Catalase HPII of *Escherichia coli* Catalyzes the Conversion of Protoheme to *cis*-Heme *d*†

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ABSTRACT: Catalase HPII from aerobically grown *Escherichia coli* normally contains heme *d* but cultures grown with poor or no aeration produce HPII containing a mixture of heme *d* and protoheme IX. The protoheme component of HPII from anaerobically grown cells is converted into heme *d* during treatment of the purified enzyme with hydrogen peroxide. It is concluded that heme *d* found in catalase HPII is formed by the *cis*-hydroxylation of protoheme in a reaction catalyzed by catalase HPII using hydrogen peroxide as a substrate. The distal His128 residue of HPII is absolutely required for the protoheme to heme *d* conversion. Two mutant enzymes, Ala128 and Asn128, are catalytically inactive and contain only protoheme, which is unaffected by hydrogen peroxide treatment. The Asn201 residue is not an absolute requirement for heme conversion. The mutant enzyme Ala201 contains predominantly heme *d* and is partially active. However, insertion of a histidyl residue to give the His201 enzyme interferes with the heme conversion reaction. This mutant form is isolated as a protoheme enzyme with limited activity, and a reversible conversion to a heme *d*-like species occurs *in vitro* in the presence of continuously generated hydrogen peroxide.

Catalase (hydrogen peroxide:hydrogen peroxide oxidoreductase, EC 1.11.1.6) has been found in virtually all aerobic organisms. It employs a two-electron-transfer mechanism in the dismutation of hydrogen peroxide to oxygen and water. A structurally diverse family of catalases exists, but the most common form is a homotetramer with one protoheme IX per subunit, as typified by the bovine liver enzyme which is active as such a tetramer of identical subunits (M_r 57 500). The crystal structure of bovine catalase has been determined at 2.5-Å resolution (Murthy et al., 1981), which allowed a detailed prediction of the active site residues and their roles in the catalytic mechanism (Fita & Rossmann, 1985). Two key residues are His74, situated over pyrrole ring III of the heme, and the adjacent Asn147; these are believed to participate directly in substrate binding and catalysis. It is suggested that the hydrogen peroxide is hydrogen bonded to these residues as it makes contact with the heme iron.

Escherichia coli produces two catalases, HPI and HPII, which are quite distinct in physical structure and catalytic properties, both from each other and from other catalases. Catalase HPI (hydroperoxidase I), encoded by *katG*, is a bifunctional catalase–peroxidase containing two protoheme IX groups in a tetramer of identical subunits (M_r 80 000) (Claiborne & Fridovich, 1979). Catalase HPII, encoded by *katE*, has been characterized as a monofunctional catalase with one *cis*-heme *d* isomer per subunit (M_r 84 200) associated in an apparent hexameric structure (Loewen & Switala, 1986). Despite the larger subunit size, apparent hexameric structure, and the unusual heme *d* component, which were suggestive of a unique catalase, striking similarities between the predicted sequence of HPII and the sequences of catalases from several plant, mammalian, and fungal sources have been noted (von Ossowski et al., 1991). All residues presumed to be involved in the active site and in binding protoheme IX in the bovine liver enzyme are conserved in HPII, albeit at different locations in the primary structure. In particular, residues His128 and Asn201 in HPII are homologues of His74 and Asn147 in the bovine enzyme.

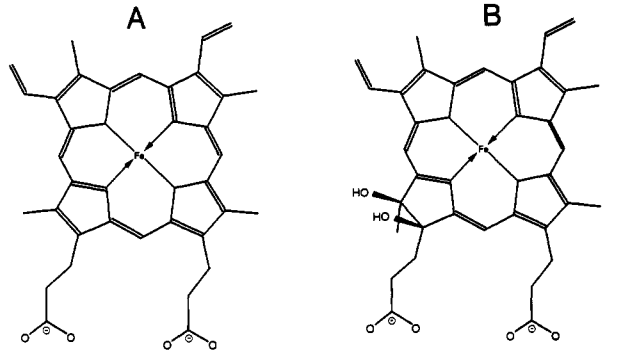

FIGURE 1: Structures of protoheme IX (A) and *cis*-heme d (B).

