Modulation of the Activities of Catalase–Peroxidase HPI of *Escherichia coli* by Site-Directed Mutagenesis[†]

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ABSTRACT: Catalase-peroxidases have a predominant catalatic activity but differ from monofunctional catalases in exhibiting a substantial peroxidatic reaction which has been implicated in the activation of the antitubercular drug isoniazid in Mycobacterium tuberculosis. Hydroperoxidase I of Escherichia coli encoded by *katG* is a catalase–peroxidase, and residues in its putative active site have been the target of a site directed-mutagenesis study. Variants of residues R102 and H106, on the distal side of the heme, and H267, the proximal side ligand, were constructed, all of which substantially reduced the catalatic activity and, to a lesser extent, the peroxidatic activity. In addition, the heme content of the variants was reduced relative to the wild-type enzyme. The relative ease of heme loss from HPI and a mixture of tetrameric enzymes with 2, 3, and 4 hemes was revealed by mass spectrometry analysis. Conversion of W105 to either an aromatic (F) or aliphatic (I) residue caused a 4–5-fold increase in peroxidatic activity, coupled with a >99% inhibition of catalatic activity. The peroxidatic-to-catalatic ratio of the W105F variant was increased 2800-fold such that compound I could be identified by both electronic and EPR spectroscopy as being similar to the porphyrin cation radical formed in other catalases and peroxidases. Compound I, when generated by a single addition of H_2O_2 , decayed back to the native or resting state within 1 min. When H_2O_2 was generated enzymatically in situ at low levels, active compound I was evident for up to 2 h. However, such prolonged treatment resulted in conversion of compound I to a reversibly inactivated and, eventually, to an irreversibly inactivated species, both of which were spectrally similar to compound I.

The class of enzymes called catalase—peroxidases are components of the oxidative defense system of bacterial (1) and fungal (2, 3) cells that function primarily as catalases to remove hydrogen peroxide before it can damage cellular components. Both catalatic and peroxidatic reactions involve a common initial step, the oxidation of the heme iron to form compound I (reaction 1). The second step of the catalatic pathway utilizes a second molecule of hydrogen peroxide as an electron donor to reduce compound I (reaction 2). The second stage of the peroxidatic pathway utilizes an electron donor(s) other than hydrogen peroxide to reduce the heme (reactions 3 and 4). The latter reactions occur only under conditions of low peroxide concentration in the presence of a suitable donor resulting in the peroxidatic reaction appearing to be slower than the catalatic reaction. Cat (Por-Fe^{III}) + H₂O₂ → compound I (Por^{•+}-Fe^{IV}=O) + H₂O (1)

compound I (Por^{•+}-Fe^{IV}=O) + H₂O₂
$$\rightarrow$$

Cat (Por-Fe^{III}) + H₂O + O₂ (2)

compound I (Por^{•+}-Fe^{IV}=O) + donor(red.) \rightarrow compound II (Por-Fe^{IV}-OH) + donor(ox.) (3)

compound II (Por-Fe^{IV}-OH) + donor(red.) \rightarrow Cat (Por-Fe^{III}) + donor(ox.) (4)

The catalase–peroxidase of *Mycobacterium tuberculosis* (MtKatG) activates the antitubercular drug isoniazid (isonicotinic acid hydrazide, INH)¹ making it the subject of intensive investigations. MtKatG oxidizes INH to an electrophilic species which binds to, and inactivates, InhA, an acyl carrier protein (4, 5) and other targets (6, 7), thereby inhibiting mycolic acid synthesis. *Escherichia coli* produces a catalase–peroxidase (EcKatG, also known as HPI or

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¹ Abbreviations: HPI, hydroperoxidase I; HPII, hydroperoxidase II; CCP, cytochrome c peroxidase; HRP, horseradish peroxidase; INH, isoniazid or isonicotinic acid hydrazide; ABTS, 2,2'-azinobis(3-ethylbenzothiazolinesulfonic acid).



