

# Catalase HP<sub>II</sub> from *Escherichia coli* Exhibits Enhanced Resistance to Denaturation<sup>†</sup>

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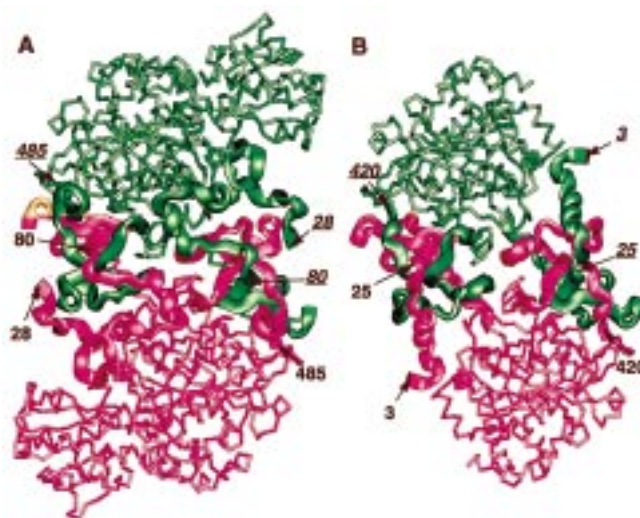
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Received December 4, 1998; Revised Manuscript Received January 11, 1999

**ABSTRACT:** Catalase HP<sub>II</sub> from *Escherichia coli* is a homotetramer of 753 residue subunits. The multimer displays a number of unusual structural features, including interwoven subunits and a covalent bond between Tyr415 and His392, that would contribute to its rigidity and stability. As the temperature of a solution of HP<sub>II</sub> in 50 mM potassium phosphate buffer (pH 7) is raised from 50 to 92 °C, the enzyme begins to lose activity at 78 °C and 50% inactivation has occurred at 83 °C. The inactivation is accompanied by absorbance changes at 280 and 407 nm and by changes in the CD spectrum consistent with small changes in secondary structure. The subunits in the dimer structure remain associated at 95 °C and show a significant level of dissociation only at 100 °C. The exceptional stability of the dimer association is consistent with the interwoven nature of the subunits and provides an explanation for the resistance to inactivation of the enzyme. For comparison, catalase-peroxidase HPI of *E. coli* and bovine liver catalase are 50% inactivated at 53 and 56 °C, respectively. In 5.6 M urea, HP<sub>II</sub> exhibits a coincidence of inactivation, CD spectral change, and dissociation of the dimer structure with a midpoint of 65 °C. The inactive mutant variants of HP<sub>II</sub> which fold poorly during synthesis and which lack the Tyr–His covalent bond undergo spectral changes in the 78 to 84 °C range, revealing that the extra covalent linkage is not important in the enhanced resistance to denaturation and that problems in the folding pathway do not affect the ultimate stability of the folded structure.

*Escherichia coli* produces two catalases, hydroperoxidase I (HPI)<sup>1</sup> and hydroperoxidase II (HP<sub>II</sub>). The former is a bifunctional catalase-peroxidase whose sequence is similar to those of many heme-containing peroxidases (1). It is expressed mainly in the exponential phase in response to oxidative stress as a part of the *oxyR* regulon (2, 3). HP<sub>II</sub> is more closely related to normal catalases with a core sequence and structure that closely resembles those of other catalases but with added C- and N-terminal extensions that make it the largest known catalase (4, 5). It is produced in stationary phase under the control of the stationary phase sigma factor encoded by *rpoS* (6).

Differences in temperature stability between the two enzymes have been noted previously and used as a tool to differentiate between the two enzymes in crude extracts (7, 8). HPI is inactivated by incubation at temperatures above 50 °C, whereas HP<sub>II</sub> retains activity during incubation at temperatures up to 70 °C. Other examples of the extreme stability of HP<sub>II</sub> are evident in the retention of activity over a broad pH range from pH 5 to 11 (9), and in urea and SDS (10).



**FIGURE 1:** Comparison of the interwoven sections of HP<sub>II</sub> (A) and BLC (B). In both panels, the residue numbers in the red subunit are in normal characters and the residue numbers in the green subunit are in italic and underlined characters. In panel A, residues 28–100 (or 28–100) and 435–485 (or 435–485) are shown as a ribbon. Residues 1–27 are not depicted because they were not visible in the crystal structure of the enzyme. Residues 1–80 (or 1–80) extend through a loop formed by residues 435–485 (or 435–485) of the adjacent subunit. In panel B, residues 3–45 (or 3–45) and 370–420 (or 370–420) are shown as a ribbon. Residues 1–25 (or 1–25) extend through a loop formed by residues 370–420 (or 370–485) of the adjacent subunit. Notice the greater interaction of residues 30–80 with the rest of the subunit in HP<sub>II</sub> as compared to the limited interactions between residues 1–25 with the rest of the subunit of BLC.

<sup>†</sup> This work was supported by a grant (OGP 9600) from the Natural Sciences and Engineering Research Council of Canada.

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<sup>1</sup> Abbreviations: HPI, hydroperoxidase I; HP<sub>II</sub>, hydroperoxidase II; BLC, bovine liver catalase; CD, circular dichroism; SDS, sodium dodecyl sulfate.

