Cys[102] and His[398] Are Required for Bleomycin-Inactivating Activity but Not for Hexamer Formation of Yeast Bleomycin Hydrolase[†]

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ABSTRACT: The bleomycin-inactivating enzyme, bleomycin hydrolase, is believed to be involved in tumor resistance to the anticancer drug bleomycin. This homohexamer is an aminopeptidase that shows homology to cysteine proteinases around the cysteine and histidine active site. The role that these residues play in hydrolyzing bleomycin and in hexamer oligomerization of bleomycin hydrolase is not known. In this study, the yeast bleomycin hydrolase gene was expressed in *Escherichia coli*, and site-directed mutagenesis was employed to precisely investigate the roles of the conserved Cys[102] and His[398] residues in its structure and enzymatic activity. Three mutants were created, in which Cys[102] was replaced by arginine or serine, and His[398] was changed to glycine. The ability of bleomycin hydrolase to oligomerize was neither affected by the subtle cysteine/serine mutation nor affected by cysteine/arginine or histidine/glycine mutations. However, the ability of bleomycin hydrolase to hydrolyze and inactivate bleomycin was totally abolished in all three mutants, suggesting that the cysteine thiol and histidine imidazole are critical for hydrolyzing bleomycin. Furthermore, in contrast to predictions from the recently reported crystal structure of this enzyme, hexamer formation is not required for the enzymatic activity of bleomycin hydrolase. Thus, these results demonstrate that Cys[102] and His[398] are required for bleomycin hydrolase activity but not hexamer formation, and that both monomer and hexamer are active forms of bleomycin hydrolase.

Bleomycins are a family of antitumor antibiotics that are used to treat human cancers, such as testicular carcinomas, lymphomas, and certain types of squamous cell carcinomas (Carter, 1985; Lazo et al., 1987; Lazo & Sebti, 1989). Bleomycins are unique anticancer drugs since they lack side effects such as liver, heart, kidney, and bone marrow toxicities that are usually associated with other antineoplastic agents. However, one of the major obstacles in the use of bleomycins as anticancer drugs is tumor resistance (Lazo & Sebti, 1989). It is believed that bleomycin hydrolase (BH),[1] a bleomycin-inactivating enzyme, is a major contributing factor in tumor resistance to bleomycin (Sebti et al., 1991; Pei et al., 1995). BH converts bleomycin to its inactive metabolite, deamidobleomycin, by hydrolyzing the carboxamide bond of the β-aminoalaninamide moiety to a carboxylic acid (Umezawa, 1979).

BH was originally identified by its ability to inactivate bleomycin in tumors and organs from mice treated with bleomycin (Umezawa, 1971; 1972). The enzyme was subsequently shown to be expressed in bacteria (Chapot-Chartier et al., 1994), yeast (Kambouris et al., 1992; Enenkel & Wolf, 1993; Magdolen et al., 1993; Xu & Johnston, 1994), birds, reptiles, and mammals (Sebti et al., 1989). The conservation throughout the evolutionary tree suggests an important function for this enzyme. Biochemical, molecular, and structural studies demonstrated that BH is a cytosolic homohexamer with a monomeric molecular mass around 50 kDa (Nishimura et al., 1987; Sebti et al., 1987; Joshua-Tor et al., 1995).

A partial-length BH cDNA was first cloned from a rabbit liver cDNA library (Sebti et al., 1989). Amino acid sequence alignment indicated that a 10-residue peptide within the BH sequence was highly homologous to a consensus sequence around the cysteine active site of cysteine proteinases such as cathepsins B, H, and L and papain (Sebti et al., 1989). Furthermore, BH exhibited cathepsin H but not cathepsin B and L enzymatic activity (Sebti et al., 1989). Subsequent to the cloning of the partial-length cDNA of mammalian BH, four independent groups isolated the full-length BH gene from yeast (Kambouris et al., 1992; Enenkel & Wolf, 1993; Magdolen et al., 1993; Xu & Johnston, 1994). The BH gene from bacteria was also recently cloned (Chapot-Chartier et al., 1994). Both the bacterial and yeast BH proteins have been crystallized (Mistou et al., 1994; Joshua-Tor et al., 1995).