FIGURE 1: Orientation of the residues in the active site of HPI based on the structure of yeast cytochrome c peroxidase. The residue numbers are for HPI, and the numbers in parentheses are for the corresponding residues in CCP. The sphere above the heme iron is a water molecule.

hydroperoxidase I) with significant (\sim 70%) sequence similarity to MtKatG. MtKatG and EcKatG have similar physical and chemical properties including comparable kinetic parameters, subunit sizes, and susceptibility to classical heme enzyme inhibitors such as cyanide. Despite these similarities, EcKatG is not as proficient in the peroxidatic oxidation of INH (8), nor are *E. coli* cells susceptible to INH, even at very high concentrations of the drug, although overexpression of MtKatG in *E. coli* results in the increased susceptibility of the cells to INH (9).

An investigation of the active site structure of EcKatG was initiated to identify residues important for the catalytic function of the protein. The crystal structure of catalase– peroxidases has so far remained elusive, and the active site residues were selected on the basis of analogy with yeast cytochrome c peroxidase for which a high-resolution structure is available (10) (Figure 1) including Arg102, Trp105, His106, and His267 (analogous to Arg48, Trp51, His52, and His175 in CCP).

MATERIALS AND METHODS

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

Strains and Plasmids. Phagemids pKS+ and pKS- from Stratagene Cloning Systems were used for mutagenesis, sequencing, and cloning. The plasmid pBT22 (11) was used as the source for the *katG* gene which was cloned into pKSto generate pAH8 used for mutagenesis. *E. coli* strains NM522 (*supE thi*)(*lac-proAB*) *hsd-5* [F' *proAB lacI*⁴ *lacZ*)15]) (12), JM109 (*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ (*lac-proAB*) (13), and CJ236 (*dut-1 ung-1 thi-1 relA1/* pCJ105 F') (14) were used as hosts for the plasmids and for generation of single-strand phage DNA using helper phage R408. Strain UM262 (*pro leu rpsL hsdM hsdR endI lacY katE1 katG12*::Tn10 *recA*) (15) was used for expression of the mutant *katG* constructs and isolation of the mutant HPI proteins.

Oligonucleotide-Directed Mutagenesis. Oligonucleotides were synthesized on a PCR-Mate synthesizer from Applied Biosystems and are listed in Table 1. The restriction nuclease fragments that were mutagenized following the Kunkel procedure (14), sequenced, and subsequently reincorporated into pAH8 to generate the mutagenized katG genes are also

listed. Sequence confirmation of all sequences was by the Sanger method (16) on double-stranded plasmid DNA generated in JM109.

Catalase, Peroxidase, Protein, and Spectral Determination. Catalase activity was determined by the method of Rørth and Jensen (17) in a Gilson oxygraph equipped with a Clark electrode. One unit of catalase is defined as the amount that decomposes 1 μ mol of H₂O₂ in 1 min in a 60 mM H₂O₂ solution at pH 7.0 at 37 °C. Initial rates of oxygen evolution were used to determine the turnover rates to minimize the inactivation caused by high $[H_2O_2]$ (18). Peroxidase activity was determined spectrophotometrically using ABTS (19). One unit of peroxidase is defined as the amount that decomposes 1 μ mol of ABTS in 1 min in a solution of 0.3 mM ABTS and 2.5 mM H₂O₂ at pH 6.0 and 25 °C. The peroxidatic substrates o-dianisidine and INH were also tested, but data are not reported. Protein was estimated according to the methods outlined by Layne (20). Absorption spectra were obtained using a Milton Roy MR3000 spectrophotometer. Samples were dissolved in 50 mM potassium phosphate, pH 7.0.

Enzyme Purification. Cultures of *E. coli* strain UM262 transformed with plasmids encoding HPI or mutant variants were grown in Luria broth containing 10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl. Growth was for 22 h at 28 or 37 °C with shaking. Cells were harvested, and HPI and its variants were isolated as previously described (*21*) involving streptomycin precipitation, ammonium sulfate precipitation, and chromatography on DEAE cellulose (Whatman).

Electrospray Injected-Time-of-Flight (ESI-TOF) Mass Spectrometry. Protein samples were prepared for ESI-TOF mass spectrometry by dialyzing extensively into 5 mM ammonium acetate. All studies were performed using an ESI-TOF mass spectrometer constructed at the University of Manitoba (22) as previously described (23, 24). Samples were continuously infused into the ion source at a flow rate of $0.4 \,\mu$ L/min using a syringe pump. Declustering voltages, which control the kinetic energy of the ions in the interface, ranged from 150 to 350 V. Data were acquired in the positive ion mode using ubiquitin for calibration.

