Hydroperoxidase II of *Escherichia coli* Exhibits Enhanced Resistance to Proteolytic Cleavage Compared to Other Catalases†

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ABSTRACT: Catalase (hydroperoxidase) HPII of *Escherichia coli* is the largest catalase so far characterized, existing as a homotetramer of 84 kDa subunits. Each subunit has a core structure that closely resembles small subunit catalases, supplemented with an extended N-terminal sequence and compact flavodoxin-like C-terminal domain. Treatment of HPII with trypsin, chymotrypsin, or proteinase K, under conditions of limited digestion, resulted in cleavage of 72−74 residues from the N-terminus of each subunit that created a homotetramer of 76 kDa subunits with 80% of wild-type activity. Longer treatment with proteinase K removed the C-terminal domain, producing a transient 59 kDa subunit which was subsequently cleaved into two fragments, 26 and 32 kDa. The tetrameric structure was retained despite this fragmentation, with four intermediates being observed between the 336 kDa native form and the 236 kDa fully truncated form corresponding to tetramers with a decreasing complement of C-termini (4, 3, 2, and 1). The truncated tetramers retained 80% of wild-type activity. The T_m for loss of activity during heating was decreased from 85 to 77 °C by removal of the N-terminal sequence and to 59 °C by removal of the C-terminal domain, revealing the importance of the C-terminal domain in enzyme stability. The sites of cleavage were determined by N- and C-terminal sequencing, and two were located on the surface of the tetramer with a third being exposed by removal of the C-terminal domain.

Catalase is a key oxidative defense enzyme that is common in aerobic organisms. Its role is to degrade hydrogen peroxide to oxygen and water ($H_2O_2 \rightarrow H_2O + O_2$) before it or its degradation products can cause cellular damage. The importance of the enzyme is evident in the evolution of three distinctly different enzymes with the same catalytic activity: the monofunctional catalases, the bifunctional catalase−peroxidases and the non-heme manganese-containing catalases (*1*). The most widespread type is the monofunctional catalase, examples of which are found in most aerobic organisms. They are typically homotetrameric with one heme per monomer, the small monomers (55−65 kDa) containing heme *b* and the large monomers (80−84 kDa) containing heme *d*. Phylogenetic analyses have grouped the small subunit catalases into one of two main groups, clade 1 or 3, and the large subunit enzymes exclusively into clade 2 (*2*).

The large and small subunit catalases share several highly conserved structural domains, which are supplemented in the large subunit enzyme with an additional 50 residues at the N-terminus and a 150-residue flavodoxin-like domain at the C-terminus. The role of these added domains remains an open question, but HPII[1] exhibits enhanced resistance to thermal denaturation (*3*), which has been attributed in part to the N-terminal extension, which forms part of the 80 residues that is overlapped by an adjacent subunit in the formation of the characteristic interweaving of subunits exhibited by all catalases. For comparison, just 30 residues of BLC and 5 residues of CatF are overlapped, and these enzymes are inactivated at temperatures almost 30 °C lower.

The catalase−peroxidases are found in bacteria, archaebacteria, and fungi and are typically homodimers or homotetramers containing about 0.5 heme *b* per monomer (*1*). Their sequence reveals similarity to the plant peroxidases, and this was confirmed by the conversion from a predominant catalase activity into a predominant peroxidase activity by changing a single tryptophan to a phenylalanine (*4*, *5*). The non-heme or manganese-containing catalases are less common and have been characterized from only a small number of organisms.

Most bacteria encode multiple catalases, and *Escherichia coli* is typical in producing two, a bifunctional catalase−peroxidase HPI (hydroperoxidase I), expressed during exponential growth phase as part of the *oxyR* regulon (*6*), and the large subunit, monofunctional catalase HPII, expressed in stationary phase as part of the *rpoS* regulon (*7*). HPII has served as a model for catalases in biochemical and structure−function studies investigating the role of active site residues

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[1] Abbreviations: SDS, sodium dodecyl sulfate; HP, hydroperoxidase; BLC, bovine liver catalase.

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and the catalytic mechanism (*1*). The enhanced thermal stability of HPII has been described (*3*), but its sensitivity to other disruptive influences has not been described. This paper describes the enhanced resistance to proteolysis exhibited by HPII in comparison to other catalases.