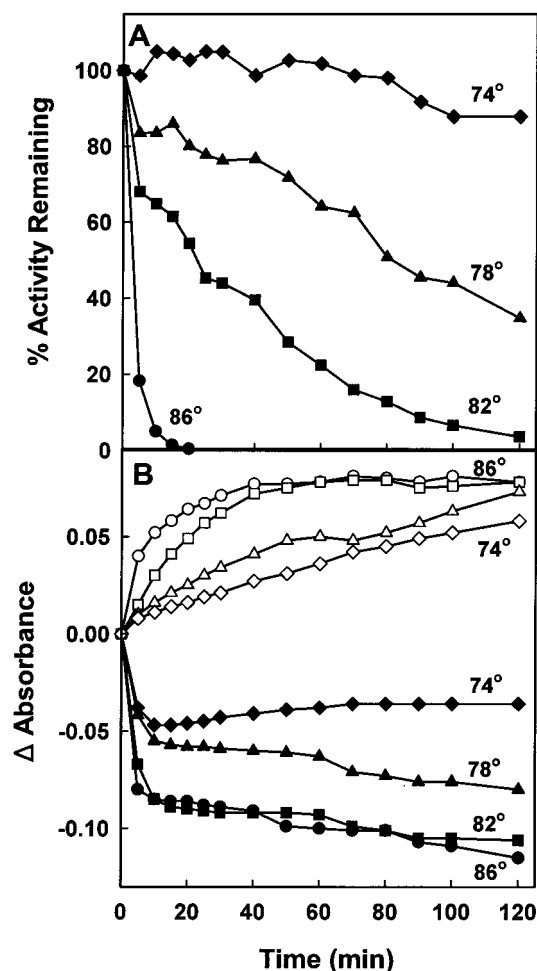


FIGURE 2: Activity changes (A) and spectral changes (B) in HPII when incubated at 74 ( $\diamond$  or  $\blacklozenge$ ), 78 ( $\triangle$  or  $\blacktriangle$ ), 82 ( $\square$  or  $\blacksquare$ ), and 86 °C ( $\circ$  or  $\bullet$ ). In panel B, the white symbols represent the change in absorbance at 280 nm and the black symbols represent the change in absorbance at 407 nm. HPII was in 50 mM potassium phosphate (pH 7). The temperatures are indicated on the figure adjacent to the appropriate line.

The crystal structure of HPII has been determined at 1.9 Å resolution, revealing a tetrameric structure with a number of unusual features that might contribute to the enhanced resistance to denaturation. Within the tetramer, there are two pairs of dimers containing interwoven subunits in which 90 residues of the N-terminal sequence of each subunit are inserted through a loop on the adjacent subunit (Figure 1). In this way, there are two overlapped segments per dimer or four per tetramer which would be expected to stabilize the quaternary structure of the enzyme (11). Other catalases exhibit a similar interweaving of subunits, but the N-terminal length of sequence that is overlapped is shorter by approximately 55 residues (12). One further feature in HPII which would lend rigidity to the structure is an unusual covalent bond between the  $C_\beta$  of Tyr415, the proximal heme ligand, and the  $N_\delta$  of His392 (13). To quantitate the enhanced stability of HPII and to relate this stability to the unique structural characteristics, a more detailed study of the denaturation kinetics of HPII, its mutant variants, and other catalases is reported.

#### EXPERIMENTAL PROCEDURES

Catalases HPI and HPII, wild type and mutant variants, were purified from transformed strains as described previ-

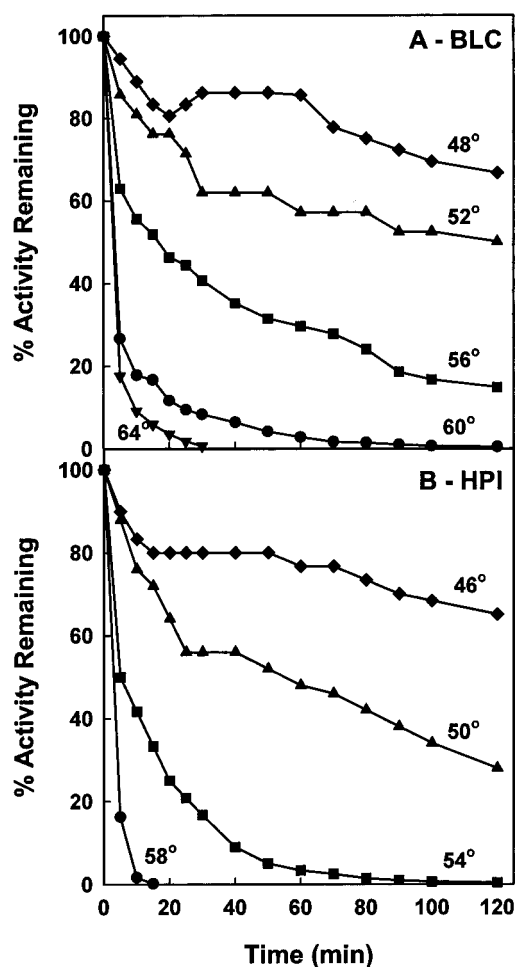


FIGURE 3: (A) Activity changes of BLC incubated at 48 ( $\blacklozenge$ ), 52 ( $\blacktriangle$ ), 56 ( $\bullet$ ), 60 ( $\blacksquare$ ), and 64 °C ( $\blacktriangledown$ ). (B) Activity changes of HPI incubated at 46 ( $\blacklozenge$ ), 50 ( $\blacktriangle$ ), 54 ( $\blacksquare$ ), and 58 °C ( $\bullet$ ). The incubations were carried out in 50 mM potassium phosphate buffer (pH 7). The temperatures are indicated on the figure adjacent to the appropriate line.