EPR Spectrometry. EPR spectra were recorded at X-band with a Bruker ESP 300E spectrometer equipped with an HP5352B microwave frequency counter and Oxford Instruments ESR900 continuous-flow cryostat. Protein samples were prepared in 50 mM potassium phosphate buffer pH 7.0, and enzyme concentrations were determined from the extinction coefficient of 143 mM⁻¹ cm⁻¹ at the Soret maximum. To prepare compound I, 3 equiv of H_2O_2 was added to 1 equiv of protein (heme) on ice. The sample was immediately frozen in liquid N₂ before transfer to the cryostat. Other experimental parameters include frequency 9.45 GHz, modulation frequency 100 kHz, modulation amplitude 4.89 G, microwave power 2 mW, and temperature 4 K except where varied in specific experiments.

RESULTS

Catalatic Properties of the HPI Variants. Purified enzyme was prepared from all of the variants of HPI except the W105C and H106L variants. These latter two variants were presumably defective in folding because they did not accumulate protein even when cultures were grown at 28

variant	codon change	oligonucleotide	restriction fragment
R102L	$CGT \rightarrow CTG$	TGTTTATT CTG ATGGCCTGG	EcoRI-ClaI (193-345)
R102C	$CGT \rightarrow TGT$	TGTTTATT TGT ATGGCCTGG	EcoRI-ClaI (193-345)
R102K	$CGT \rightarrow AAA$	TGTTTATT AAA ATGGCCTGG	EcoRI-ClaI (193-345)
H106C	$CAC \rightarrow TGC$	TGGCCTGG TGC GGCGCGGGG	EcoRI-ClaI (193-345)
H106Y	$CAC \rightarrow TAC$	TGGCCTGGTACGGCGCGGGG	EcoRI-ClaI (193-345)
H106L	$CAC \rightarrow CTG$	TGGCCTGG CTG GGCGCGGGG	EcoRI-ClaI (193-345)
H267Y	$CAT \rightarrow TAC$	GCGGGTGGT TAC ACGCTGGGT	ClaI-BamHI (345-1085)
W105C	$TGG \rightarrow TGT$	GTATGGCC TGT CACGGCGCG	EcoRI-ClaI (193-345)
W105L	$TGG \rightarrow CTG$	GTATGGCCCTGCACGGCGCG	EcoRI-ClaI (193-345)
W105F	$TGG \rightarrow TTT$	GTATGGCCTTTCACGGCGCG	<i>Eco</i> RI- <i>Cla</i> I (193-345)

Table 2: Catalatic and Peroxidatic Specific Activities of the Purified Variants of I
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	catalatic activity		peroxidatic activity			
variant	units/mg	units/nmol of heme ^c	units/mg	units/nmol of heme ^c	peroxidas	e/catalase ^b
wild-type HPI	1900	300	660	106	0.35	(1)
MtKatG ^d	970	150	2500	402	2.7	(8)
R102L	19	22	85	99	4.5	(13)
R102C	10	8	63	51	6.3	(18)
R102K	35	26	85	63	2.4	(7)
H106C	0.25	0.06	11	2.4	44	(125)
H106Y	0.16	0.08	18	9.2	110	(321)
H267Y	0.95	3.9	12	49	12	(36)
W105L	5.9	2.5	1300	543	210	(612)
W105F	1.9	0.43	1900	422	980	(2800)

^{*a*} Insufficient protein was produced from the H106L and W105C variants to allow isolation and characterization. ^{*b*} The numbers in parentheses are for comparison with the wild-type ratio normalized to 1.0. ^{*c*} The normalization to heme content was based on the $A_{270/408}$ ratio assuming that a ratio of 0.5 represents 0.5 heme/subunit (30). ^{*d*} MtKatG, KatG protein from *M. tuberculosis*.

Table 3: Optical Absorbance Maxima and Calculated Heme/Subunit Ratio of the Purified Variants of HPI

variant	Soret (nm)	α-charge transfer (nm)	β -charge transfer (nm)	A _{407/280} (heme/subunit)
wild type	408	640	510, 540	0.51
R102L	408	650	540	0.07
R102K	408	640		0.11
R102C	412	660	530	0.10
H106Y	408	645	510, 540	0.16
H106C	407	635	510, 540	0.37
H267Y	411	655		0.02
W105L	409	630		0.19
W105F	409	630	500, 540	0.36

°C. The lower incubation temperature has been shown to be effective in promoting the accumulation of some, but not all, variants of HPII, the monofunctional catalase of *E. coli*, by reducing the rate of proteolysis and providing a greater chance for correct folding to occur (25).