MATERIALS AND METHODS

Materials. BLC, trypsin, chymotrypsin, proteinase K, and common biochemicals were obtained from Sigma Chemical Co. Catalase HPII and catalase−peroxidase HPI were purified from transformed strains as previously described (*8*, *9*).

Enzyme and Protein Determinations. Catalase activity was determined by the method of Rørth and Jensen (*10*) in a Gilson oxygraph equipped with a Clark electrode. One unit of catalase is defined as the amount that decomposes 1 μmol of H_2O_2 in 1 min in a 60 mM H_2O_2 solution at pH 7.0 at 37 °C. Protein was estimated according to the methods outlined by Layne (*11*). Absorption spectra were obtained using Pharmacia Biotech Ultrospec 4000 spectrophotometer. Samples were dissolved in 50 mM potassium phosphate, pH 7.0. Thermal denaturation studies were done using a Neslab Instruments Model RTE 111 programmable water bath. The temperature was increased at 0.6 °C/min from 50 to 90 °C (*3*). N-Terminal sequencing was performed by the Protein Microsequencing Laboratory at the University of Victoria. C-Terminal sequencing was performed by Dr. J. Keen of the School of Biochemistry and Molecular Biology, University of Leeds.

Gel Electrophoresis. Visualization of catalase activity on an 8.5% polyacrylamide gel was done according to Gregory and Fridovich (*12*) with modifications as described previously (*13*). The method of Hedrick and Smith (*14*) was used to determine the nondenatured molecular weights of the truncated variants of HPII on different acrylamide gel concentrations of 7%, 8%, and 9%. The gels were stained for protein with Coomassie brilliant blue dye. SDS−polyacrylamide gel electrophoresis was performed on 8% gels according to the method of Laemmli (*15*).

Conditions of Proteolysis. Limited digestions of HPII, BLC, and HPI were performed at 37 °C in 50 mM potassium phosphate, pH 7.0, for various times with trypsin, chymotrypsin, and proteinase K. The ratio of catalase to protease (w/w) is described for each experiment. Proteolysis was stopped by the addition of phenylmethanesulfonyl fluoride (1 mM).

Electrospray Ionization Time-of-Flight Mass Spectrometry (ESI TOF MS). All spectra were acquired on a noncommercial orthogonal time-of-flight mass spectrometer constructed at the University of Manitoba (*16*, *17*). Before mass spectrometric analysis, pure catalase protein was dialyzed into 100 mM ammonium acetate (Aldrich 99.999%) by ultrafiltration in a Centricon 50 (Amicon). To determine the mass of the subunit, an aliquot of protein was denatured by diluting to about 10 μM in 2% acetic acid and 50% methanol and analyzed by electrospray ionization with a declustering voltage of 150 V and nitrogen as the curtain gas. To examine the intact complex, a further aliquot of the protein was diluted to 5 μM subunit in 100 mM ammonium acetate. After proteolysis, samples were again dialyzed into 100 mM ammonium acetate, using a Centricon 50 to remove the protease and the phosphate buffer. These samples were diluted in the same manner as the intact protein. For each sample, a new New Objective PicoTip was cut to the required length, rinsed with a small amount of sample, and then loaded with 3 μL of sample. Spectra were acquired with SF_6 as the curtain gas, and the declustering voltage was varied from 100 to 400 V in order to assess the stability of the complexes.