ously (14, 15). BLC was obtained from Sigma Chemical Co. Catalase activity was determined by the method of Rørth and Jensen (16), using a Gilson oxygraph equipped with a Clark electrode. One unit of catalase is defined as the amount that decomposes 1  $\mu$ mol of  $H_2O_2$  in 1 min at 37 °C. Gel electrophoresis of proteins was carried out on SDS-polyacrylamide gels as described previously (17, 18). Absorbance spectra and readings were made using a Milton Roy MR3000 spectrophotometer. CD spectra were obtained using a JASCO J-500A spectropolarimeter calibrated with (+)-10-camphorsulfonic acid. Spectra were measured at a scan rate of 0.5 nm/min and with a time constant of 32 s. The temperature in the spectrophotometer cuvettes was maintained using a Haake thermocirculator. CD spectra were deconvoluted using the convex constraint algorithm of Perczel et al. (19).

#### RESULTS

**Denaturation of HPII.** As a first step in relating the structural characteristics of HPII to its enhanced resistance to thermal inactivation, we incubated HPII at different temperatures between 74 and 86 °C (Figure 2A). Incubation at 74 °C initially caused a small activation of the enzyme followed by a 10% loss of activity after 2 h, whereas

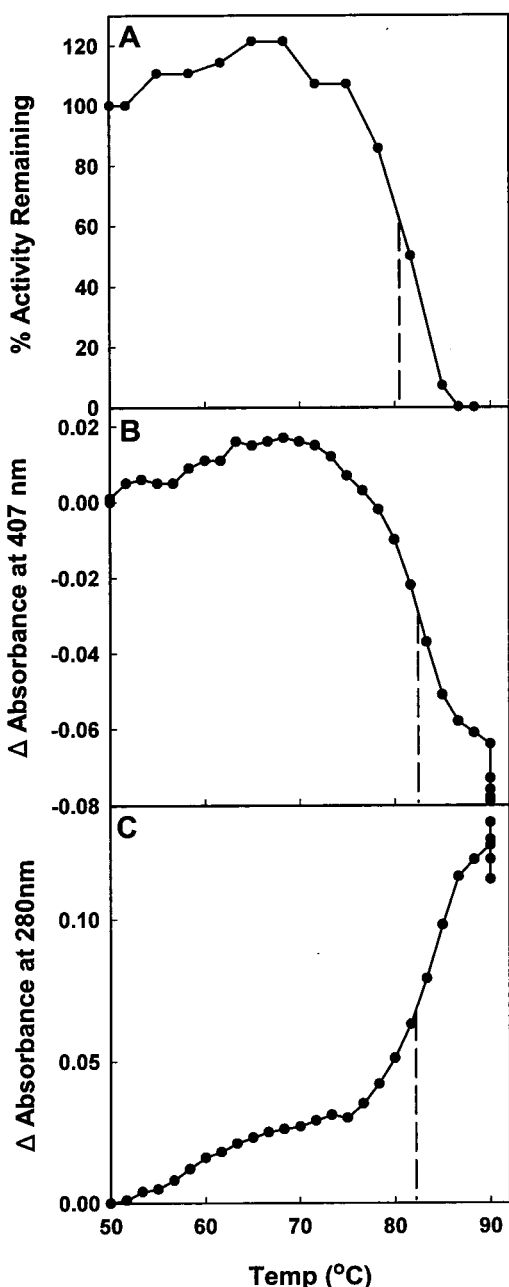


FIGURE 4: Activity changes (A) and absorbance changes at 407 (B) and 280 nm (C) as the temperature is increased at a rate of 0.6 °C/min from 50 to 90 °C. HP11 was in 50 mM potassium phosphate buffer (pH 7). The dashed lines indicate the midpoints or temperatures of 50% change.

complete inactivation occurred within 20 min at 86 °C. Increases in absorbance at 280 nm ( $\Delta A_{280}$ ) and decreases in the Soret peak ( $\Delta A_{407}$ ) accompanied the loss in activity, confirming that changes in protein structure were occurring (Figure 2B). Smaller absorbance changes were observed even at 74 °C where only minor changes in activity had occurred, suggesting that some degree of structural change is possible that does not affect activity. At higher temperatures, the changes in absorbance accompanying inactivation were greater and occurred more rapidly.

