Mutants That Alter the Covalent Structure of Catalase Hydroperoxidase II from Escherichia coli*

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The three-dimensional structures of two HPII variants, V169C and H392Q, have been determined at resolutions of 1.8 and 2.1 Å, respectively. The V169C variant contains a new type of covalent bond between the sulfur atom of Cys169 and a carbon atom on the imidazole ring of the essential His128. This variant enzyme has only residual catalytic activity and contains heme b. The chain of water molecules visible in the main channel may reflect the organization of the hydrogen peroxide substrates in the active enzyme. Two alternative mechanisms, involving either compound I or free radical intermediates, are presented to explain the formation of the Cys-His covalent bond. The H392Q and H392E variants exhibit 75 and 25% of native catalytic activity, respectively. The Gln192 variant contains only heme b, whereas the Gln392 variant contains a mixture of heme b and cis and trans isomers of heme d, suggesting of a role for the cis residue in heme conversion. Replacement of either Gln392 and Ser414, both of which interact with the backbone of the heme, affected the cis:trans ratio of spirolactone heme d. Implications for the heme oxidation mechanism and the His-Tyr bond formation in HPII are considered.

Catalase (hydroperoxide:hydroperoxide oxidoreductase, EC 1.11.1.6) is an ubiquitous component of the defense system against oxidative stress that virtually all cells growing under aerobic conditions possess. The crystal structures of six heme-containing catalases have now been solved revealing a common highly conserved core in all enzymes. These structures include three prokaryote enzymes, Micrococcus luteus catalase (1), Proteus mirabilis PR (2), and Escherichia coli hydroperoxidase II (HPII) (3, 4), and three eukaryote enzymes, Penicilium vitale (PVC) (5, 6), catalase A from Saccharomyces cerevisiae (7), and bovine liver catalase (8, 9).

Catalase catalyzes the dismutation of hydrogen peroxide by one of two reaction pathways depending on conditions. Both pathways begin with the formation of compound I, an intermediate which holds two oxidation equivalents (basically an oxygen-ferryl group with the iron in the Fe^4+ state and a porphyrin cation radical) (10, 11) (Reaction 1).

\[
\text{Cat}(\text{Por-Fe}^{III}) + \text{H}_2\text{O}_2 \rightarrow \text{Comp. I}(\text{Por}^\cdot\text{-Fe}^{IV} = \text{O}) + \text{H}_2\text{O} 
\]

Reaction 1

Compound I is an active oxidant which can either react with a second molecule of H2O2 to give oxygen and water, completing the catalatic mechanism (Reaction 2), or oxidize another small substrate molecule, for example ethanol, completing the peroxidatic mechanism (Reaction 3).

\[
\text{Comp. I}(\text{Por}^\cdot\text{-Fe}^{IV} = \text{O}) + \text{H}_2\text{O}_2 \rightarrow \text{Cat}(\text{Por-Fe}^{III}) + \text{H}_2\text{O} + \text{O}_2 
\]

Reaction 2

\[
\text{Comp. I}(\text{Por}^\cdot\text{-Fe}^{IV} = \text{O}) + \text{CH}_3\text{CH}_2\text{OH} \rightarrow \text{Cat}(\text{Por-Fe}^{III}) + \text{CH}_3\text{CHO} + \text{H}_2\text{O} 
\]

Reaction 3

Under certain conditions, compound I can be reduced by a one-electron addition resulting in the formation of compound II (a formal Fe^4+ state) which can lead to inactivation of the enzyme. Small subunit catalases (12), utilize NADPH to prevent the accumulation of compound II while the large subunit enzymes, HPII and PVC, do not form compound II, which may explain why they do not require NADPH.

The structure of native HPII, solved and refined at 1.9-Å resolution contains a cis-hydroxochlorin γ-spirolactone heme d and a novel type of covalent bond joining the C\(^\equiv\) of the essential Tyr115 and the N\(^\circ\) of His392 (4, 13). As part of an ongoing study of catalases, HPII mutant variants with changes in Val168, Asp197, His192, His392, Ser414, and Gln119 were produced. The HPII variant V169C, designed to investigate the effect of changes in the major channel leading to the active site, was found to contain an unusual covalent bond between the essential histidine (His128) and the substituted residue (Cys169). Variants in other positions were produced to study the mechanism of heme oxidation and Tyr-His bond formation in HPII. In particular, a variant of His392 was designed to change the residue to its Glu (14) counterpart in PVC which has heme d but not the His-Tyr bond. We report here the enzymatic characterization of 12 mutant variants of HPII and the structure determination of two of them, V169C and H392Q, refined at 1.8- and 2.1-Å resolution, respectively. Implications of the biochemical and structural peculiarities found for these variant enzymes are discussed.
**EXPERIMENTAL PROCEDURES**

**Materials**—Standard chemicals and biochemicals were obtained from Sigma. Restriction nucleases, polynucleotide kinase, DNA ligase, and the Klenow fragment of DNA polymerase were obtained from Life Technologies, Inc.