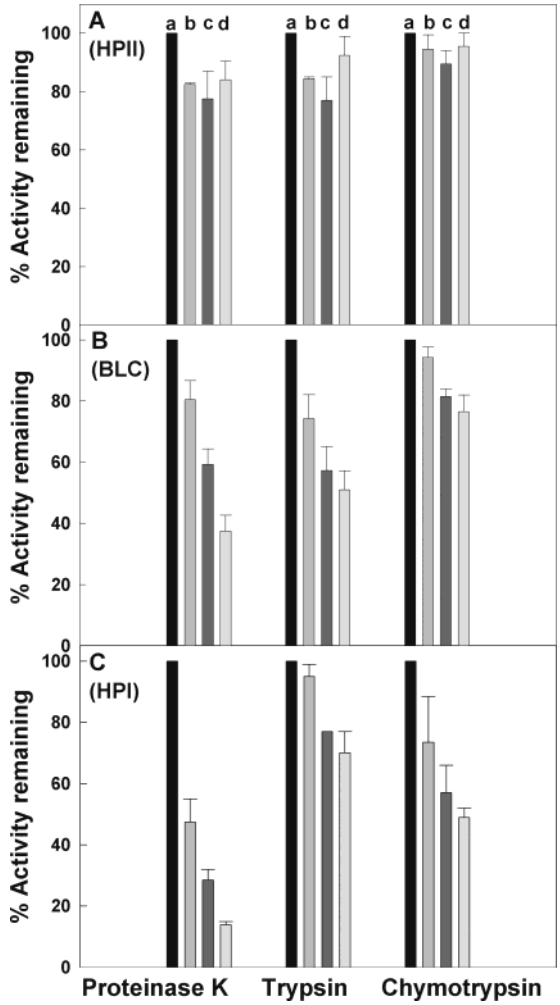

FIGURE 1: Effect of treatment with proteinase K, trypsin, and chymotrypsin on the activity of HPII (A), BLC (B), and HPI (C). Mixtures of protease and catalase at 0 (a), 1:100 (b), 1:50 (c), and 1:20 (d) (w/w) were incubated for 1 h at 37 °C and quenched with trypsin inhibitor or PMSF (1 mM). Error bars indicate the standard error of three individual assays.

RESULTS

Sensitivity of Catalases to Proteinase K, Trypsin, and Chymotrypsin. The three catalases, HPII, BLC, and HPI, exhibited varying sensitivities to digestion with proteinase K, chymotrypsin, and trypsin (Figure 1) at ratios (w/w) of protease to catalase from 1:100 to 1:20. HPII (Figure 1A) was the least sensitive to digestion by all three proteases, followed in order of increasing sensitivity by BLC (Figure 1B) and HPI (Figure 1C). Of the three proteases, proteinase K was the most effective, causing the loss of more than 75% of HPI activity after just 1 h of incubation with a 1:20 ratio of protease to catalase. This is consistent with the observation that HPI was rapidly proteolyzed to a 35 kDa fragment containing the inactive C-terminal portion (*19*).

Time Course of Digestion of HPII. Because low protease to HPII ratios did not seem to have a significant effect on

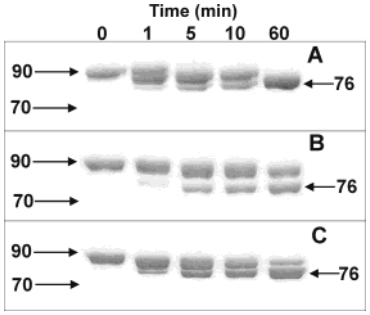

FIGURE 2: Products of proteolytic cleavage of HPII by trypsin (A), chymotrypsin (B), and proteinase K (C). HPII and protease (2:1 w/w) were incubated at 37 °C for the times indicated (in minutes), and the mixtures were separated by electrophoresis on 8% SDS−polyacrylamide gel. The protein was visualized by staining with Coomassie brilliant blue. Size markers indicated as $\times 10^3$ Da were determined using the GIBCO protein ladder. The 76 kDa product is indicated with the arrow labeled with 76.

HPII, the time courses of digestion of HPII by a 1:2 ratio (w/w, protease to catalase) were followed by SDS−polyacrylamide gel electrophoresis, revealing an initial cleavage by all three proteases of the 84 kDa subunit via several intermediate-sized fragments to a relatively stable 76 kDa fragment (Figure 2). The formation of the 76 kDa fragment was slowest with trypsin (Figure 2B) compared to chymotrypsin and proteinase K (Figure 2A,C). Longer digestion with proteinase K at a 1:1 ratio generated a transient 59 kDa band and, eventually, two bands at 26 and 32 kDa (Figure 3C). The third band at approximately 35 kDa corresponds to proteinase K. Very little smaller material was observed from chymotrypsin and trypsin digestions even after 16 h of digestion at a 1:1 ratio (data not shown). In contrast to HPII, digestion of BLC with any of the proteases did not produce distinguishable partial digest fragments (data not shown). Digestion of HPI produced only the expected 35 kDa C-terminal fragment after treatment with a 1:100 ratio of protease to catalase, and higher ratios of protease eliminated even this fragment (data not shown).