The origin of the heme *d* in HPII has not been determined. However, it has been suggested (Timkovich & Bondoc, 1990) that protoheme is first bound to the HPII apoenzyme and that the subsequent heme hydroxylation is catalyzed by HPII utilizing one of the first H_2O_2 molecules bound to the enzyme (Figure 1). As His128 is situated over pyrrole ring III, the site of hydroxylation, and Asn201 is in close proximity, a role for these residues in the hydroxylation reaction seemed likely. The data in this paper confirm that a protoheme to heme *d* conversion is catalyzed by HPII and that His128, but not Asn201, is required for the reaction.

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EXPERIMENTAL PROCEDURES

Materials. Standard chemicals and biochemicals were obtained from Sigma Chemical Co. Restriction enzymes, polynucleotide kinase, DNA ligase, and the Klenow fragment of DNA polymerase were obtained from either GIBCO-BRL or Pharmacia.

The plasmid pAMkatE72 (von Ossowski et al., 1991) was used as the source for the *katE* gene. Phagemids pKS+ and pKS- from Stratagene Cloning Systems were used for mutagenesis, sequencing, and cloning. *E. coli* strain NM522 *supE thi* Δ(*lac-proAB*) *hsd-5* [F'*proAB lacI*^{q} *lacZ*Δ*15*] (Mead et al., 1985) was used as host for the plasmids and for generation of single-strand phage DNA using helper phage R408. *E. coli* strain UM255 *pro leu rpsL hsdM hsdR endI lacY katG2 katE12*::Tn*10 recA* (Mulvey et al., 1988) was used for expression of the mutant *katE* constructs and isolation of the mutant HPII proteins.

Oligonucleotide-Directed Mutagenesis. Oligonucleotides were synthesized on a PCR-Mate synthesizer from Applied Biosystems. For His128 replacements, the sequence CAT at 1202 (von Ossowski et al., 1991) was changed to GCT (Ala128) using 5'-GCTATTGTTGCTGCACGCGG and AAT (Asn128) using 5'-GCTATTGTTAATGCACGCGG. A 1.2-kb fragment containing the CAT codon, corresponding to nucleotides 1–1247 of the *katE* sequence (von Ossowski et al., 1991), generated by *Pst*I and *Hin*dIII, was cloned into pKS- for mutagenization according to the method of Kunkel (1985). For Asn201 replacements, the sequence AAT at 1421 was changed to GCT (Ala201) using 5'-CTCGTTG-GCGCTAACACGCCA, GAT (Asp201) using 5'-CTCGT-TGGCGATAACACGCCA, CAT (His201) using 5'-CTCGT-TGGCCATAACACGCCA, and CAA (Gln201) using 5'-CTCGTTGGCCAAAACACGCCA. A 0.6-kb fragment containing the AAT (Asn201) codon, corresponding to nucleotides 1247–1856 of the *katE* sequence, generated by *Hin*dIII and *Eco*RI was cloned into pKS- for mutagenization. Sequence confirmation was by the Sanger method (Sanger et al., 1977) on single-stranded DNA from the same phagemids. The mutagenized fragments were then reincorporated into pAMkatE72 to generate the mutagenized *katE* genes and transformed into UM255 for expression.

Plasmid Expression. Cultures of *E. coli* UM255 transformed with plasmids carrying one of the mutant *katE* genes were grown in LB medium containing 10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl for 16 h at 37 °C with shaking. Cells were harvested and HPII was isolated as described by Loewen and Switala (1986) with the modification that DEAE-cellulose DE52 (Whatman) was used in place of DEAE-Sephadex A-25. Where there was little or no catalase activity associated with the HPII protein, the purification was followed by SDS–polyacrylamide gel electrophoresis (Weber et al., 1972). The protein was usually more than 90% pure following the ion-exchange chromatography step, and when an additional purification step was required, either a second passage through DEAE-cellulose or gel filtration through Bio-Gel A-1.5 m was employed. The specific activities of the various His128 and Asn201 replacement mutants are shown in Table I.

Catalase Assay and Protein Determination. Catalase activity was determined by the method of Rorth and Jensen (1967) 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 (1957).