Two other related enzymes, the catalase-peroxidase HPI from *E. coli* and bovine liver catalase (BLC), were found to be rapidly inactivated even at 74 °C (data not shown). This led to an investigation of temperatures between 46 and 64

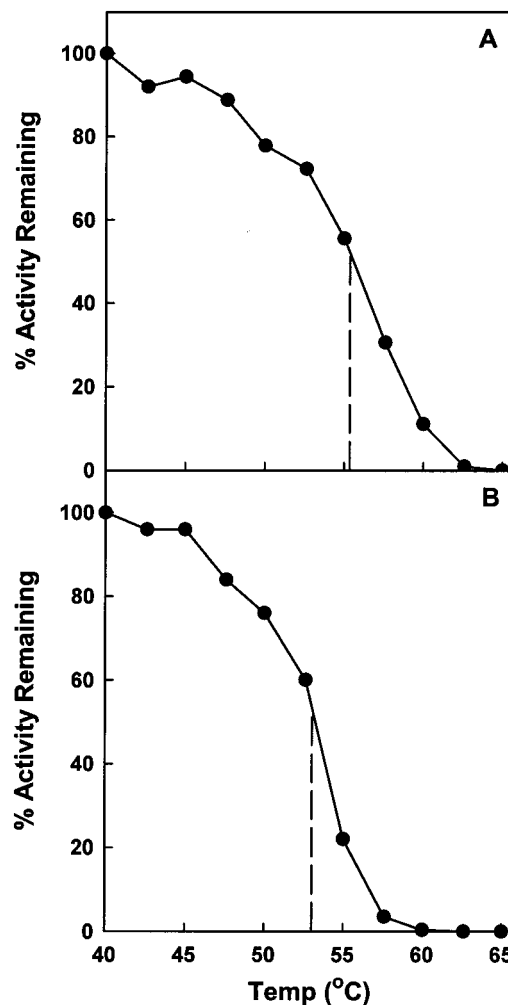


FIGURE 5: Activity changes in BLC (A) and HPI (B) as the temperature is increased at a rate of 0.6 °C/min from 40 to 65 °C. The enzymes were in 50 mM potassium phosphate buffer (pH 7). The dashed lines indicate the midpoints or temperatures of 50% change.

°C (panels A and B of Figure 3 for BLC and HPI, respectively) which revealed that BLC was completely inactivated within 30 min at 64 °C and HPI was completely inactivated within 15 min at 58 °C.

**$T_m$  Determination.** The “melting” temperature, or temperature of 50% inactivation, of HP11 was determined by following activity changes (Figure 4A), the decrease in  $A_{407}$  (Figure 4B), and the increase in  $A_{280}$  (Figure 4C). In all three cases, a  $T_m$  of approximately 82 °C was observed. The  $T_m$  proved to be independent of the rate of temperature rise between 0.3 and 0.6 °C/min over the range of 50–90 °C. Initially, a 20% increase in activity was observed as the temperature was increased from 50 to 70 °C, and this was accompanied by small increases in both  $A_{407}$  and  $A_{280}$ . Consistent with the data in Figure 2B, the  $A_{407}$  subsequently decreased. For comparison, the  $T_m$ s for BLC and HPI were determined to be 56 °C (Figure 5A) and 53 °C (Figure 5B), respectively.

**CD Spectral Changes.** The circular dichroism spectrum of HP11 was determined to see if changes in secondary structure could be correlated with the changes in activity and absorbance. The CD spectra of HP11 at 40 and 92 °C after boiling (Figure 6A) reveal a change in molar ellipticity in the region below 230 nm that was irreversible. Deconvolution

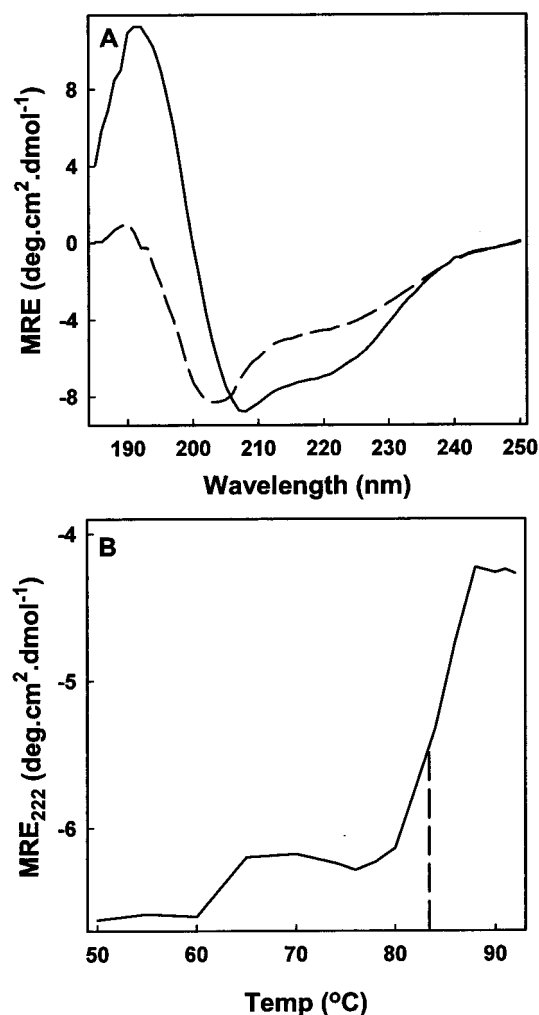


FIGURE 6: (A) CD spectra of HPII determined at 40 (solid line) and 92 °C (dashed line) in 5 mM potassium phosphate buffer (pH 7). MRE is the molar residue ellipticity. (B) The change in MRE determined at 222 nm as a function of temperature as it is raised from 50 to 92 °C at a rate of 0.6 °C/min. The dashed line indicates the midpoint or temperature of 50% change.