Whereas denaturing gels presented a picture of at least three cleavage sites within HPII resulting in sequential cleavage to 26 and 32 kDa fragments, 20% of the original activity remained in the digest mixture, suggesting that much of the tertiary and quaternary structure had not been significantly affected. This was confirmed in the time course of cleavage of HPII with proteinase K visualized by Coomassie brilliant blue (Figure 3B) and activity (data not shown) staining after electrophoresis on nondenaturing gels. There was a rapid conversion of the 336 kDa tetramer to a band of approximately 310 kDa, composed principally of 76 kDa subunits. This was followed by the appearance and disappearance of bands at approximately 290, 270, and 240 kDa. The subunit sizes associated with these conversions included bands at 76, 59, 32, and 26 kDa. All HPII was eventually converted to a band of protein of approximately 220 kDa, containing equal amounts of the 32 and 26 kDa fragments.

HPII samples digested for either 1 h (N-termini removed) or 16 h (N- and C-termini removed) were purified by size exclusion chromatography. Both forms of the truncated enzyme exhibited similar specific activities of 14800 and 14400 units/mg (compared to 17100 units/mg for the native enzyme), indicating that loss of the N- and C-terminal

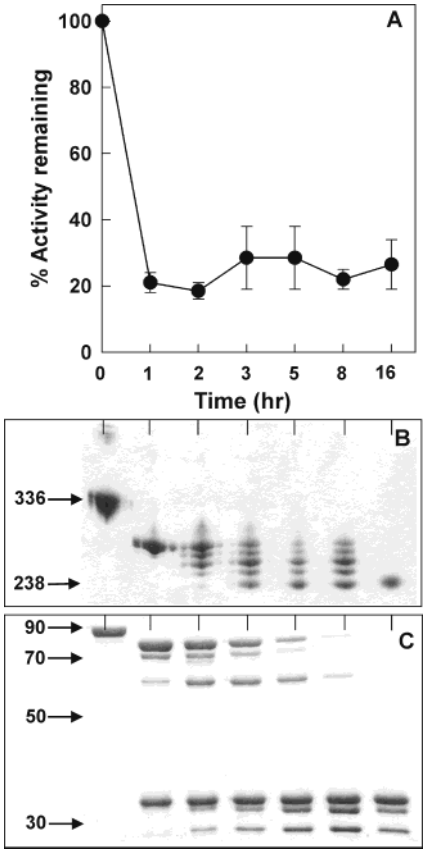

FIGURE 3: Correlation of product size and activity during proteolytic cleavage of HPII by proteinase K. Samples were incubated in a 1:1 ratio (w/w) with proteinase K at 37 °C for the times indicated. Catalase activities were determined, and the activity remaining is shown in (A). Samples were separated on a nondenaturing 8% gel (B) or a denaturing 8% gel (C), and the protein was visualized by staining with Coomassie brilliant blue. Size markers indicated as $\times 10^3$ Da were determined using HPII (336 kDa), bovine liver catalase (238 kDa), and HPI (160 kDa, not visible) in panel B and the GIBCO protein ladder in panel C.

domains had little effect on the function of the deeply buried active site. Therefore, the rapid reduction in activity to an apparent residual 20% (Figure 3A) is most likely an artifact of the high concentration of proteinase K (1:1 w/w) in the mixture interfering with catalase activity. Heme exhibits a negligible 62 units/μmol compared to 1.4×10^6 units/μmol of heme in HPII, too low a level for the small amount of heme released to have contributed to the residual activity observed.

N- and C-Terminal Sequence Analysis of Protease Fragments. To identify the sites of cleavage in HPII, the various fragments separated on SDS−polyacrylamide gels were subjected to N-terminal sequencing. The 76 kDa fragments generated by all three proteases had very similar sites of cleavage (Table 1), with N-terminal residues at Lys73, Gly74, and Ser75 for chymotrypsin, trypsin, and proteinase K, respectively. The unusual cleavage by chymotrypsin is most likely the result of a trypsin contaminant in the chymotrypsin. The 59 kDa fragment generated by proteinase K also had Ser75 at the N-terminus, and the shorter size arose from cleavage after Tyr591 which removed the C-terminal domain (Table 1). The 26 and 32 kDa bands had Ser75 and Lys309 at their N-termini, respectively, revealing a third site of proteinase K cleavage in HPII.