Table I: Specific Activities of the Wild-Type and Mutant HPII Catalases[a]

mutant	activity (units/mg)	mutant	activity (units/mg)
WT[b]	15200	N201H	100
H128A	<0.1	N201D	1700
H128N	<0.1	N201A	1300
		N201Q	50

[a] Enzyme preparations were assayed at 37 °C in 50 mM potassium phosphate, pH 7.0, using 60 mM H_2O_2. [b] Wild type.

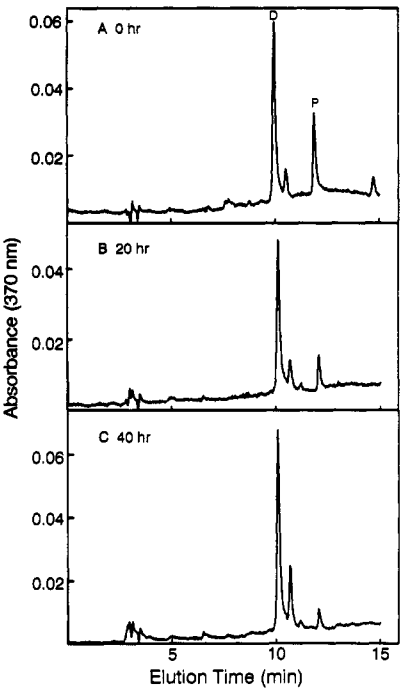

FIGURE 2: Reversed-phase HPLC chromatograms for the heme isolated from HPII catalase before (A), and following 20 hr (B) or 40 hr (C) of incubation with 100 mM ascorbate at 20 °C. The peak of heme *d* is indicated by D, and the protoheme peak is indicated by P.

Spectral Determinations. Absorption spectra were obtained using a Milton Roy MR3000 spectrophotometer and a Beckman DU-7HS spectrophotometer. Samples were dissolved in 50 mM potassium phosphate, pH 7.0, unless otherwise stated.

Hemochromogen Characterization. The hemochromogen from various fractions was extracted in acetone containing 0.13% by volume concentrated HCl. After the precipitated protein was removed by centrifugation, an appropriate volume of a Na_2CO_3 solution was added to neutralize the HCl. The sample was evaporated to dryness and separated on a 4.6 × 250 mm column packed with Whatman 5 μM ODS III (C_{18} coating) eluted with a gradient of 24:24:48:4 to 48:48:0:4 acetonitrile/methanol/water/acetic acid in a LKB HPLC system with optical detection at 370 nm.

RESULTS

Heme Content of HPII. The heme component of wild-type HPII has been characterized as *cis*-heme *d* (Chiu et al., 1989). However, a recent HPLC analysis of some HPII preparations revealed that as much as 10–20% of the heme could be protoheme. The elution profile for a typical preparation (Figure 2 A) shows two main peaks, one of heme *d* (labeled D) and a second smaller peak of protoheme (labeled P). The protoheme peak was eluted coincident with authentic protoheme from either catalase HPI or bovine catalase. The

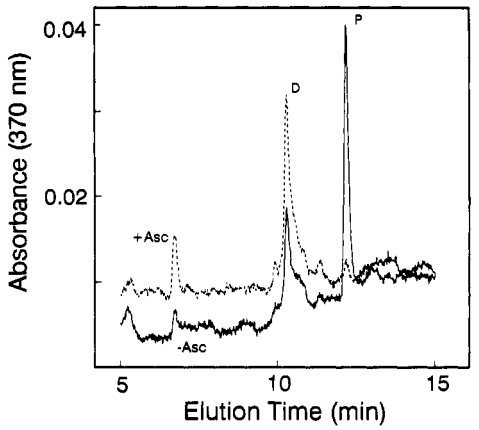

FIGURE 3: Reversed-phase HPLC chromatogram for the heme isolated from HPII isolated from anaerobically grown UM255-[pAMkatE72] before (dashed line) and after (solid line) treatment with 100 mM ascorbate for 60 min. The peak of heme *d* is indicated by D, and the peak of protoheme is indicated by P.

relative size of the two peaks varied from preparation to preparation. No protoheme at all was present in a few preparations as described by Chiu et al., (1989). Timkovich and Bondoc (1990) have proposed that the HPII heme *d* may arise from the *in situ* hydroxylation of protoheme (Figure 1). If correct, this would suggest that protoheme was present in some preparations of HPII because of incomplete conversion. This was tested by treating HPII samples containing protoheme with ascorbate, the oxidation of which generates low levels of H_2O_2 (Richter & Loewen, 1981) which can be used as a substrate for the enzyme. As shown in Figure 2B,C, the protoheme peak disappeared during the treatment, consistent with its conversion to heme *d*. However, because only small amounts of protoheme relative to heme *d* were originally present, it was not possible to confirm complete conversion.