**Strains and Plasmids**—The plasmid pAMkatE72 (15) was used as the source for the katE gene. Phagemids pKS and pKS15 from Stratagene Cloning Systems were used for mutagenesis, sequencing, and cloning. E. coli strains MM22 (supE thi 2-lac-proAB lacZ15), JM109 and JM109 (recA1 supE44 endA1 hisD17 gyrA96 relA1 thi-1 lac-proAB; Ref. 17) were used as hosts for the plasmids and for generation of single-strand plasmid DNA using helper phage R408. Strain UM255 (pro leu rpsL hsdM hsdR endA1 lacI2 KatE2; Tn10 recA; Ref. 19) 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 and are listed in Table I. The restriction nuclease fragments, that were mutagenized following the Kunkel procedure (18), were sequenced and subsequently reincorporated into pAMkatE72 to generate the mutagenized katE genes, are also listed. Sequence confirmation of all sequences was by the Sanger method (20) on double-stranded plasmid DNA generated in JM109. Subsequent expression and purification were carried out as described previously (21).

**Catalase Assay and Protein Determination**—Catalase activity was determined by the method of Rar and Jensen (22) in a Gilson oxi
graph equipped with a Clark electrode. One unit of catalase is defined according to the method of Rørth and Jensen (22) in a Gilson oxy
described previously (21).

**RESULTS**

| Mutant | Sequence change | Oligonucleotide | Restriction fragment |
|--------|----------------|----------------|---------------------|
| V189A  | (GTT-GCT)      | TTCTGACGGTTGTC  | HindIII-EcoRI (1246–1861) |
| V186S  | (GTT-ACT)      | TTCTGACGGTTGTC  | HindIII-EcoRI (1246–1861) |
| V186C  | (GTT-TGT)      | TTCTGACGGTTGTC  | HindIII-EcoRI (1246–1861) |
| D197A  | (GAC-GCC)      | GAGGTATTTTTGCTGGTTGTTG  | HindIII-EcoRI (1246–1861) |
| D197S  | (GAC-AGC)      | GAGGTATTTTTGCTGGTTGTTG  | HindIII-EcoRI (1246–1861) |
| H392A  | (CAT-GCT)      | GCGTCTTGCCTGATGGCAT  | EcoRI-ClaI (1856–3466) |
| H392Q  | (CAT-CAG)      | GCGTCTTGCCTGATGGCAT  | EcoRI-ClaI (1856–3466) |
| H392E  | (CAT-GAA)      | GCGTCTCAGCCTGATGGCAT  | EcoRI-ClaI (1856–3466) |
| H392D  | (CAT-GAT)      | GCGTCTTCAGCCTGATGGCAT  | EcoRI-ClaI (1856–3466) |
| H395A  | (CAT-GCT)      | ATCTGGGATCTGGCCG  | EcoRI-ClaI (1856–3466) |
| H395Q  | (CAT-CAG)      | ATCTGGGATCTGGCCG  | EcoRI-ClaI (1856–3466) |

**Oligonucleotides and katE restriction fragments used in oligonucleotide-directed mutagenesis of katE**

**Data collection and structural refinement statistics**

| Protein | V169C | H392Q |
|---------|-------|-------|
| A, B, C |       |       |

**TABLE II**

| Protein | V169C | H392Q |
|---------|-------|-------|
| A, B, C |       |       |