Table 1: Sequences at Protease Cleavage Sites in HPII

Fragment	Protease	Sequence	Mass (calc) (Da)
A. N-terminal sequences			
HPII sequence		V R K G S E N Y A L T T N Q G 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85	
76 kDa	chymotrypsin	K G S E N Y A L T T N Q G	76,464
76 kDa	trypsin	G S E N Y A L T T N Q G	76,335
76 kDa	proteinase K	S E N Y A L T T N Q G	76,279
59 kDa	proteinase K	S E N Y A L	59,155
26 kDa	proteinase K	S E N Y A L T T N Q	26,512
B. N-terminal sequence at the central site			
HPII sequence		K L T G R D P D F H 309 310 311 312 313 314 315 316 317 318	
32 kDa	proteinase K	K L T ? R D ? D F H	32,661
C. C-terminal sequence at C-terminal domain cleavage site			
HPII sequence		L S L Y A I P D 588 589 590 591 592 593 594 595	
59 kDa	proteinase K	L Y	59,155

The sizes of the truncated variants of HPII were confirmed by electrophoresis on nondenaturing gels, correlating the rate of migration with molecular weights on a series of different percentage gels (*14*) (data not shown), and by electrospray ionization mass spectrometry (Figure 4). Denatured HPII protein was measured by mass spectrometry to be 84035 Da (data not shown), which agrees well with the expected mass of 84031 Da for the full-length protein lacking the N-terminal methionine residue. On the basis of this value, the expected size of the tetramer with heme is 338596 Da [4(84031 + 618)], and the major ion in the deconvolution of the spectrum (Figure 4A) is centered at 339514, with the extra mass arising from water and buffer salts bound to the protein (*20*). The secondary ion centered at 337295 Da has probably lost two heme residues and some of the buffer and salts (calculated mass of 337364 Da). The smallest and least well resolved ion is the tetramer without heme. After proteolysis and inactivation of the proteinase K with phenylmethanesulfonyl fluoride, a majority of the proteinase K was removed by rapid filtration, allowing the ions from catalase to be clearly apparent at *m*/*z* 7000−8000 at 200 V declustering voltage. Resolution was not as good as for the native protein complex (Figure 4B), but deconvolution gives a major ion with a measured mass centered at 223012 Da, smaller by 13648 Da than the expected 239092 Da [4(59155 + 618)]. This discrepancy may have arisen from loss of heme from a more easily denatured protein and from additional cleavage during extensive proteinase K digestion that removed small fragments totaling about 3400 Da from each subunit in the complex. Such small fragments would have been lost from the complex during the extensive dialysis used in sample preparation, and it is not possible to say whether this small amount of protein was removed in a single or multiple fragments. The locations of the three main cleavage sites on the tetrameric structure of HPII (Figure 5) reveal that the first two sites at S75 and Y591 are located on the surface of the protein while the site at K309 becomes exposed when the C-terminal domains are removed.