Although, BH contains the conserved cysteine and histidine active site of cysteine proteinases, the role of these residues in the hydrolysis of bleomycin is not known. There are several features that distinguish BH from the cysteine proteinase family and that makes it intriguing to investigate the role of Cys[102] and His[398] in the bleomycin-inactivating activity and cysteine proteinase activity of yeast BH. First of all, cathepsin B, a typical cysteine proteinase, does not hydrolyze bleomycin (Sebti et al., 1989). Furthermore, the homology between BH and cysteine proteinases is restricted to 10 and 6 amino acids around the active site cysteine and

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[1] Abbreviations: BH, bleomycin hydrolase; GST, glutathione *S*-transferase; PCR, polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; YCP1, yeast cysteine proteinase.

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Table 1: PCR Primers

primers		DNA sequences
1	sense	5′TCt TTG GAT CCA TGC TTC CTA CT3′
2	antisense	5′GCT GAG TCG ACT TAT TTG GCC AA3′
3(Arg)	antisense	5′ATT CAA GCG CAG TTG ATT GGT AGC GGC AAA CAA CCA AC**G** TCT ACC3′
4(Ser)	antisense	5′ATT CAA GCG CAG TTG ATT GGT AGC GGC AAA CAA CCA AC**T** TCT ACC3′
5(Gly)	sense	5′GTA TTA GGT ACC ATG AAA GTT TGA TGA CT**G G**TG CTA TG3′
6	antisense	5′ACT TTC ATG GTA CCT AAT ACG3′

histidine, respectively. BH also contains a substantially longer stretch of 296 amino acids between the active site cysteine and histidine as compared to 135−165 amino acids in the papain-like cathepsin family (Dufour, 1988). Differences in the pH optimum which is neutral for BH and acidic for cathepsins as well as the fact that BH but not cysteine proteinases oligomerize into a homohexamer also prompted us to investigate the role of Cys^{102} and His^{398} in the hydrolysis of bleomycin. In the present study, we report the functional expression of the yeast BH in *E. coli*, and site-directed mutagenesis of the yeast BH to precisely investigate the roles of the highly conserved cysteine and histidine residues in its structure and enzymatic activities.

EXPERIMENTAL PROCEDURES

Plasmid Constructs and Site-Directed Mutagenesis. The yeast cysteine proteinase (YCP1) gene *ycp1* (Kambouris et al., 1992) was used in this study. The primers used for cloning of the wild-type and mutated YCP1 proteins were ordered from GIBCO BRL Custom Primers. The entire coding region of *ycp1* from plasmid pUT533a-ycp1 (Pei et al., 1995) was amplified by the polymerase chain reaction (PCR), and subcloned into the bacterial vector pGEX-4T-1 which contains the entire coding sequence for GST (Pharmacia, Piscataway, NJ). The two primers used, primers 1 and 2 as shown in Table 1, incorporated a *Bam*HI restriction site immediately 5' to the initiation codon and a *Sal*I restriction site immediately 3′ to the termination codon, respectively (Figure 1). Following PCR, the amplified fragment was digested with *Bam*HI and *Sal*I, isolated, and ligated into the *Bam*HI−*Sal*I site on digested pGEX-4T-1 vector. The *ycp1* gene was expressed as a fusion protein to GST which is under the control of the *tac* promoter (Smith & Johnson, 1988).