of the spectra confirmed that the  $\alpha$ -helix content had decreased from 24 to 9% while  $\beta$ -configuration content had remained relatively unchanged at 40%. Plotting the spectral changes at 222 nm as a function of temperature reveals no ellipticity change below 60 °C (Figure 6B). Between 60 and 80 °C, there is a small transition that coincided with the increase in activity over the same temperature range (see Figure 4A). Above 80 °C, there is a rapid change in ellipticity with a midpoint at 82 °C (Figure 6B) which is consistent with the melting temperature determined by the loss of activity and the change in absorbance.

For comparison, the CD spectra of HPI and BLC at 35 and 65 °C are presented in Figure 7A and Figure 8A, respectively, revealing small changes in secondary structure similar to those observed for HPII. Deconvolution of the spectra revealed changes similar to those for HPII with a reduction in the amount of  $\alpha$ -helix and a small increase in the amount of  $\beta$ -configuration. The  $T_m$  for the ellipticity transition at 222 nm was 53 °C for HPI (Figure 7B) and 56 °C for BLC (Figure 8B), in good agreement with the  $T_m$  data determined using activity measurements (see Figure 5).

**Dissociation of Dimers.** To determine if the changes in absorbance and the loss of activity that occurred between

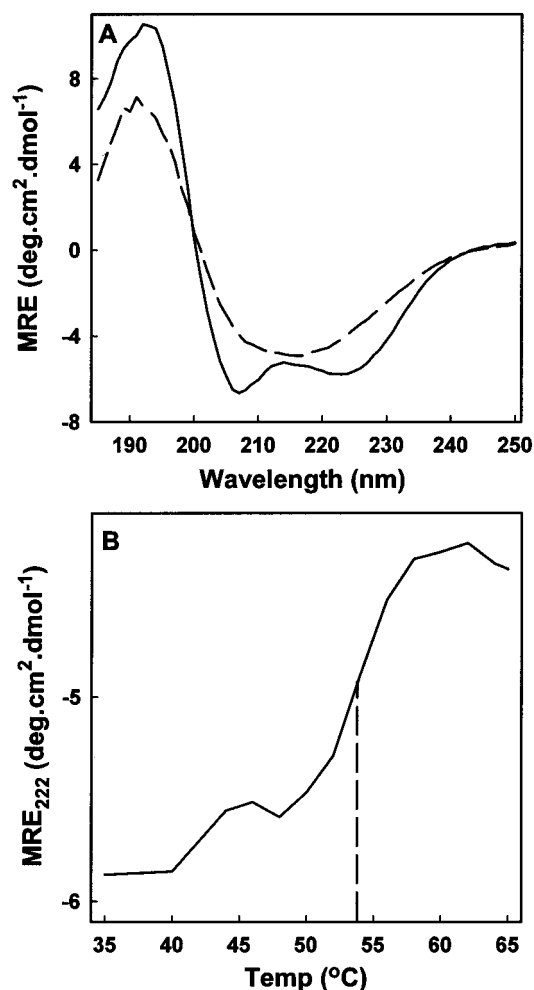


FIGURE 7: (A) CD spectra of HPI determined at 35 (solid line) and 65 °C (dashed line) in 5 mM potassium phosphate buffer (pH 7). MRE is the molar residue ellipticity. (B) The change in MRE determined at 222 nm as a function of temperature as it is raised from 50 to 65 °C at a rate of 0.6 °C/min. The dashed line indicates the midpoint or temperature of 50% change.

80 and 85 °C arose simply from changes in secondary structure or were accompanied by dissociation of the multimeric protein, the electrophoretic mobility of the protein on urea-SDS-polyacrylamide gels was investigated. Incubation in buffer at temperatures up to 95 °C prior to electrophoresis caused only minor dissociation of the dimer (Figure 9A), and boiling of the solution was required for complete dissociation. It should be noted that the dimer of HPII was stable in 1% SDS-7 M urea loading buffer at room temperature, making this assay possible. By contrast, BLC and HPI multimers dissociated in the loading buffer at room temperature.

**Stability of Mutant Variants of HPII.** A large number of mutant variants of HPII have been constructed by site-directed mutagenesis as part of an ongoing characterization of the enzyme. Many of the mutant enzymes accumulated in only small amounts, if at all, when the cells were grown at 37 °C. However, growth of the cultures at 28 °C resulted in a significant increase in enzyme yield. The low yield at the higher temperature was attributed to a disruption in the folding pathway allowing proteolytic degradation of the enzyme. At 28 °C, the slower growth rate provided a longer