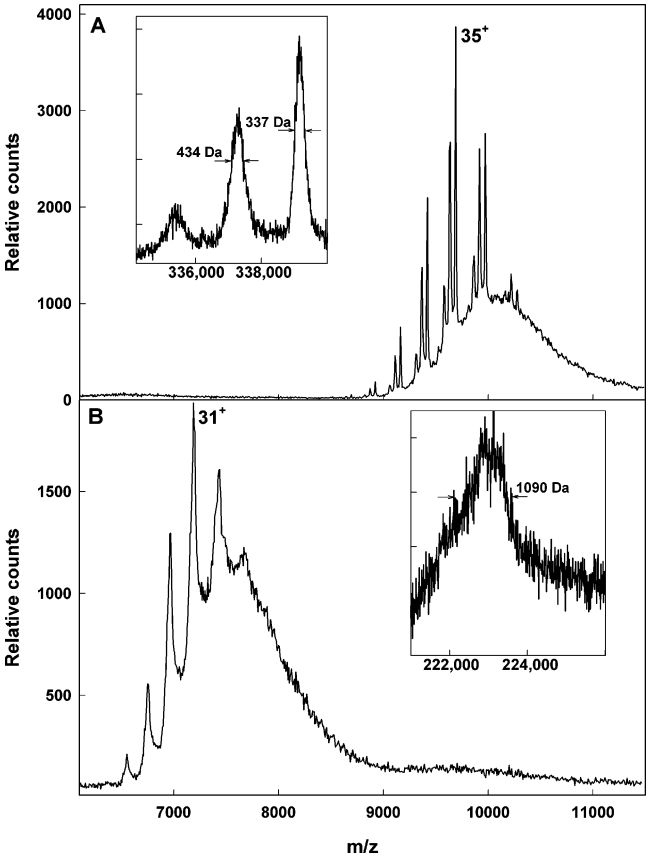

FIGURE 4: Mass analysis by nanospray ionization of HPII protein before (A) and after (B) treatment with proteinase K. Deconvolutions of the data from each spectrum are shown as insets with the errors of measurement shown as fwhm. Both spectra were acquired at 200 V with SF6 as the curtain gas. At lower voltage, the ions of the tetramer were not as well resolved. Higher voltage did not change the spectra.

Thermal Stability of the Truncated HPII Variants. HPII is relatively insensitive to thermal denaturation, exhibiting a T_m for loss of activity of 83−84 °C (*3*). Furthermore, heating above 95 °C was necessary to dissociate a dimer association, evident on SDS−urea denaturing gels (*3*). A thermal denaturation study of the truncated variants of HPII was undertaken to determine the role, if any, of the N- and C-terminal domains in enhancing the stability of HPII. The 310 kDa multimer, composed of N-terminally truncated 76 kDa subunits, showed an 8 °C reduction in the T_m for activity to 77 °C (Figure 6), and the stability of the dimer association was reduced, requiring heating to only 80 °C for dissociation (Figure 7). The T_m of the truncated form was independent of the protease used. By contrast, the 220 kDa multimer, composed of 26 and 32 kDa fragments lacking both the N- and C-termini, exhibited a 22 °C reduction in the T_m for activity loss to 59 °C (Figure 6), and the multimer dissociated upon mixing in SDS−urea loading buffer at room temperature, indicating that the stability of the dimer association was significantly reduced. Both properties are similar to those of BLC, which exhibits a T_m of 56 °C (*3*) and dissociates at room temperature in loading buffer.

DISCUSSION

HPII exhibits unusual resistance, compared to other catalases, not only to proteases such as trypsin and chymotrypsin with greater substrate specificity but even to the broad