Because the quantity of protoheme varied among preparations, the effectiveness of aeration during growth may have determined the extent of conversion. This was confirmed when HPII isolated from anaerobically grown cells was found to contain predominantly (>75%) protoheme. Upon treatment of this "anaerobic" HPII with ascorbate, there was a rapid conversion of the protoheme into heme *d* (Figure 3).

Isolation of Mutant HPII Catalases. The core of the predicted amino acid sequence of HPII is very similar to the sequences of a number of eukaryotic catalases (von Ossowski et al., 1991) including the bovine liver enzyme. Sequence similarity is particularly striking in the central region containing predicted active site residues including His128 and Asn201 (Figure 4). In the eukaryotic enzyme, His74 and Asn147 were predicted to be involved in binding the substrate H_2O_2. The roles of their HPII homologues, His128 and Asn201, in heme conversion were tested using site-directed mutants as outlined in Figure 4. Of the His128 replacements, only the Ala128 and Asn128 enzymes were sufficiently stable to accumulate protein; of the Asn201 replacements, the Ala201, Asp201, His201, and Gln201 mutants all formed stable, isolable proteins.

Spectral Properties of the Mutant Catalases. HPII containing heme *d* has a spectrum with characteristic absorption maxima at 590 and 715 nm (Loewen & Switala, 1986). A protoheme-containing enzyme such as HPI has these absorption maxima shifted to 535 and 630 nm, respectively. The absorption spectra of the mutant proteins were consistent with the presence of one predominant heme species, either protoheme in the Ala128, Asn128, and His201 mutant proteins or heme *d* in the Ala201, Gln201, and Asp201 mutant proteins. In the latter three mutants, shoulders on the main absorption peaks were consistent with the presence of some protoheme, but accurate quantitation from the spectra was not possible. The spectral data for these peaks, normalized to the main Soret absorption at 407 nm, are summarized in Table II.

The Ala128, Asn128, and His201 mutant enzymes containing predominantly protoheme were incubated with ascorbate to study the ease of protoheme to heme *d* conversion. There was no change in either activity or spectra for both the Ala128 and Asn128 enzymes. However, there was an increase in specific activity of the His201 mutant enzyme (Figure 5A) and a change in its predominantly protoheme-like spectrum to one that was predominantly heme *d*-like (Figure 6). Thus, protoheme hydroxylation was apparently taking place in the active site of the mutant, resulting in a more active enzyme. A similar conversion of the spectrum was observed using glucose oxidase plus glucose as the source of H_2O_2 (data not shown). However, HPLC analysis of the heme components of the His201 enzyme before and after ascorbate treatment revealed no permanent conversion of the protoheme despite the spectral changes (Figure 7).

Effect of 2-Mercaptoethanol. Thiol compounds in aerobic solution inhibit eukaryotic catalase activity (Takeda et al., 1980) by a mechanism that may involve the reduction of O_2 by thiols to the reactive superoxide radical. If the rate of enzyme inactivation by superoxide is slower than the rate of dismutation of superoxide to H_2O_2, the His201 mutant should exhibit an increase in activity upon treatment with thiols. This increase in activity should be similar to, but less than, that observed during ascorbate treatment because of the competing inactivation reaction. This is indeed what was observed, with the His201 mutant being activated by 5 mM 2-mercaptoethanol (Figure 5B) to a lesser extent than by ascorbate (Figure 5A). As expected, the wild-type HPII was inhibited 40% during a 1-h incubation with 5 mM 2-mercaptoethanol.