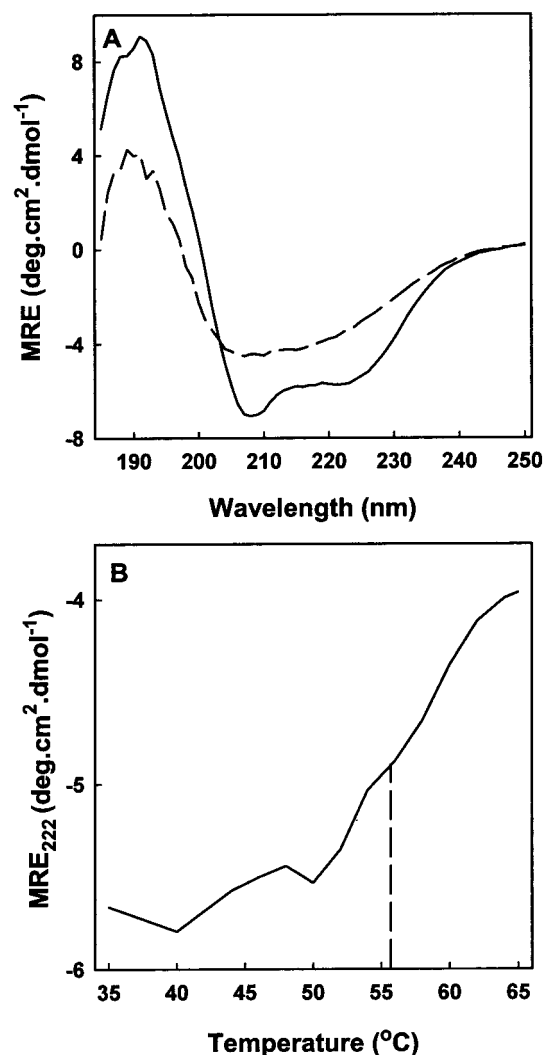


FIGURE 8: (A) CD spectra of BLC determined at 35 (solid line) and 65 °C (dashed line) in 5 mM potassium phosphate buffer (pH 7). MRE is the molar residue ellipticity. (B) The change in MRE determined at 222 nm as a function of temperature as it is raised from 50 to 65 °C at a rate of 0.6 °C/min. The dashed line indicates the midpoint or temperature of 50% change.

time for folding and lower rates of proteolysis, giving rise to a greater accumulation of the mutant enzymes (20).

To determine if there was any relationship between the apparent abnormalities in the folding pathway and a less stable structure, the denaturation properties of two mutant variants of HP<sub>II</sub> were studied. Specifically, the His128Ala and Asn201His mutant variants, which have no detectable activity and less than 1% of the wild type activity, respectively, and which accumulate protein only when grown at 28 °C, were studied. These two mutant enzymes, which lack the His392–Tyr415 covalent bond, also provided an opportunity to investigate the importance of the unusual bond as a stabilizing factor for the enzyme (13). Because of the very low or complete absence of catalytic activity in the mutant enzymes, spectral changes were used to determine the  $T_m$ s which differed only slightly from the  $T_m$  of the wild type enzyme at 78 °C for the His128Ala variant (Figure 10A) and 84 °C for the Asn201His variant (Figure 10B). This indicated that the susceptibility to denaturation was not increased by the apparent difficulties in folding or by the lack of the Tyr415–His392 covalent bond.

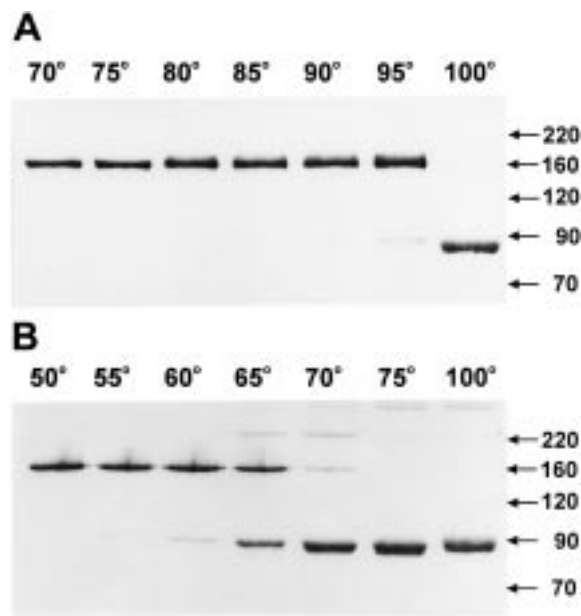


FIGURE 9: Conversion of HP<sub>II</sub> dimers to monomers. In panel A, HP<sub>II</sub> was incubated for 10 min at 70, 75, 80, 85, 90, 95, and 100 °C in 50 mM potassium phosphate buffer (pH 7). In panel B, HP<sub>II</sub> was incubated for 10 min at 50, 55, 60, 65, 70, 75, and 100 °C in 50 mM potassium phosphate (pH 7) and 5.6 M urea. The temperatures of incubation are indicated above each lane. In both panels A and B, 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% polyacrylamide gel. Size markers (indicated as 10<sup>3</sup> Da) were the GIBCO Protein Ladder.