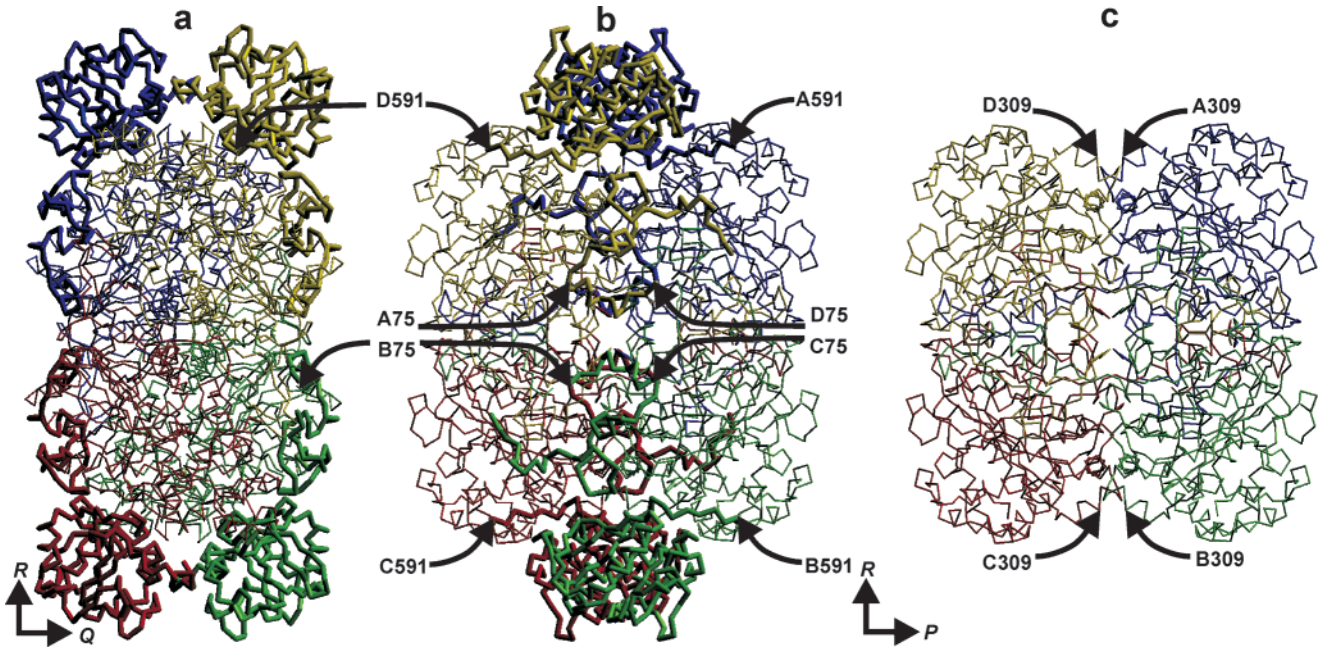

FIGURE 5: Location of the three main locations on the HPII tetramer that are sensitive to proteolytic cleavage. HPII is shown in panels a and b rotated 90° around the *R* axis with respect to each other to show the surface locations of the cut sites at residues 75 and 591 in each of subunits A (blue), B (green), C (red), and D (yellow). The N- and C-terminal fragments removed by these cuts are presented as heavier lines in panels a and b and are removed in panel c where the protein has the same orientation as in panel b. The surface locations of the third main cut site at residue 309, exposed by the removal of the excised domains, are evident in panel c. The figure was prepared using SETOR (*27*).

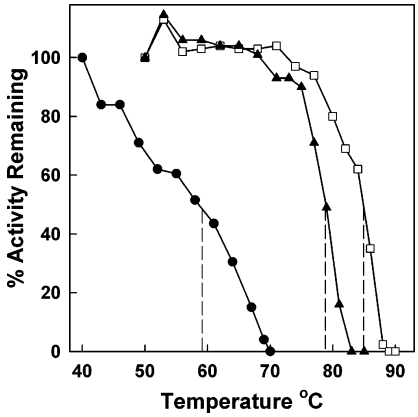

FIGURE 6: Activity changes in HPII (open squares) and HPII treated for 1 h (solid triangles) and 16 h (solid squares) at 37 °C with 1:1 (w/w) proteinase K as the temperature is increased at 0.6 °C/min from 40 to 90 °C. The enzymes were in 50 mM potassium phosphate buffer (pH 7). The dashed lines indicate the midpoints or temperatures of 50% inactivation.

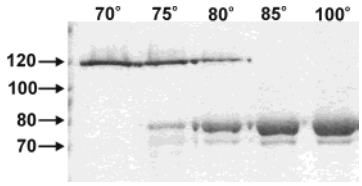

FIGURE 7: Conversion of HPII dimers to monomers. HPII was treated with proteinase K in a 1:1 ratio (w/w) for 1 h at 37 °C. Samples were then incubated for 10 min at 70, 75, 80, and 100 °C in 50 mM potassium phosphate buffer (pH 7). The temperatures of incubation are indicated above each lane. Samples were removed, cooled to room temperature, and added to SDS−urea loading buffer. Samples were loaded and run without further heating on an 8% SDS−polyacrylamide gel. Size markers indicated as $\times 10^3$ Da were from the GIBCO protein ladder. The band at 120 kDa is a dimer of two HPII subunits that are resistant to thermal denaturation (*3*). As the temperature of incubation is increased, the dimer dissociates into monomers at 76 kDa.

substrate range protease, proteinase K. Globular proteins generally exhibit some level of resistance to proteolysis at least initially because the tertiary structure of the protein chain imparts sufficient inflexibility that it cannot fit into the protease active site. Such resistance to proteolysis is usually demonstrated under conditions of limited digestion involving protease to substrate ratios of 1:100 and temperatures around 25 °C (*21*). What makes HPII unusual is its resistance to cleavage even at a very high (1:1) ratio of protease to HPII at 37 °C. This property must be considered in the light of another unusual property of HPII, its enhanced thermal stability (*3*).