**Major Channel Variants of HPII**—The side chain of Val169 is situated immediately above the essential residue His128 where the major access channel enters the distal heme pocket (Fig. 1A). This valine is fully preserved among all known catalase
FIG. 1. Stereo views of the heme distal side pocket and of the final part of the major channel in wild type HPII (A), the V169C variant (B), and the H392Q variant (C). The corresponding electron densities are shown for the three cases. In the V169C variant structure, a chain of well defined solvent molecules reach the distal pocket as compared with the native and H392Q structures where solvent molecules are...
sequences and it is placed in the narrowest and most hydrophobic section of the channel. Changing the equivalent residue in catalase A from *S. cerevisiae* to a smaller residue (V111A) resulted in an enzyme with reduced catalatic activity but enhanced peroxidatic activity for larger substrates (34). This was attributed to a disruption in the flow of H_{2}O_{2} in the enlarged channel and to easier access for the larger peroxidatic substrates. The rationale behind changing Val^{169} → Cys in HPII was that subsequent selective reaction of the sulfhydryl group might allow progressive blockage of the access channel. This objective was precluded by the finding that the purified V169C mutant had only about 0.1% of the native HPII activity, and contained heme b as determined by HPLC and spectral analyses (Table III). In order to define if these dramatic and unexpected changes were a result of steric factors or another property specific to the cysteine residue, the V169A and V169S mutant variants were constructed. These two variant enzymes contained heme d and exhibited about 25% of wild type activity, similar to the change in activities of the equivalent Val mutants in catalase A from *S. cerevisiae* and significantly higher than the activity of the V169C variant. No o-dianisidine peroxidatic activity was detected. From this, it was concluded that the Cys residue was a determining factor in the reduced catalatic activity and in the lack of conversion of heme b to heme d.

A recent study (30) of the peroxidatic activity of the V169C variant of HPII showed that the enzyme had only about 0.1% of the native HPII activity, and contained heme b as determined by HPLC and spectral analyses. The remarkable reduction in activity was attributed to a disruption in the flow of H_{2}O_{2} in the enlarged channel and to easier access for the larger peroxidatic substrates. This disruption of catalatic activity is consistent with the hypothesis that the equivalent residue in catalase A from *S. cerevisiae* can be replaced with a smaller residue (V111A) without significantly affecting catalatic activity, but enhancing peroxidatic activity for larger substrates. The rationale behind changing Val^{169} → Cys in HPII was that subsequent selective reaction of the sulfhydryl group might allow progressive blockage of the access channel. This objective was precluded by the finding that the purified V169C mutant had only about 0.1% of the native HPII activity, and contained heme b as determined by HPLC and spectral analyses. In all cases, the enzymes contained one heme per subunit.

### TABLE III

| Mutation         | Specific activity | Heme | His^{392,Tyr^{415}} |
|------------------|------------------|------|---------------------|
| Wild type        | 14.322           | d    | Yes                 |
| V169A            | 3.788            | d    | Yes                 |
| V169S            | 3.703            | d    | Yes                 |
| V169C            | 16.8              | b    | No                  |
| D197A            | 14.354           | d    | Yes                 |
| D197S            | 14.721           | d    | Yes                 |
| D197S/H395Q      | 15.473           | d    | Yes                 |
| H392A            | 8.875            | b    | No                  |
| H392Q            | 7.368            | b    | No                  |
| H392E            | 4.507            | b + d’| No                  |
| H392D            | 2.857            | b    | No                  |
| H395A            | 9.821            | d    | Yes                 |
| H395Q            | 9.224            | d    | Yes                 |
| S414A            | 5.511            | b + d’| Yes                 |
| Q419A            | 7.317            | d’  | Yes                 |
| Q419H            | 11.494           | d’  | Yes                 |

a Heme composition was determined by spectral and HPLC analyses. In all cases, the enzymes contained one heme per subunit.

b Presence of the His^{392}-Tyr^{415} covalent bond was determined by MALDI-MS analysis of tryptic digest mixtures.

c There was an increased percentage of the trans isomer in these variants.

The most striking feature evident in the final electron density maps of the V169C variant is the presence of a covalent bond between the sulfur atom of Cys^{169} and the imidazole ring of His^{128} (Fig. 1B and Fig. 2). The shape of the electron density and the refinement behavior strongly suggest that, within the limitations of the resolution available, the sulfur atom from Cys^{169} together with all the atoms from the imidazole ring of His^{128} are in a common plane. Perfect planarity implies that the atoms in the imidazole ring retain the sp^{2} hybridization state which imposes strong constraints on the possible chemical mechanisms that can produce the covalent bond. The modified imidazole ring is rotated about 30° relative to its position in wild type HPII and its orientation can be unambiguously defined by the proximity of a water molecule within 3.1 Å of the imidazole N° atom and by the bifurcated hydrogen bond formed by N° with both O° from Ser^{167} and main chain carbonyl oxygen from Thr^{168} (Fig. 2). This leaves the C° of the imidazole ring situated just 1.84 Å from the cysteine sulfur atom, a distance consistent with a covalent bond. The rigidity introduced by the new Cys-His covalent bond that would likely raise the pK_{a} of the imidazole ring together with the altered imidazole-heme stacking, should both contribute to the diminished catalytic activity of the V169C variant.