Site-directed mutagenesis was also carried out by PCR. For mutant C102R, in which Cys^{102} was replaced by arginine, the mutation was created by designing an arginine mutation primer (primer 3 in Table 1, the single point mutation is shown in *boldface*) with a unique *Cfo*I restriction site (Figure 1). Primer 3 corresponds to nucleotides 952−996 in *ycp1* where nucleotide C was substituted for nucleotide T at position 958 to create the cysteine/arginine mutation. The PCR-amplified primer 1/primer 3 fragment (using the wild-type *ycp1* plasmid as a template) was digested with *Bam*HI and *Cfo*I, and then, along with the *Cfo*I-*Sal*I fragment from the wild-type, was ligated into the pGEX-4T-1 vector. The mutant C102S, where the Cys^{102} was replaced with serine, was created by a similar strategy but using primer 1 and primer 4 (Table 1). Primer 4 corresponds to the same sequence of *ycp1* as primer 3 except that the nucleotide T at position 958 was replaced by nucleotide A. To make mutant H398G, the His^{398} was replaced with glycine. The mutation was prepared by introducing a new unique *Kpn*I restriction site, where the codon AGA (encodes Arg^{390}) was changed to AGG that still encodes arginine. Based on this, two primers were designed: primer 5 (Table 1), which corresponds to sequence 1817−1854 in *ycp1*, had the *Kpn*I restriction site and the glycine mutation (codon CAT to GGT), and the other primer (primer 6) had the *Kpn*I restriction site only (Table 1). The PCR-amplified primer 1 and 6 product digested with *Bam*HI and *Kpn*I and the PCR-amplified primer 5 and 2 product digested with *Kpn*I and *Sal*I were then ligated into the pGEX-4T-1 vector. The cloning and mutations were verified by sequencing (Automated DNA Sequencing Facilities, School of Medicine, University of Pittsburgh, PA).

Expression and Purification of YCP1 Proteins. The plasmids encoding wild-type and mutated YCP1 proteins were transformed into *E. coli* strain BL-21 (Pharmacia). Cells were grown in 2× YTA medium (tryptone 16 g/L, yeast extract 10 g/L, and NaCl 5 g/L) with ampicillin (100 μg/mL) at 37 °C. When grown to an absorbance of 1−1.2 (at 600 nm), cells were transferred to room temperature and incubated for 1 h. Isopropyl β-thio-D-galactopyranoside was then added at a final concentration of 0.08 mM to induce the expression of GST−YCP1 fusion proteins. Cells were grown for another 3 h at room temperature and then harvested.

The GST-fusing proteins were then purified by mildly sonicating cells and treating with Triton X-100 at a final concentration of 2% for 1 h to solubilize the fusion proteins. The supernatants were then collected and added to a 50% slurry of glutathione−sepharose 4B. The GST−YCP1 proteins were eluted by the addition of reduced glutathione buffer (Pharmacia). GST-free YCP1 proteins were obtained by adding the protease thrombin directly to the cell lysate/glutathione−sepharose 4B mixture, and the YCP1 proteins were eluted with PBS buffer (1.4 M NaCl, 27 mM KCl, 101 mM Na_2HPO_4, and 18 mM KH_2PO_4, pH 7.3). Proteolytic cleavage affording release of GST-free YCP1 could be accomplished because the pGEX-4T-1 vector contains a sequence between GST and YCP1 that encodes the cleavage site for thrombin.

Gel Electrophoresis. PAGE analysis was performed on a Bio-Rad system. Crude bacterial pellets and purified samples were analyzed by 10% SDS−PAGE. Gels were stained with Coomassie blue. For native PAGE, no SDS and other denaturants were used in gels and running buffers.

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) (Pei et al., 1995). Briefly, 2 μg of pure YCP1 or GST−YCP1 proteins was incubated with 60 μM bleomycin A_2 (Bristol-Myers Squibb Laboratories) in a 50 μL reaction buffer (20 mM Tris, pH 7.5) at 37 °C for 3 h. The reactions were stopped by adding 40 μL of methanol and 10 μL of 7.5 mM $CuSO_4$, and injected into a C_8 reverse-phase HPLC column (25 cm x 4.6 mm; 5 um particle size; Rainin Dynamax). The bleomycin A_2 metabolites 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. Bleomycin A_2 and dA_2 were detected by the absorbance at 292 nm.

