBH structure in an unbiased manner initially using a yeast two-hybrid approach. Among the several proteins that interacted with hBH in the yeast two-hybrid system, we found an N-terminal truncated form of hBH. Using both two-hybrid and in vitro binding assays, we identified a region on the N-terminus of hBH, namely, hBH_{1-82}, that interacted with two nonoverlapping parts of hBH located on the N-terminus (hBH_{14-103}) and C-terminus of hBH ($hBH_{358-455}$). The contact of hBH_{1-82} with the C-terminus, which contains the two other catalytically essential amino acids, His^{372} and Asn^{396}, might be necessary for formation of the active site cleft or oligomerization of hBH.

The aminopeptidase activity of hBH is well-known, but only chicken aminopeptidase H has established endopeptidase activity (*16*). The absence of endopeptidase activity in yBH was explained by the projection of the carboxyl terminus into active site cleft of yBH, thus limiting substrate access (*14*). Therefore, our observation that hBH had endopeptidase activity is significant and supported by initial reports that truncated yeast BH may also have some endopeptidase activity (*23*). The amino- and endopeptidase enzymatic activities of hBH appeared to be regulated differently. There is a highly conserved amino acid sequence in hBH that was not found in any other cysteine proteases reported to date and which was shared by all BH-like enzymes, namely, the BHYD (Figure 1A). Loss of BHYD resulted in a sharp decrease in aminopeptidase activity but not in endopeptidase activity, suggesting that the C-terminus may play a role in the substrate binding. The reduced aminopeptidase activity was not associated with a loss of oligomerization, although deletion of a much larger portion of hBH that comprised 40 amino acids did destroy oligomer formation. Moreover, Pei and Sebti (*11*) proposed that oligomerization was not essential for bleomycin metabolism using yBH. Finally, replacement of the deleted amino acids with irrelevant amino acids did not restore activity, thus excluding reduced size as a cause.

In summary, we have shown that (a) BH can form higher order structures in vivo; (b) hBH can form tetramers in vitro; (c) several sites of homomeric interactions exist; (d) the BHYD is important for aminopeptidase activity; (e) hBH has endopeptidase activity; and (f) the C-terminus is important for oligomerization. This information could help facilitate the design of specific inhibitors and lead to a better understanding of the biological function of BH-like enzymes.

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