Azide Binding to HPII. Like eukaryotic catalases, HPII and the Asn201 mutants formed spectroscopically distinct high-spin complexes with azide (Figure 8A), confirming that azide can bind at the active site of HPII. Azide also reacts with complex I of eukaryotic catalases to produce several inactive ferroenzyme species (Nicholls, 1964). A similar reaction takes place with HPII. Titration of the azide–HPII complex in a CO-saturated buffer with H_2O_2 resulted in formation of the (carbonmonoxy)ferroenzyme complex. The spectral change involved is shown in Figure 8A. In the cases of the wild-type enzyme (Asn201) and the Ala201 and Gln201 mutants, such titrations were almost stoichiometric (Figure 8B). Only one H_2O_2 molecule is required to convert one heme group, as with the eukaryotic enzyme. But the titration of the azide–His201 complex required a far larger amount of H_2O_2 to achieve a similar conversion (Figure 8B). Therefore, the formation of complex I by this mutant is probably more difficult or less efficient than in the wild-type enzyme or the other mutants. This is consistent with the idea that the presence of protoheme rather than heme *d* diminishes the enzyme's specific activity relative to that of the wild-type enzyme.

DISCUSSION

E. coli is capable of controlling heme biosynthesis so that it can produce much larger than normal amounts of protoheme or heme *d*. Such large amounts are required in cells overproducing plasmid-encoded catalases HPI and HPII. The

msqhneknphqhqsplhdsseakpgmdslapedgshrpaaeptppgaqptapqSlkapDtrnEkLNsleDvRkGSENyaLTT

(A,Q,E,N)$_{128}$

NQGvrIADDqNSLRAGsRGPtLLDEFILrEKItHFDHERIPERIVHARGSAAHGYFQpyKSLSDITKAdFLsDpnKiTPVFV

(A,D,H,Q,R)$_{201}$

RFSTVqGGaGSADTVRDiRGFATKFYTEEGiFDLVGNNTPIFFIqDAhKFPdFVHAvKPEpHwaiPQgqsahDTFWDYVSLQ

PETLHNVMWamSDRGIPrSYRTMEGFGIHTFRLINAEGKATFVRFHWKPlaGkaSLvwDEAqKLTGrDPDFHrRELWEAIEA

GDFPEYELgfQLIpEEDEfKFDFDLLDpTKLiPeelVPVqRVGKMVLNRNPDNFFAENEQAAFhPGHIVPGLDFTNDPLLQG

RLFSYTDTQisRLGgPNFhEIPINRPtCPYHNFQRDGMhrMGiDTNpaNYePNSiNDNWpreTpPgPKrggfesyQerVeGN

kVrERsPsfgEYyshPRLfwlsQtpFeqrHIVDGFsFelsKvVRpYiRErvvDqLAHIDltLaQaVAKnlGIeLtDdqlnIT

pppdVNglkkDpslslyaipdGdvkgrvvaillndevrsadllailkalkakgvhakllysrmgevtaddgtvlpiaatfag

apsltvdavivpcgniadiadngdanyylmeaykhlkpialagdarkfkatikiadqgeegiveadsadgsfmdelltlmaa

hrvwsripkidkipa

FIGURE 4: Amino acid sequence of catalase HPII. Capitalized residues are conserved in the bovine liver catalase and include the active site residues indicated with an asterisk or an arrow. The two residues changed for the current study, His128 and Asn201, are indicated with arrows pointing at the replacement residues.

Table II: Absorption Spectral Data for Wild-Type and Mutant Catalases[a]

mutant	A_{535}/A_{407}	A_{590}/A_{407}	A_{630}/A_{407}	A_{715}/A_{407}
HPI (WT)	0.13		0.08	
HPII (WT)		0.17		0.06
H128A	0.11		0.07	
H128N	0.14		0.09	
N201H	0.11		0.08	
N201D		0.14		0.04
N201A		0.17		0.08
N201Q		0.19		0.09

[a] Spectral determinations were on 1 mg/mL protein solutions in 50 mM potassium phosphate, pH 7.0.