Assays for Cysteine Proteinase Activities. The assays were described previously (Sebti et al., 1989). The pure YCP1 or GST−YCP1 proteins were diluted to 500 μL with 0.1% Brij 35 solution (Sigma) and added to 250 μL of reaction buffer (352 mM KH_2PO_4, 48 mM Na_2HPO_4, 4 mM Na_2-EDTA, and 8 mM dithiothreitol). The assays were started by adding 250 μL of the various substrates (dissolved in 20 μM in 0.1% Brij 35 solution) and incubating the mixtures for 30 min. For cathepsin H, the substrate used was arginine 4-methyl-7-coumarylamide (Sigma) at 30 °C, and the pH for the reaction buffer was 6.8. Benzyloxycarbonylphenylalanylarginine 4-methyl-7-coumarylamide was used as a substrate for cathepsin L at 30 °C and pH 5.5, whereas benzyloxycarbonylarginylarginine 4-methyl-7-coumarylamide was used for cathepsin B enzyme activity at 40 °C and pH 6.0. 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 fluorometer at an excitation wavelength of 370 nm and an emission wavelength of 460 nm. The picomoles of products generated by YCP1 or GST−YCP1 proteins was calculated from a standard curve of 7-amino-4-methylcoumarin fluorescence (Sebti et al., 1989).

Gel Filtration. The molecular mass of the oligomer was determined by gel filtration on a Superose 12 HR 12/30 column (Pharmacia). The column was equilibrated with PBS buffer. The molecular mass standards (Pharmacia) were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), and ribonuclease A (14 kDa). The blue dextran (200 kDa) was used for the void volume. Fractions (0.4 mL) were collected, and protein amounts were determined by the Bradford protein assay (Bradford, 1976) using Bio-Rad protein assay dye solution (Bio-Rad Laboratories, Hercules, CA).

RESULTS

Expression and Purification of Wild-Type and Mutated YCP1 Proteins in E. coli. The entire coding region of the yeast BH gene, *ycp1*, was cloned into the fusion protein vector pGEX-4T-1 as described under Experimental Procedures. In order to determine whether the conserved cysteine and histidine residues are involved in the catalysis associated with bleomycin hydrolase and cysteine proteinase activities of YCP1, we employed site-directed mutagenesis to mutate Cys^{102} either to a basic amino acid arginine or to the closely related serine (Figure 1). His^{398} was also mutated to glycine as described under Experimental Procedures and shown in Figure 1. The wild-type and mutated YCP1 proteins were expressed as GST fusion proteins in *E. coli* strain BL21. YCP1 proteins were purified from bacterial lysates by affinity chromatography using glutathione-conjugated Sepharose-4B beads. YCP1 proteins were cleaved from the GST by the protease thrombin, and their purity and molecular mass were checked by SDS−PAGE as described under Experimental Procedures. As shown in Figure 2, the purified wild-type and mutants all migrated as single bands with apparent molecular masses of 55 kDa. There were no significant differences in the expression level and yield among the wild-type and mutated YCP1 proteins.

Bleomycin Hydrolase and Cysteine Proteinase Activities of the Wild-Type and Mutated YCP1 Proteins. The expressed

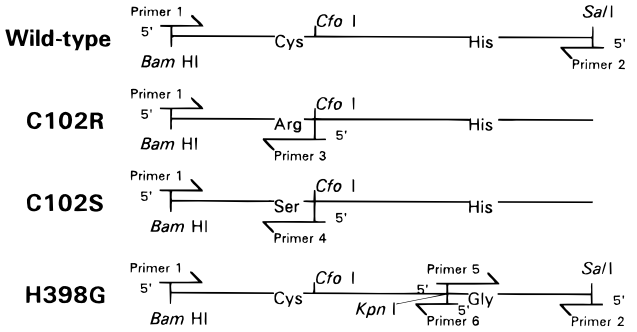

FIGURE 1: Schematic representation of cloning and site-directed mutagenesis (not to scale). Details are described under Experimental Procedures. All primers are listed in Table 1. Cys, cysteine[102]; His, histidine[398]; Arg, arginine; Ser, serine; Gly, glycine.