**Susceptibility to Chemical Denaturation.** The sensitivity of HP<sub>II</sub> to the common chemical denaturants urea, guanidinium, and SDS was investigated, revealing an enhancement of enzyme activity following a short incubation in both 1% SDS and 7 M urea, but a partial inactivation in 5 M NaCl and 4 M guanidinium hydrochloride (Table 1). For comparison, BLC was more sensitive to all but 5 M NaCl, and HPI exhibited an intermediate sensitivity. Short periods in guanidinium hydrochloride solutions of up to 5 M (Figure 11A) caused a partial loss of up to 50% of the activity of HP<sub>II</sub>. Solutions above 5 M guanidinium ion caused a loss of activity within minutes. By contrast, BLC and HP<sub>II</sub> were rapidly inactivated in 2.25 and 2.75 M guanidinium ion, respectively. Surprisingly, HPI suffered less inactivation than HP<sub>II</sub> in low concentrations of the denaturant, but HP<sub>II</sub> retained a low level of activity even after 24 h in 4 M guanidinium ion (data not shown). The time course of inactivation of the enzymes in 7 M urea reveals a complete inactivation of BLC within 10 min, very little effect on HP<sub>II</sub> even after 30 min, and a slow inactivation of HPI (Figure 11B). HP<sub>II</sub> retained 75% of the activity even after 42 h. Yeast catalase A responded in a manner similar to that of BLC (data not shown).

As noted above, the CD spectra of HP<sub>II</sub> revealed a loss of helical structure with a midpoint of 82 °C and the dimers remained associated at temperatures up to 95 °C. The CD spectral changes of HP<sub>II</sub> in 5.6 M urea were similar to those determined in buffer as shown in Figure 6, but the midpoint for the change in molar residue ellipticity at 222 nm was lowered almost 20 °C to 65 °C (Figure 12). The presence of urea significantly destabilized the dimer structure, resulting in a sharp transition from the dimer to monomer with a

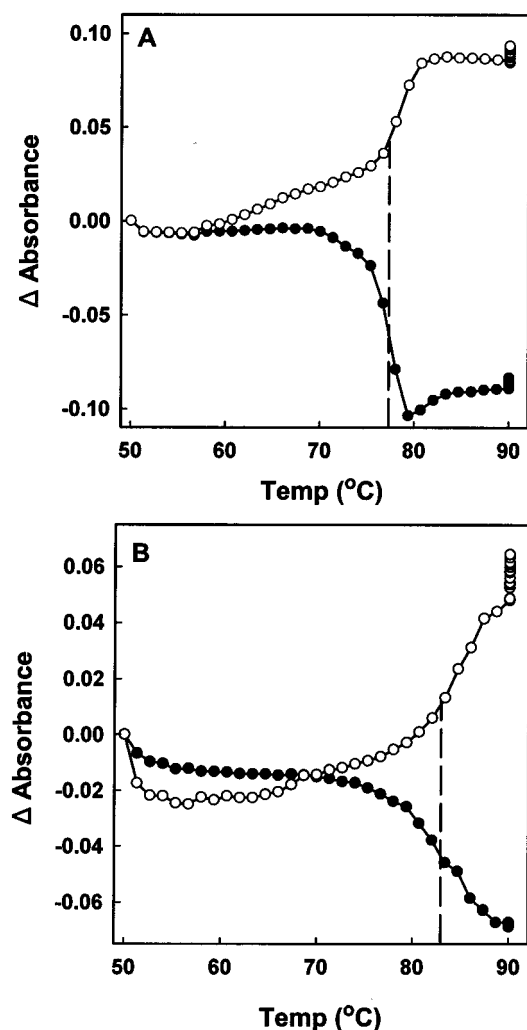


FIGURE 10: Change in absorbance at 280 (○) and 407 nm (●) for the His128Ala mutant variant (A) and the Asn201His mutant variant (B) of HPII as the temperature is raised from 50 to 90 °C at a rate of 0.6 °C/min. The dashed lines indicate the midpoints or temperatures of 50% change.

Table 1: Effect on Catalase Activity of Exposure of HPI, HPII, and BLC for 1 min at 20 °C to Various Denaturants

reagent	% activity retained		
	HPII	BLC	HPI
1% SDS	137	0	119
7 M urea	113	18	96
7 M urea and 1% SDS	137	0	104
5 M NaCl	44	44	58
4 M guanidinium hydrochloride	61	0	0

midpoint of 65 °C (Figure 9B) as compared to the value of >95 °C in the aqueous buffer.

## DISCUSSION

Catalase HPII exhibits a surprising resistance to denaturation for an enzyme derived from an enteric bacterium such as *E. coli* which has a normal temperature for growth in the 30–37 °C range. HPII suffers nonreversible inactivation only above 75 °C in aqueous buffer and retains activity in 5.6 M urea at temperatures up to 60 °C. The inactivation is accompanied by absorbance changes at 280 and 407 nm and by changes in the CD spectrum consistent with there being