biosynthetic pathway leading to protoheme IX is known, but the enzymes responsible for the *cis*-hydroxylation of protoheme on ring III to produce heme *d* have not been identified. This led Timkovich and Bondoc (1990) to propose that the reaction may be catalyzed by HPII itself. According to this hypothesis, only protoheme binds to the HPII apoenzyme during enzyme assembly; it is subsequently converted to heme *d* using one of the first few H_2O_2 molecules to bind in the active site as substrate. The present paper describes the direct observation of heme conversion in purified HPII, supporting the hypothesis. Poor culture aeration results in HPII containing some protoheme, and the ratio of protoheme to heme *d* could be significantly increased by growing the cells anaerobically. If the amount of *in situ* H_2O_2 generated were lower in poorly aerated cells, this would limit the amount of available substrate. Consistent with this hypothesis, the O_2 limitation results in incomplete conversion of protoheme to heme *d*. Overproduction of plasmid-encoded HPII will increase the effectiveness of O_2 limitation with far greater than normal amounts of the enzyme being present in the cell. Subsequent treatment of the protoheme-containing HPII results in a rapid conversion of the protoheme to heme *d*.

The crystal structure of bovine catalase places His74 just above pyrrole ring III of protoheme and the Asn147 residue

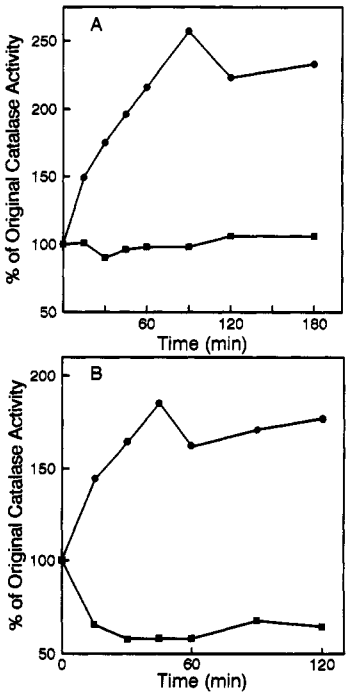

FIGURE 5: Effects of 5 mM ascorbate (A) and 5 mM 2-mercaptoethanol (B) on the activity of wild-type HPII (■) and the His201 mutant enzyme (●). The enzymes were incubated at 37 °C, and samples were removed at the times indicated for assay.

in close proximity. Both play a role in the model for the dismutation of H_2O_2 proposed by Murthy et al. (1981). As the site of HPII heme hydroxylation is also pyrrole ring III, this suggests that the counterparts of His74 and Asn147 in HPII, His128 and Asn201, may be involved with the heme conversion as well. Replacement of His128 with Ala128 or Asn128 results in mutant HPIIs containing only protoheme, confirming that His128 is absolutely required for heme conversion.

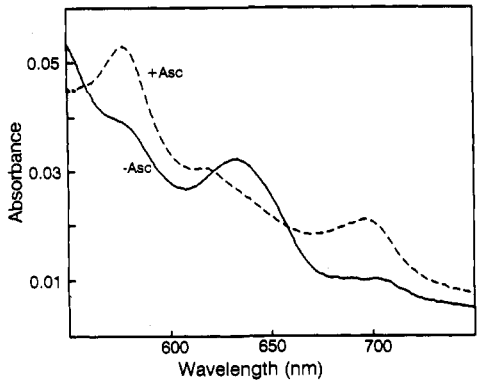

FIGURE 6: Effect of 16-h incubation at 20 °C without (solid line) and with 100 mM ascorbate (dashed line) on the absorption spectrum of the His201 mutant enzyme.

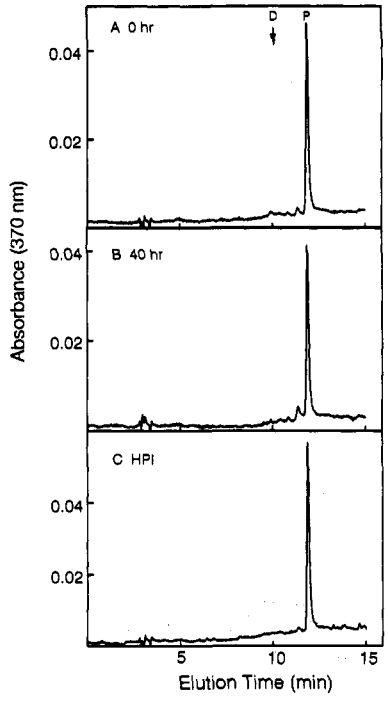

FIGURE 7: Reversed-phase HPLC chromatograms for the heme isolated from the His201 mutant enzyme before (A) and after (B) a 40-h incubation with 100 mM ascorbate at 20 °C and from catalase HPI (C) without incubation. The position expected for eluting heme *d* is indicated by D, and the protoheme peak is indicated by P.