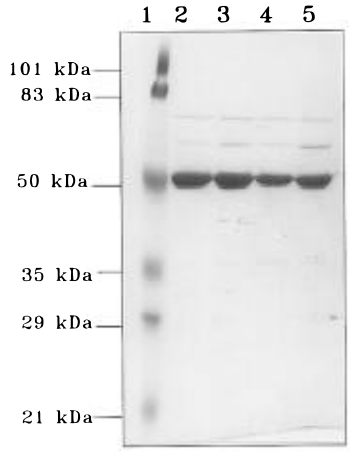

FIGURE 2: SDS−PAGE analysis of purified YCP1 proteins. Lane 1, molecular mass markers; lane 2, wild-type; lane 3, mutant C102R; lane 4, mutant C102S; lane 5, mutant H398G.

wild-type and mutated YCP1 proteins were first examined for their ability to metabolize bleomycin A_2 to its inactive metabolite dA_2. YCP1 proteins were purified to homogeneity (Figure 2) and incubated with bleomycin A_2. The reaction was then stopped, and bleomycin A_2 was separated from dA_2 by HPLC as described under Experimental Procedures. As shown in Figure 3A, wild-type YCP1 protein metabolized bleomycin to a great extent, resulting in the conversion of 61% of A_2 to dA_2 within a period of 180 min. Thus, functional expression of wild-type YCP1 protein with bleomycin hydrolase activity was achieved. However, replacement of Cys^{102} with its closely related amino acid serine which resulted in substitution of a thiol by a hydroxyl resulted in total abolishment of bleomycin hydrolase activity of YCP1 (Figure 3C). Mutation of Cys^{102} to arginine also abolished bleomycin hydrolase activity (Figure 3B). Similarly, mutation of His^{398} to glycine which resulted in substituting the imidazole for a hydrogen also abolished bleomycin hydrolase activity (Figure 3D). These results indicate that the cysteine thiol and the histidine imidazole play a key role in the bleomycin hydrolase catalytic activity of YCP1 protein.

We next determined whether Cys^{102} and His^{398} were also critical for the cysteine proteinase activity of YCP1. Previously, we had shown that rabbit lung BH is able to cleave the substrate of cathepsin H but not that of cathepsins B or L (Sebti et al., 1989). Here we demonstrated that the yeast BH homologue, YCP1, also had cathepsin H but not cathepsins B or L activity (Table 2). This cathepsin H

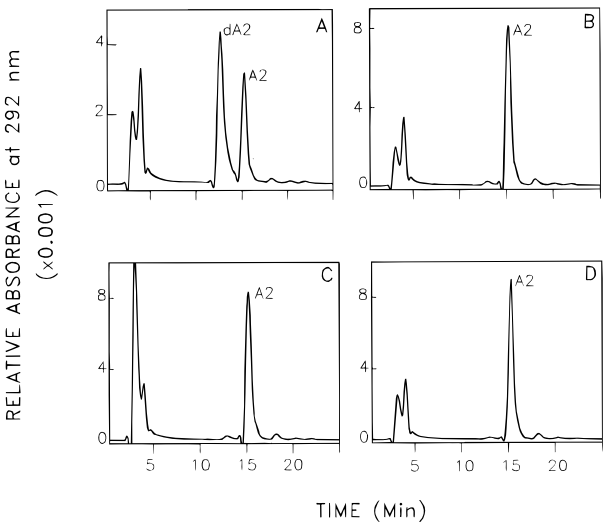

FIGURE 3: Bleomycin hydrolase activity. YCP1 proteins were purified and incubated with bleomycin A_2. The amount of bleomycin A_2 metabolized to bleomycin dA_2 was determined by HPLC as described under Experimental Procedures. A, Wild-type; B, mutant C102R; C, mutant C102S; D, mutant H398G. Data are representative of three independent experiments.