The catalatic specific activities of all the variants are significantly reduced compared to the wild-type enzyme (Table 2), in part because they contain less heme than the wild type enzyme. However, even when corrected for heme content, the activities of the mutants are all lower than wild type. This confirms that the lower activity is a result of the residue changes affecting catalysis as well as the folding process and heme binding. For comparison, the HPI catalatic activity (1900 units/mg of protein) is approximately $\frac{1}{8}$ of the specific activity of catalase HPII, also from *E. coli*, probably a reflection of a less than optimal active site. The further reduction of the catalatic activity in the variants makes them very weak catalases. The specific activity of KatG from *M. tuberculosis* is also included in Table 2 for comparison.

Small variations in the location of the Soret maxima between 407 and 412 nm were observed among the variants,

Table 4: Apparent $K_{\rm m}$ and $k_{\rm cat}$ Calculated for the Purified Variants of HPI

	catalase		peroxidase		
variant	<i>K</i> _m (mM H ₂ O ₂)	$k_{\text{cat}} \ (\mathrm{s}^{-1})^a$	K _m (µM ABTS)	$k_{\text{cat}} \ (\mathrm{s}^{-1})^a$	
wild type	5.9	5.3×10^{3}	55	1.7×10^4	
R102L	33	9.0×10^{2}	48	6.6×10^{2}	
R102K	12	2.1	400	3.7×10^{3}	
R102C	31	53.5	100	7.1×10^{2}	
H106Y	100	6.1×10^{-3}	330	1.1	
H106C	28	8.9×10^{-2}	80	1.6	
H267Y	53	2.6	nd ^b	nd	
W105L	77	23	240	1.4×10^{5}	
W105F	91	1.1	480	2.1×10^5	
w 103F	71	1.1	400	2.1 X 10°	

^{*a*} Corrected for differing heme content based on the $A_{407/280}$ ratio, assuming 2 hemes/tetrameric holoenzyme. ^{*b*} nd = not determined.

as were shifts in the α -charge-transfer band between 630 and 660 nm (Table 3) which may be indicative of small differences in the distal ligand bond length and hydrogen bond changes (unpublished data). The apparent $K_{\rm m}$ and $k_{\rm cat}$ values for the catalatic reaction, normalized for the heme content of the variants, are presented in Table 4. They confirm a substantial decrease in affinity for peroxide and in turnover rates for all of the variants.

Heme Content As Determined by Mass Spectrometry. HPI was originally characterized as having approximately one heme per dimer (26). It was not clear if this was an average value resulting from a heterogeneous mixture with varying occupancies or a reflection of each dimer having only one heme binding site. In view of the variants binding even less heme, the heme composition of HPI was investigated by electrospray mass spectrometry, revealing that there is a heterogeneity in heme composition among the tetramer molecules (Figure 2). As the declustering voltage increases,



FIGURE 2: Mass analysis by electrospray mass spectrometry of wildtype HPI. The solid trace was obtained at a declustering voltage of 200 V, and the dotted line, at a declustering voltage of 350 V. The inset figures show the deconvoluted data from the dimeric (A and B) and tetrameric (C and D) portions of the spectrum. As peaks in the insets differ by approximately 600 Da and as the declustering voltage increases, there is an increase in the amount of dimer and tetramer with less heme.

the amount of tetrameric enzyme (m/z > 7500) decreases and the amount of monomeric form (m/z = 2000-4000) and free heme (m/z = 616) increases suggesting a fairly fragile enzyme. Furthermore, the deconvoluted data of the dimer and tetramer regions (insets A and B, respectively, of Figure 2) reveal peaks differing, in both cases, by 590 \pm 30 Da, within experimental error for the 617 Da mass of a heme. The amount of bound heme decreases with increasing declustering voltage, but even at the lowest voltage, a mixture of species differing by approximately 600 Da is present along with a small amount of heme, consistent with HPI being a heterogeneous mixture of tetrameric molecules containing 2, 3, and 4 heme groups per tetramer. Similarly, myoglobin retains a full complement of heme at low voltages and begins to lose heme as the declustering voltage is raised (27). The small amount of dimeric enzyme most likely arises from the declustering or excitation process and confirms the heterogeneous nature of the enzyme with peaks containing 2, 1, and 0 hemes per dimer being evident. Unfortunately, because even the lowest declustering voltage can cause some heme removal, it is not possible to determine precisely the relative proportions of the various components. However, there seems to be a preponderance of species with 2, 3, and 4 hemes making the original estimate of 2 per dimer somewhat low.