Asn147 in bovine catalase was predicted to form a hydrogen bond with H_2O_2, promoting bond polarization and breakage. While this residue is also in the vicinity of the hydroxylation site, its role in the heme conversion is not clear. The Ala201 mutant enzyme, in which the methylcarbonylamide group is replaced by a methyl group, contains predominantly heme *d*, showing that the asparagine side chain is not required for the heme conversion. Similarly, the Gln201 mutant enzyme contains predominantly heme *d*, indicating that an increase in the length of the side chain by one methylene group does not affect heme conversion. However, the imidazole group in the His201 mutant prevents heme conversion and the mutant enzyme as isolated contains only protoheme. Apparent conversion of the protoheme in the His201 mutant with H_2O_2 was successful as judged by spectral changes and an increase in enzyme activity. However, only protoheme could be isolated from the "converted" enzyme, indicating that the hydroxylation mechanism had perhaps been blocked at an intermediate stage that could rapidly reverse to protoheme upon heme extraction. Whether this blockage in the conversion is purely a steric

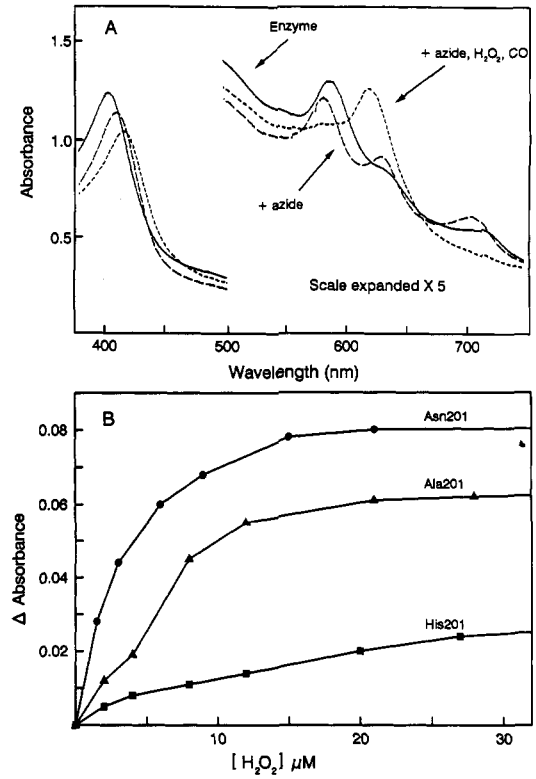

FIGURE 8: Effect of azide and peroxide on the spectrum of HPII and titrations of wild-type and mutants with H_2O_2. (A) Spectra of HPII, azide–HPII, and the azide–CO–H_2O_2 complex. Approximately 10 μM HPII was dissolved in 17 mM potassium phosphate, pH 6.5,, at 30 °C (solid line), plus 1.33 M sodium azide (long-dashed line) or plus 1.3 M azide, saturating (approximately 1 mM) carbon monoxide, and 30 μM H_2O_2 (short-dashed line). Scale from 375 to 500 nm (Soret region) is 0.0–1.5 A and from 500 to 750 nm (visible region) is 0.0–0.3 A. (B) Titrations of wild-type (Asn201) HPII (●), Ala201 mutant (▲), and His201 mutant (■) azide complexes with H_2O_2. Enzyme samples as in Figure 7A were titrated with successive aliquots of 1.0 mM H_2O_2 and the spectral changes monitored in the visible and Soret regions. The plotted values are the differences between peak and trough absorbances for the change in the visible region (582–620 nm) in each case. All solutions were CO-saturated to ensure stoichiometric trapping of the reduced species, presumably as the $Fe^{2+}CO$ complex (Nicholls, 1964).

effect of the imidazole ring or a consequence of a more substantive change in H_2O_2 binding cannot be deduced from these results. The solution of the crystal structure of HPII (Tormo et al., 1990) may answer this question and should also clarify differences between the active sites of HPII and the bovine enzyme that facilitate heme *d* formation in HPII.

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