Table 2: YCP1 Cysteine Proteinase Activities

Ycp1 proteins[a]	cysteine proteinase activity[b]					
	cathepsin B		cathepsin H		cathepsin L	
	expt 1	expt 2	expt 1	expt 2	expt 1	expt 2
wild-type	0.96	1.10	107.33	101.00	0.50	0.67
C102R	0.20	0.20	0.20	0.20	0.73	0.56
C102S	0.20	0.20	0.20	0.20	0.53	0.56
H398G	0.20	0.20	0.28	0.36	0.57	0.56

[a] Wild type and mutated YCP1 proteins were purified, and their ability to hydrolyze cathepsin B, H and L substrates was determined as described under Experimental Procedures. [b] Cysteine proteinase activity is defined as the amount (picomoles) of aminomethylcoumarin released from the substrates per minute (pmol min^{-1}). The activity of no enzyme controls varied between 0.1 and 0.3 pmol min^{-1}.

activity was also abolished by the cysteine and histidine mutations (Table 2).

Oligomerization of the Wild-Type and Mutated YCP1 Proteins. Based on recent crystal structure studies, the yeast BH was shown to be a hexamer (Joshua-Tor et al., 1995). In order to determine whether YCP1 protein could still form a hexamer when expressed in bacteria, and whether the cysteine and histidine mutations had any effect on YCP1 oligomerization, the molecular mass of native wild-type and mutated YCP1 proteins was determined. YCP1 proteins were purified and loaded onto a native polyacrymide gel as described under Experimental Procedures. Figure 4 shows that the wild-type and the three mutants all migrated as a single band of about 330 kDa. Figure 4 coupled with Figure 2 which shows a 55 kDa monomer under denatured conditions indicates that these proteins are hexameric in nature. Their molecular mass was further examined by gel filtration on a Superose 12 column. As shown in Figure 5, the wild-type YCP1 protein, as well as the mutants, eluted as a single peak of molecular mass of about 330 kDa. The results suggest that the cysteine and histidine residues have no effect on YCP1 oligomerization, even in the case of the C102R mutant where the cysteine was mutated to the positively charged arginine.

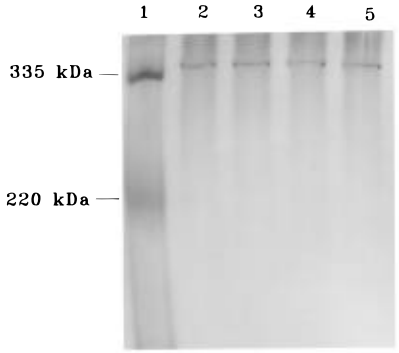

FIGURE 4: Native PAGE analysis of purified YCP1 proteins. The YCP1 protein were run on a 7% native acrymide gel overnight as described under Experimental Procedures. Lane 1, molecular mass markers; lane 2, wild-type; lane 3, mutant C102R; lane 4, mutant C102S; lane 5, mutant H398G.

Oligomerization Is Not Required for Bleomycin Hydrolase and Cysteine Proteinase Activities of YCP1 Protein. We next determined whether the oligomerization state was required for the enzymatic activities of the wild-type and mutant YCP1 proteins fused to GST. GST−YCP1 fusion proteins were purified from bacterial lysates as described under Experimental Procedures except that the GST was not cleaved off by thrombin. SDS−PAGE analysis of these GST−YCP1 proteins indicated that they all had, under denatured conditions, an apparent molecular weight of 84 KDa (data not shown). This is consistent with a fusion protein made of YCP1(55 kDa) and GST (29 kDa). In contrast to the GST-free YCP1 protein which formed a hexamer under native conditions, the GST−YCP1 fusion protein did not. Figure 5e,f shows that the wild-type GST−YCP1 eluted from the Superose 12 gel filtration column with a molecular mass of 84 kDa. Thus, the wild-type GST−YCP1 fusion protein eluted from the Superose 12 column as a monomer. The three GST−YCP1 mutants also eluted as monomers (data not shown). We next determined whether the monomer form of YCP1 could still carry out bleomycin hydrolase and cysteine proteinase activities. The GST protein alone had no bleomycin hydrolase and cysteine proteinase activities (data not shown). Figure 6A shows that the bleomycin hydrolase specific activity of the wild-type GST−YCP1 protein was similar to that of wild-type YCP1 protein (Figure 3A). Figure 6B shows that the cathepsin H specific activity of wild-type YCP1 and GST−YCP1 proteins was also similar. The results indicate that oligomerization is not required for both enzymatic activities of YCP1.