The quaternary structure of HPII might be described as a dimer of dimers in which monomers first associate by forming an interwoven structure in which the 80 N-terminal residues of each subunit are threaded through a loop formed by the wrapping domain of the adjacent subunit (subunit A with subunit C and subunit D with subunit B in Figure 5). Such interweaving was suggested to be an explanation for the enhanced stability of the dimers that dissociate only above 95 °C and for the elevated T_m for thermal inactivation, 84 °C (*3*). A recent comparison of the properties of 16 different catalases, however, observed that there was no correlation between the length of the N-terminal extension involved in the interweaving and the thermal stability (*23*). The current work supports this conclusion by demonstrating that removal of the N-terminal domain, leaving only seven residues overlapped in the interwoven structure, reduced the T_m by only 6 °C, to 77 °C. Furthermore, the truncated dimers still required incubation at over 80 °C to dissociate. On the other hand, removal of the C-terminal domain caused a significant reduction in the T_m for thermal inactivation to 59 °C. An even more striking result of the double truncation was the

effect on the stable dimer association, which dissociated on mixing with gel loading buffer, even without heating, making it impossible to assess the temperature of dissociation.

While the C-terminal domain appears to be a key factor in enhancing the thermal stability of HPII, it was not possible to assess the effect of C-terminal domain removal in the presence of the N-terminal region, because the latter domain was more sensitive to proteolysis and was rapidly removed. Attempts have been made to address this question through the construction of truncated genes, but the truncated variants did not fold correctly and protein did not accumulate (*22*). The conclusion that the C-terminal domain is more important than subunit interweaving in dimer stability calls into question the previous surmise that the interweaving was the reason for the enhanced stability of HPII dimers. Rather, the current work suggests that it is the interaction of subunits along the *R* axis (subunit A with subunit D and subunit B with subunit C in Figure 5), specifically the wrapping of the C-terminal domain around the β-barrel core of the adjacent subunit, that is the major determinant of dimer stability in HPII.

A globular protein must change conformation to fit into the active site of proteases, and resistance to proteolysis arises in less flexible proteins. In the case of HPII, a combination of interweaving and wrapping of domains on adjacent subunits undoubtedly creates a structure that is much less flexible than that of small subunit catalases. Consequently, the very same structural features that enhance thermal stability may be responsible for the enhanced resistance to proteolysis. In addition, the presence of the unusual covalent linkage between the C_β of Tyr415 and N_δ of His 392 may serve to enhance this rigidity (*24*).

Are these two unusual properties of HPII, enhanced thermal stability and protease resistance, related evolutionarily? Resistance to thermal denaturation is unusual in an enzyme from a bacterium that does not survive exposure to temperatures anywhere near the 83 °C required to inactivate the enzyme. Examples of lateral gene transfer of catalase genes are becoming more common, and it is possible that *katE*, encoding HPII, originated in an organism with a thermophilic background. Given that *katE* is typical of other *E. coli* genes in its nucleotide composition and codon usage pattern (*25*, *26*), any such lateral transfer event must have either been from an organism with composition and codon usage similar to that of *E. coli* or happened long enough ago that the original nucleotide and codon patterns had been lost. Curiously, the phenotype of thermal stability was retained, despite the absence of thermal stress as an evolutionary determinant. By contrast, the property of enhanced resistance to proteolysis in HPII can be easily explained in terms of bacterial physiology. The expression of *katE* is induced in early stationary phase, and HPII accumulates in stationary phase cells. This is a period of rapid protein turnover as cells adapt to a period of slow metabolism, and resistance to proteolysis would be important to allow HPII to survive and function in its role as a protective enzyme. Because both thermal stability and protease resistance may have a common basis in a very stable and rigid structure, the original properties of thermal stability in HPII may have been adapted to the physiological need for protease resistance in the niche of stationary phase metabolism. For comparison, the more protease-sensitive HPI is expressed mainly in log phase where protease resistance would not be as important.

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