Peroxidatic Properties of the HPI Variants. Three different organic electron donors were evaluated as substrates for the peroxidatic reaction, including *o*-dianisidine, INH, and ABTS, and the latter was found to support a 200-fold higher reaction rate. Therefore, ABTS was used as the peroxidatic substrate in all subsequent assays including those reported in Table 2 which show a significant reduction in peroxidatic activity for all but two of the variants. The peroxidatic activity resulting in a general increase in the peroxidatic to catalatic activity ratio of from 7- to 320-fold among the variants, again excluding the two W105 replacement variants.

Table 5: Cyanide and Azide Binding and Inactivation of the Variants of HPI

variant	[KCN] causing 50% inhibition (µM)	[NaN ₃] causing 50% inhibition (µM)	<i>K</i> _d (μM KCN)
wild type	90	8	50
R102K	390	10	16
R102L	55	95	100
R102C	75	180	180
H106Y	210	230	13
H106C	40	190	1000
W105L	65	8	7
W105F	12	250	12

Similar trends were evident when *o*-dianisidine and INH were used as substrates (data not shown).

The two W105 variants, W105L and W105F, differ dramatically from the others in having peroxidatic activities 4- and 5-fold higher (on a per heme basis), respectively, than the wild type enzyme, and this is accompanied by a significantly reduced catalatic activity. The biggest difference between the two variants is that the W105F variant contains about twice as much heme as the W105L variant indicating that the bulkier aliphatic group may interfere with heme binding. These changes result in the peroxidatic/catalatic ratios being increased 612- and 2800-fold respectively for W105L and W105F. For comparison, the variants are still not as efficient as HRP, despite the optimization, which has a 70-fold higher specific activity of 1.2×10^5 units/mg of protein.

The apparent peroxidatic $K_{\rm m}$ and $k_{\rm cat}$ values of the variants are presented in Table 4 and confirm a decrease in affinity for substrate in all variants except R102L. The apparent turnover rate for the W105F and W105L variants increased substantially to give rise to the increased specific activity.

Sensitivity to and Affinity for Common Inhibitors. Differing sensitivities to the common heme-containing enzyme inhibitors, cyanide and azide, for both catalatic and peroxidatic reactions were apparent among the variants (Table 5). Some of the variants, particularly R102L, were less sensitive than others, but no trend was apparent. Affinities for cyanide were determined on the basis of spectral changes in the Soret region and are also summarized in Table 5. The R102L, R102C, and H106C variants exhibit an increase in K_d indicating a lower affinity whereas all other mutants exhibit enhanced cyanide binding consistent with enhanced accessibility to the heme resulting from the incorporation of smaller residues and from modified hydrogen-bonding interactions.

Characterization of the Peroxidatic Reaction of the W105F and W105L Variants. The similarity in properties of the two W105 variants indicates that removal of the indole ring is the primary determinant in changing the enzyme from being a reasonably strong catalase with peroxidatic activity to being a stronger peroxidase with weak catalatic activity. The identity of the replacing residues whether aliphatic or aromatic is secondary. The fact that the peroxidatic activity is enhanced suggests that compound I formation is not adversely affected by the mutations and may even be enhanced. Compound I is normally a difficult species to identify in catalase—peroxidases because of the potential for rapid reduction in both the catalatic and peroxidatic processes. Slowing of the catalatic process opened an avenue



 H_2O_2 (mM)

FIGURE 3: Dependence of compound I formation on hydrogen peroxide concentration. HPI was mixed with increasing concentrations of H_2O_2 and incubated for 10 s. An aliquot was then added to a solution of ABTS for activity determination.