The moderate increase in the heme channel volume that results from the Val to Cys replacement can only explain in part the presence of four extra solvent molecules in the variant enzyme (Figs. 1, B and D, and 2). These solvent molecules form a continuous hydrogen bonded chain that extends the full-length of the channel from the molecular surface to the distal pocket, ending with the water molecule above the heme iron. The presence of this continuous chain of solvent molecules reaching the active center proves that the low activity of this mutant is not a result of steric hindrance to substrate access. In native HPII, the solvent chain is interrupted in the vicinity of Val-169.

The V169C variant crystal structure confirmed the presence of heme b, rather than heme d as had already been suggested by HPLC analysis, and also confirmed the mass spectroscopic analyses that showed the absence of the Tyr^{415}-His^{392} covalent bond found in wild type HPII. The absence of the two covalent modifications in the V169C variant is similar to the situation found in the structure of the inactive or weakly active variants, H128A, H128N, and N201H (Fig. 3) (12). In these structures, Glu^{319} is rotated forming a hydrogen bond with the heme propionate side chain and with a water molecule located near Thr^{116}. Furthermore, the main chain from residues 414 to 417 is shifted with respect to the native model, and Ile^{136}, on the heme distal side, exists in two conformations likely due to the restrictions imposed by its proximity to the propionate chain (Fig. 3).

**Mass Analysis of Tryptic Peptides—** An independent confirmation of the presence of the His^{128}-Cys^{169} covalent bond was provided by a mass analysis, using MALDI-MS, of trypsin digests of wild type HPII and of the V169C variant. Complete digestion of HPII by trypsin should generate a mixture of 75 peptide products, which will be further complicated, particu-
larly below 3000 Da, by the presence of partial digest products. His128 and Val169 (or Cys169) are situated on separate tryptic peptides (Fig. 4B). The His128-containing peptide extends from Ile126 to Arg130 and has a predicted mass of 596 Da. The Val169-containing peptide extends from Phe166 to Arg180, and replacement of Val with Cys would increase the predicted mass from 1453 to 1457 Da. Covalent linkage of the His128- and Cys169-containing peptides through the imidazole-sulfur bond would produce a 2051-Da peptide, assuming that 2 hydrogens were lost as part of the covalent bond formation. The mass spectrum of a tryptic digest of wild type HPII (Fig. 4A) confirms the absence of significant peptide peaks in the 2000 to 2100 Da range. By comparison, the mass spectrum of a tryptic digest of the V169C variant (Fig. 4B) shows a prominent peak in this range with a mass of 2051, consistent with the presence of the His128-Cys169 covalent linkage.

Heme Proximal Side Variants of HPII—The conversion of heme b to heme d and of the formation of the His392-Tyr415 covalent bond in HPII have been linked in a concerted mechanism that requires the formation of compound I. The proposed mechanism was supported by the observation that neither modification is found in the catalytically inactive variants of HPII, H128A, H128N, and N201H previously reported (21). In PVC, the His-Tyr bond cannot be formed because the residue that would correspond to the histidine is a glutamine. However, PVC does form the V169C variant (Fig. 3). Additional changes around the mutated residue, Gln419, included the hydrogen bonding of Gln419 with O from Ser417 and O from Thr168 departs about 30° from the orientation found in the active enzyme. The geometry of this Cys-His bond imposes strong restraints on the possible mechanisms of formation.

Crystal Structure of H392Q—Determination of the crystal structure of the H392Q variant of HPII was undertaken because it differed significantly from wild type HPII in lacking both the His-Tyr bond and heme d despite retaining near wild type activity. The structure, determined and refined following a similar approach to the one described for the V169C variant, gave crystallographic agreement factors R = 14.4% and Rfree = 21.0% for data at 2.1-Å resolution collected with a conventional rotating anode x-ray source and a graphite monochromator. A number of structural changes with respect to native HPII, including a few rearrangements in the solvent structure and the presence of the heme b group were evident in the electron density (Fig. 1C). Changes directly related to the presence of heme b, such as the double conformation of Ile126 on the heme distal side and the rotation of Gln193 on the heme proximal side, are consistent with changes already described for the V169C structure (Fig. 3). Additional changes around the mutated residue, Gln193, included the hydrogen bonding of Gln193 with both the propionate side chain of the heme group and a molecule of water that is also hydrogen bonded with the amide nitrogen atom of Gln193 (Fig. 3). This water, not present in the native HPII structure, is found in PVC and in the HPII variants, V169C and H392Q. Gln419 has the same disposition in the structures of all the HPII variants containing heme b regardless of whether residue 392 is Gln or His (Fig. 3).