DISCUSSION

Biochemical, molecular, and structural studies showed that the yeast BH contains the cysteine and histidine active sites of the papain-like family of enzymes, papain, actinidin, calotropin, and cathepsins B, H, and L (Joshua-Tor et al., 1995). These cysteine proteinases are monomers (Baker, 1980; Heinemann et al., 1982; Kamphuis et al., 1984; Musil et al., 1991), but all BHs found so far are hexamers (Nishimura et al., 1987; Sebti et al., 1987; Mistou et al., 1994; Joshua-Tor et al., 1995). Furthermore, the role of the conserved cysteine and histidine in the hydrolysis of bleomycin by BH is not known. Therefore, the understanding of the relationship among the catalytic domain, the hexamer structure, and the enzymatic activities of BH is of great

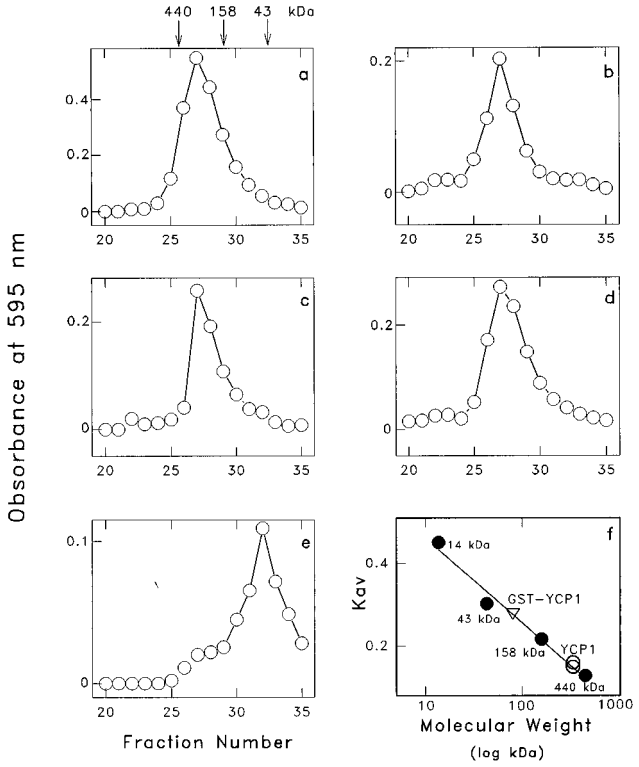

FIGURE 5: Molecular weight determination by gel filtration. Wild-type and mutated YCP1 proteins were purified and analyzed by a Superose 12 column as described under Experimental Procedures. a, The elution profile of wild-type YCP1; b, mutant C102R; c, mutant C102S; d, mutant H398G; e, wild-type GST−YCP1; f, molecular weight determination. $K_{av} = (V_e - V_o)/(V_t - V_o)$, where V_e is the elution volume for the protein, V_o is the column void volume, and V_t is the total bed volume.