FIGURE 4: Rate of decay of compound I. Compound I was formed by the addition of a 3-fold excess of H_2O_2 . Changes in the Soret peak at 408 nm (A) were monitored at 1.6 ms intervals for 2 min. Changes in activity (B) were monitored at 5 s intervals for 2 min.

for generating and studying compound I in the W105 variants.

The amount of compound I formed, as determined by its ability to oxidize ABTS, increases with H_2O_2 concentration up to 2 mM H_2O_2 (Figure 3). A single treatment of the W105F variant with a 3-fold excess of H_2O_2 results in an extremely rapid hypochromic red shift that is complete within 0.1 min and decays back to the ground state within 1-2 min (Figure 4A). This rapid formation and subsequent decay of compound I is also evident in the direct activity assay (Figure 4B).

To study the spectral changes inherent in compound I and the long-term stability of compound I, the W105F and



FIGURE 5: Spectral changes in the W105F variant of HPI (A), the W105L variant (B), horseradish peroxidase (C), and wild-type HPI (D) induced by H_2O_2 generated in situ by glucose and glucose oxidase. To enzyme at 1 mg/mL (3 mg/mL for W105L) in 50 mM potassium phosphate pH 7.0 was added 10 μ g/mL glucose oxidase and 4 mM glucose. Spectra were determined at 5 min intervals for 15 min. Arrows show the direction of the spectral shift.



FIGURE 6: Inactivation of compound I of the W105F variant in the presence of glucose and glucose oxidase. The reaction was started as in Figure 5, and aliquots were removed for reaction with ABTS at various times. Additional 4 mM glucose was added after 2 and 4 h as indicated by the arrows (closed circles). In a parallel experiment, additional 4 mM glucose was added at 1, 2, and 3 h (open circles).

W105L variants were treated with glucose/glucose oxidase, which generates low levels of H₂O₂ in situ. A hypochromic red shift of the Soret peak is evident (Figure 5A,B), and the overall spectral changes are similar to those of HRP (Figure 5C). For comparison, the wild-type HPI (Figure 5D) undergoes only a very small hypochromic shift on the shoulder of the Soret peak below 400 nm. The spectral changes shown in Figure 5 were recorded after only 15 min but persisted for periods of up to 24 h (data not shown). The activity assay (Figure 6) confirms the slow generation of compound I over 15 min, a result of the low levels of H_2O_2 generated by glucose and glucose oxidase. The assay also reveals that compound I is not stable for long periods as suggested by the spectra but decays over the period of 2 h. Part of the decay is a result of glucose depletion and the resulting drop in H₂O₂ levels, and the addition of more glucose results in an increase in compound I levels. However, with each succeeding addition of glucose, the plateau amount of active compound I is reduced (Figure 6), and the enzyme is converted to an inactive species which is spectrally similar to compound I. If a second addition of glucose is delayed for 2 h, the increase in active compound I is much reduced, suggesting that the irreversible inactivation of compound I occurs slowly throughout the incubation period.

EPR Spectroscopy. Confirmation of the formation of a porphyrin radical-containing compound I was achieved using EPR spectroscopy. The heme iron in both wild-type HPI and the W105F variant is predominantly high spin as indicated by g values of 6 and 2. The observed anisotropic \mathbf{g} tensors also show that the heme iron is a mixture of species with axial and rhombic symmetry, with the variant being less rhombic showing decreased intensities near g = 6.8 and 5.0. This increased axial character of the variant could result from coordination at the distal side of the heme iron. In the presence of H_2O_2 , a strong free radical signal is evident at g = 2.010 for the W105F variant (Figure 7A) and g = 2.008for theW105L variant (data not shown). Also shown is the signal for the wild-type HPI which is also at g = 2.0, but with weaker intensity, presumably because of lower levels of compound I arising from its rapid turn over in the presence of substrate peroxide. No other changes in the spectrum of the W105F variant were evident that might suggest the presence of a radical elsewhere in the protein. The EPR spectra of both wild type and W105F variant enzymes were unaffected by the presence of INH with or without H₂O₂ present. This is unlike the effect of INH on the spectra of the M. tuberculosis KatG (28) and is consistent with the weaker affinity of HPI for INH as compared to MtKatG revealed in functional studies (8) and confirmed spectrally (data not shown). The power saturation profiles of the radicals formed by these enzymes (Figure 7B) suggest that the radical formed from the wild-type HPI is more easily saturated and exhibits a slower spin-lattice exchange than the radical of the variant. The intensity of the peroxideinduced radicals in wild-type HPI and the W105F variant are temperature dependent, although whether this is due to line broadening or thermal instability of the radical is unclear (Figure 7C).