A water molecule in PVC and in the H392Q variant of HPII occupies the space that corresponds to the imidazole group in the native HPII structure. This water is in direct contact with the Cα atom of the essential tyrosine (Tyr350 in PVC and Tyr415 in HPII) suggesting a strong polarization of this region. Otherwise, the solvent organization on the heme proximal side in the H392Q variant is very similar to that of active HPII. The solvent structure on the distal side of the heme is similar to wild type HPII with no solvent molecules being present in the final part of the major channel which indicates that neither the Cα atom of the essential tyrosine (Tyr350 in PVC and Tyr415 in HPII) suggests a strong polarization of this region.
FIG. 3. Stereo views of the heme proximal side in native HPII (A), PVC (B), the variant V169C (C), and the variant H392Q (D). The native HPII structure contains a modified heme d and also presents a covalent bond between the C$^\alpha$ of Tyr$^{415}$ and N$^\delta$ of His$^{392}$. The structure of the HPII variant H392Q contains an unmodified heme b cofactor but retains a high catalatic activity. The structure of PVC also contains a modified heme d group, although the configuration in the vicinity of the essential tyrosine is very close to the one determined for the HPII variant H392Q.
presence of heme b nor the double conformation of Ile\textsuperscript{126} affect the solvent organization in the major channel. The presence of only one solvent molecule in the pocket immediately above the heme (Fig. 3C; corresponding to W0 in native HPII) rather than two found in native HPII could indicate that accessibility to the iron is somewhat diminished in the H392Q structure. Whether this is due to Ile\textsuperscript{126}, to the heme b, or to the water being a weaker ligand in the heme b environment is not yet clear.

Involvement of Other Proximal Side Residues—The concerted mechanism that couples heme oxidation to His-Tyr bond formation implicated His\textsuperscript{395} and Asp\textsuperscript{197} as playing a role in the reaction (13). The H395A, H395Q, D197A, and D197S variants of HPII were constructed to test this hypothesis. These four mutants all retained near wild type levels of activity and contained both heme d and the His-Tyr bond (Table III). The only difference from wild type was the apparently slightly slower heme conversion rate in the case of the H395A variant indicating that His\textsuperscript{395} and Asp\textsuperscript{197} do not have a significant role in heme conversion or His-Tyr bond formation.

Both Ser\textsuperscript{414} and Gln\textsuperscript{419} are located in close proximity to the heme. Ser\textsuperscript{414} is hydrogen bonded with the hydroxyl group on ring III of the heme d, and its interaction with the incoming hydroxyl group might potentially influence the oxidation reaction. Gln\textsuperscript{419} is hydrogen bonded with the carbonyl group of the spiro lactone on ring III in HPII and with the propionate side chain in the HPII variants that contain heme b. It seems likely that the association continues throughout the oxidation and cyclization reaction providing the Gln residue with an opportunity to influence the heme modification pathway. The mutant variants S414A, Q419A, and Q419H were constructed to test these hypotheses. The three variants retained 40% or more of wild type activity (Table III) indicating that the residues were not critical to the catalytic function of the enzyme. Spectral and HPLC analyses revealed a distribution of hemes quite different from that of wild type HPII (Fig. 5). For the two Gln\textsuperscript{419} mutant variants, there was a larger than normal peak of the trans isomer of heme d (~30% of the total as compared with 10% in the native HPII) indicating that changing the Gln residue affected the direction of lactone cyclization. Even more dramatic was the elution pattern of heme from the S414A variant which contained more trans than cis isomer and some heme b indicating that the Ser\textsuperscript{414} residue has important cata-

Both in PVC and in H392Q, a water molecule fills the space occupied by the imidazole ring of His\textsuperscript{392} in HPII. The green trace in B, C, and D indicates the location of the equivalent atoms in native HPII.
lytic roles both in directing the path of reaction and in facilitating the reaction.