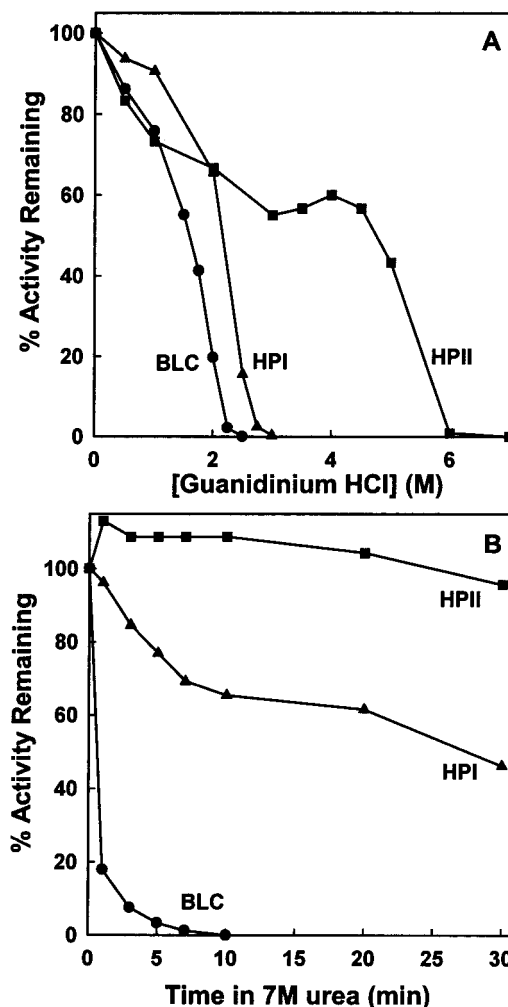


FIGURE 11: (A) Activity of HPII (■), HPI (▲), and BLC (●) remaining after 5 min in various concentrations of guanidinium hydrochloride buffered to pH 7.0 with potassium phosphate. (B) Change in activity of HPII (■), HPI (▲), and BLC (●) during incubation in 7 M urea and 50 mM potassium phosphate buffer (pH 7).

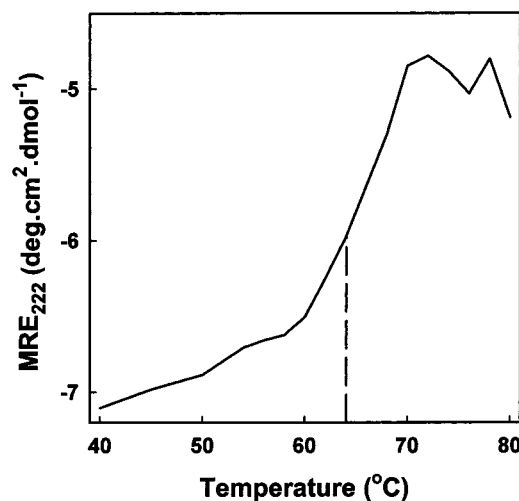


FIGURE 12: Change in molar residue ellipticity (MRE) at 222 nm of HPII in 50 mM potassium phosphate buffer (pH 7.0) and 5.6 M urea as the temperature was raised from 40 to 80 °C at a rate of 0.6 °C/min. The dashed line indicates the midpoint or temperature of 50% change.

irreversible changes in secondary structure. Surprisingly, the quaternary structure is not disrupted in parallel with these

changes, and dimers remain associated even at 95 °C in aqueous buffer.

By contrast, BLC, which has a core sequence and structure very similar to those of HP11, and HPI, the second catalase produced by *E. coli*, are much less stable in that they suffer inactivation at temperatures almost 30 °C lower than does HP11 and are more sensitive to chemical denaturants. HP11 has a much larger subunit than BLC, and the approximately 300 additional residues in HP11 are divided between extensions at the N-terminus (90 residues) and the C-terminus (200 residues). All small subunit catalases have an interwoven structure in which subunits related about the P and Q axes (Figure 1) have approximately 25 N-terminal residues of each subunit folded underneath the "hinge" region leading to the C-terminus of the adjacent subunit. In the case of HP11, the N-terminal extension results in more than 80 residues being trapped, and the trapped residues form many more interactions than the 25 trapped residues in BLC (Figure 1). In addition, the C-terminal domain of HP11 presents a more organized and potentially stable structure than the shorter BLC, and this may contribute further to the stability. The resistance of HP11 dimers to dissociation at 95 °C in buffer and 60 °C in urea, as compared to that of BLC dimers which dissociate to monomers in urea at 25 °C, confirms that the extended sequence and the interwoven structure have an important role in stabilizing the dimers of HP11.

The unusual covalent bond between the C $\beta$  of the essential Tyr415 and the N $\delta$  of the adjacent His392 in HP11 is another structural feature which could potentially contribute to the rigidity and stability of HP11. However, the absence of the linkage in the His128Ala and Asn201His mutant enzymes did not significantly affect their denaturation temperatures, demonstrating that the covalent bond was not an important factor in stabilizing the enzymes. Furthermore, the fact that the mutant variants were as resistant to denaturation as the wild type enzyme and yet experienced difficulty in folding indicates that the mutations compromise the *in vivo* folding pathway but not the ultimate *in vitro* stability of the folded protein.

A recent phylogenetic analysis grouped the 80 known catalase sequences along species lines with enzymes from animals, plants, fungi, and bacteria, forming distinct branches. Only the branch of large subunit catalases was an exception in that it contained enzymes from both fungi and bacteria (including HP11 from *E. coli*) that lacked apparent organiza-

tion along species lines. This was interpreted in terms of horizontal gene transfer between fungi and bacteria, creating the possibility of a nonbacterial origin or at least a nonproteobacterial origin for HP11. By this rationale, HP11 may have originated in an organism capable of survival under more extreme conditions than those for which *E. coli* is adapted. Whether all large subunit catalases exhibit such enhanced stability remains to be determined.

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BI982863Z