DISCUSSION

Residues in the putative active site of the catalaseperoxidase of E. coli were identified initially by analogy with the active site of yeast cytochrome c peroxidase for which the X-ray structure has been solved (10). The fact that changes to these residues can elicit both increases and decreases in activity strongly suggests that they are located in the active site and have a catalytic role. Furthermore, the properties of the HPI variants are generally consistent with the properties of variants of CCP and HRP with changes in equivalent residues. For example, changing either of the distal side His (42 in HRP and 52 in CCP) or Arg (38 in HRP and 48 in CCP) caused significant reductions in peroxidatic activity (29-31), and the W51F variant of CCP decreased the k_{cat} by only 2–4-fold and increased the K_m for substrate (32). The opposite F41W mutation in HRP which would result in HRP more closely resembling HPI, caused a reduction in peroxidatic activity to 5% of wild type (33). Unfortunately, whether this HRP variant exhibited catalatic activity was not reported. Mutation of equivalent residues in the M. tuberculosis KatG protein to produce R104L and



FIGURE 7: EPR spectroscopy of HPI and the W105F variant following addition of a 3-fold excess of H₂O₂. (A) X-band EPR spectra of HPI and the W105F variant. Protein concentrations were 0.102 and 0.146 mM for wild type and W105F variant, respectively. Signals were averaged over 5 and 8 scans for the W105F and wildtype enzymes, respectively. (B) Power saturation profiles exhibited by wild-type HPI and the W105F variant. Each data point was averaged over 5 and 8 scans for the W105F variant and wild-type enzymes, respectively: open circles, W105F variant; closed circles, wild-type HPI. The data for HPI are shown $\times 4$ to illustrate clearly the difference between the two curves. (C) Temperature dependence of the intensity of the peroxide induced radicals of wild-type HPI and the W105F variant. Experimental parameters were as described but at a power of 0.1 mW. Each data point was averaged over 5 scans for the W105F variant and 16 scans for wild-type HPI enzyme: open circles, W105F variant; closed circles, HPI.

H108Q variants were only paritally characterized but did show reduced catalatic and peroxidatic activity (*34*).

Most of the variants contain significantly less heme than native HPI suggesting that heme binding in catalase– peroxidases is easily disrupted. Indeed the affinity of HPI for heme has been a question ever since the heme content of HPI was determined to be approximately 1 heme per dimer (26). Were half of the subunits unable to bind heme or was the binding sufficiently weak that full occupancy was never achieved? The mass spectral analysis shows that the enzyme is a mixture of tetrameric species containing 2, 3, and 4 hemes. Because even the lowest declustering or excitation voltage causes the loss of some heme from the complex, it is not possible to define precisely the proportions of the different heme containing species, but it is possible to conclude that all subunits are capable of binding heme and that the incomplete occupancy probably arises from limited heme availability. This observation may also explain the difficulty experienced in obtaining crystals of HPI and other catalase—peroxidases.

Arguably, the most interesting variant of HPI in this study is W105F which is a peroxidase with weak catalatic activity, a result of increased peroxidatic activity and reduced catalatic activity. A comparison of the active sites of HRP and CCP reveals that the phenyl ring in HRP (equivalent to Phe105 in the W105F variant) is coplanar with and occupies a portion of the same space as the indole ring in CCP (equivalent to Trp105 in HPI). It is unlikely, therefore, that the Trp to Phe changes in W105F will result in very great changes in the active site of HPI aside from a small increase in cavity volume and loss of the indole nitrogen. The small increase in the size of the active site cavity resulting from the tryptophan to phenylalanine change may account for the increased peroxidatic activity through easier binding of the aromatic substrates.

Removal of the indole ring in the W105F variant does not seem to affect the formation of compound I. Indeed, the loss of catalatic activity allows compound I to accumulate in the absence of organic electron donors revealing that it is formed so rapidly that only the final part of the reaction can be captured in a stopped-flow apparatus following the addition of H_2O_2 .