DISCUSSION

The report of unusual covalent modifications in proteins is becoming increasingly common as accurate information about large structures is becoming available. Examples in redox related systems include the methionine sulfone found in the active site of catalase from *P. mirabilis* (2), the cysteine-sulfenic acid in the NADH peroxidase from *Streptococcus faecalis* (35), the oxidized tryptophan residue in lignin peroxidase (36), the modified cysteine in catalase HPII (37), the internal cyclization of the peptide backbone in the green fluorescent protein (38), and the His-Tyr covalent bond in catalase HPII (13). The bond between the C_e of the imidazole ring of His128 and the sulfur of Cys169 in the V169C mutant variant of HPII is yet another example of an unexpected and unusual covalent bond located in the core of a protein. The presence of this linkage, with essentially full occupancy, is well supported by both the electron density of the crystal structure and the sizes of fragments in the tryptic digest maps. The strong peptide peak in the mass spectrum indicates that the bond is relatively stable and does not break down readily under trypsin digest or MALDI-MS conditions.

Two possible mechanisms to explain the formation of the imidazole-sulfur bond can be proposed involving either an acid-catalyzed, nucleophile-mediated reaction (39) or a free radical mediated reaction. The protonation of His128 may facilitate the nucleophilic attack of the thiol group of Cys169 on the C_e of the imidazole ring (Fig. 6A). This would yield an intermediate with the C_e converted to sp^3 hybridization which could return to sp^2 hybridization through release of a hydride ion to the oxyferryl group of compound I. The same mechanism could be revised slightly such that the protonation of His128 at the initial stage of compound I formation triggers the nucleophilic attack by the thiol group (Fig. 6B).

Free radical addition of thiols to double bonds can be initiated by peroxides (40) providing several pathways for a free radical mechanism. The initial oxidation of the thiol group of Cys169 by H_2O_2 would yield a reactive thiyl radical which could then attack the C_e of His128 as shown in Fig. 6C. The participation of compound I as the oxidant of the thiol seems less likely given the 7.9 Å distance between the Cys-SH and the heme iron.

The absence of heme modification and of the His-Tyr covalent bond in the V169C mutant variant, both of which require compound I formation, suggest that compound I is not formed in this HPII variant, apparently invalidating mechanisms such as those in Fig. 6, A and B. The solution to this dilemma lies in the likelihood that the formation of the His-Cys bond is a more rapid reaction than either the heme modification or the His-Tyr bond formation. Reaction with the sulfur may occur in the first few catalytic rounds before any other modification occurs resulting in rapid modification of the essential His128 imidazole ring. Once His128 is modified, compound I cannot be formed and further modification cannot take place. A second explanation involves the replacement of compound I as primary oxidant by hydrogen peroxide as a hydride acceptor in the nucleophilic mechanism or the free radical mechanism (Fig. 6C).

The concerted mechanism that combines heme oxidation with His-Tyr bond formation in HPII seems to be corroborated...
by the properties of the H392Q variant of HPII. This variant was surprising in retaining a high percentage of native activity despite the presence of heme b and the absence of the His-Tyr bond which demonstrated that neither heme d nor the His-Tyr bond is essential for the activity of HPII. The H392E variant which contains some heme d but no His-Tyr bond appears to contradict the assumption of a linkage between the heme modification and His-Tyr bond formation and confirms that an alternate mechanism is possible in HPII. However, the slower rate of heme conversion and the abnormal heme profile as compared with native HPII suggest that the alternate mechanism may be different from that normally operating in the native enzyme. By the same criteria, the mechanism in the H392E variant would appear to be different from that operating in PVC.

Both Gln\textsuperscript{119} and Ser\textsuperscript{414} have roles in controlling the heme oxidation reaction as is evident in the fact that changing either residue causes changes in the cis:trans ratio of the spirolactone. The most dramatic change results from removal of Ser\textsuperscript{414}, consistent with it being hydrogen bonded with the OH on ring III. Interaction with the Ser\textsuperscript{414} side chain must stabilize the hydroxyl group on the proximal side of the heme, presumably limiting isomerization to the more stable trans isomer and ensuring formation of the cis isomer. The S414A variant contains the His-Tyr bond even when retaining some of the unmodified heme b group. All the inactive HPII variants analyzed lacked the two covalent modifications (13), which suggest that compound I formation is required for these modifications to take place.

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Mutants That Alter the Covalent Structure of Catalase Hydroperoxidase II from
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