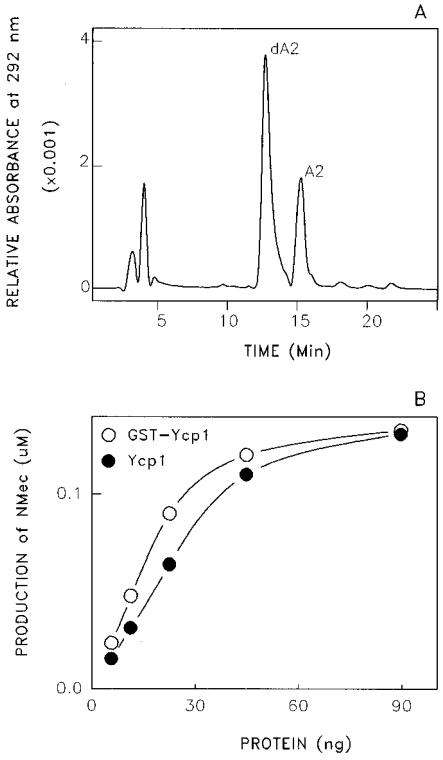

FIGURE 6: Enzymatic activities of the wild-type GST−YCP1 protein. A, Bleomycin hydrolase activity; B, cysteine proteinase (cathepsin H) activity. Data are representative of three independent experiments.

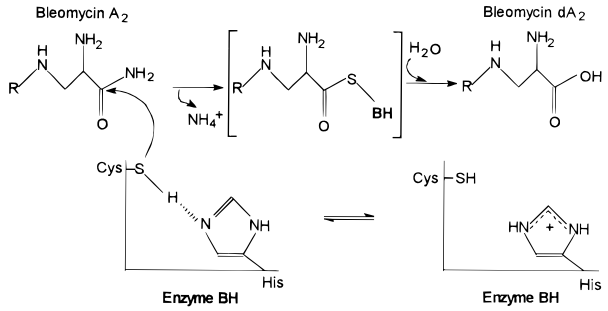

FIGURE 7: Hypothetical model of the mechanism for bleomycin degradation by bleomycin hydrolase.

significance. In the present studies, we used site-directed mutagenesis to define this relationship.

We substituted the highly conserved cysteine with two different amino acids (arginine or serine), and histidine with glycine. All three mutants lost their bleomycin hydrolase and cathepsin H activities (Figure 3 and Table 2). Although serine and cysteine differ by only one atom (sulfur for cysteine and oxygen for serine), the thiol group of the cysteine residue is critical, not only for the cysteine proteinase activity, but also for the bleomycin hydrolase activity of yeast BH. An important feature of cysteine proteinases in general is the high nucleophilicity of the sulfur atom of the active site cysteine residue (Husain & Lowe, 1968; Vernet et al., 1995). It is believed that, in cysteine proteinases, the active form of the enzyme consists of a thiolate−imidazolium ion pair (Polgar, 1974; Lewis et al., 1976, 1981). Our data suggest that the thiolate−imidazolium ion pair may also be critical for degrading bleomycin (Figure 7). Therefore, mutation of any one of the cysteine or histidine residues, which destroys the thiolate−imidazolium ion pair, totally abolishes the ability to degrade bleomycin.

Our gel filtration studies showed that, although the mutants had no cysteine proteinase and bleomycin hydrolase activities, they could still oligomerize into hexameric structures like the wild-type protein (Figures 4 and 5). However, our gel filtration studies showed that the wild-type GST−YCP1 fusion protein was not able to oligomerize, but still had bleomycin hydrolase activity (Figures 5 and 6). The results indicate that hexamer formation is not required for YCP1 catalytic activity. The fact that the GST−YCP1 fusion protein did not oligomerize is not surprising since the N-terminus to which GST was fused makes up most of the oligomerization domain of yeast BH (Joshua-Tor et al., 1995). Since the monomeric wild-type GST−YCP1 fusion protein had similar bleomycin hydrolase and cathepsin H specific activities to the hexameric wild-type YCP1 protein, the formation and stabilization of the thiolate−imidazolium ion pair in the active site of yeast BH may be independent from protein oligomerization. Interestingly, papain-like monomeric cysteine proteinases appear to require formation of several disulfide bonds for proper folding that stabilizes the active site. Even though yeast BH contains no disulfide bonds (Joshua-Tor et al., 1995), proper folding is achieved in both monomeric and hexameric forms of the enzyme.

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