Compound I can also be formed by prolonged exposure to low levels of H₂O₂, generated in situ by glucose and glucose oxidase, but its fate is quite different. The rate of compound I formation is much slower, a result of limiting H₂O₂ concentrations, and full activity is maintained for up to 15 min. This is followed by a slow decay to an inactive form that takes over 1 h to reach completion. During the early phase of decay, a portion of the inactive enzyme can be reactivated by the addition of glucose which would cause transient increases in H₂O₂. The apparent reversible inactivation could be explained in terms of a decay of compound I back to the resting state, but this is not consistent with the lack of spectral change for over 24 h and repeated glucose additions. After 2-3 h of exposure to H_2O_2 , the irreversible inactivation is complete and reactivation is not possible. These data suggest the presence of at least two inactive species which are spectrally similar to compound I, a reversibly inactivated species which can be converted back to an active compound I and an irreversibly inactivated species. Whether the transiently inactivated species is an intermediate on the way to compound II (route A in Figure 8) or is the product of a separate decay pathway (route B in Figure 8) is not clear.

The active sites of monofunctional catalases and catalase– peroxidases, the latter deduced from plant peroxidase structures, differ significantly, suggesting that there may also be significant differences between the catalatic mechanisms operating in the two classes of enzymes. First, there is a very different complement of residues in the two enzymes. There is a distal side histidine in both active sites, but in



FIGURE 8: Summary of the effect of the W105F mutation on the combined peroxidatic and catalatic activities. The conversion of the resting state enzyme (Fe^{III}) to compound I (Cpd I) is common to both the catalatic reaction, proceeding to the left, and the peroxidatic reaction, proceeding to the right from compound I. The W105F mutation effectively blocks the catalatic pathway as indicated by the black bar. Compound I of the W105F variant can also decay into one of two inactive forms. The species labeled, Inactive Species^R, can be converted back to compound I by H₂O₂, but long-term incubation with H₂O₂ results in conversion to a nonrecoverable form labeled Inactive Species. Whether this irreversible inactivation occurs via the reversibly inactivated form (path A) or directly from compound I (path B) is unknown.

monofunctional catalases it is essential to the catalatic mechanism (35), whereas variants of HPI lacking the residue retain a low level of activity. In peroxidases, the imidazole ring is oriented perpendicular to the plane of the heme and is 6 Å away whereas in monofunctional catalases it is oriented parallel to and just 3.5 Å above the plane of the heme. Furthermore, the active site asparagine of monofunctional catalases is missing in the catalase-peroxidases, which contain arginine and tryptophan instead (Figure 1). Second, whereas the active sites of monofunctional catalases are deeply buried, the active sites of the catalase-peroxidases are probably near the surface. By analogy with CCP and HRP, the active site of HPI is adjacent to a large cavity accessible to the exterior of the protein which is the binding site of the organic peroxidatic substrates. The presence of such a large surface exposed cavity should make access for the small substrate hydrogen peroxide and exhaust of the oxygen and water products relatively easy compared to monofunctional catalases.

The data presented in this paper suggest that the three active site residues of HPI, Arg102, Trp105, and His106, all have a role in the catalatic mechanism which can be modeled on the original peroxidatic mechanism (36, 37). The first stage of both the catalatic and peroxidatic processes is the oxidation of the resting state enzyme (Figure 9A) to compound I (Figure 9C). H₂O₂ is the oxidant and is shown in Figure 9B associated with Arg102 and His106. Trp105 seems to have no role in this reaction. The second stage of the catalatic process is the reduction of compound I (Figure 9C) back to the resting state (Figure 9A), utilizing a second molecule of H₂O₂ as the reducing agent. This H₂O₂ is shown in Figure 9D associated with His106 and Trp105. We propose the involvement of Trp105 in this stage rather than Arg102 on the basis of the phenotype of the W105 variants wherein the indole ring is essential for the catalatic process but not for either compound I formation or the peroxidatic reduction stage. The involvement of different residue pairs in the two stages, the His-Arg pair in the oxidation stage and the His-Trp pair in the reduction stage, in the catalase-



FIGURE 9: Scheme showing the involvement of key active site residues of HPI in the oxidation of the resting state enzyme shown in (A) to compound I (C) and reduction back to the resting state (A). The substrate hydrogen peroxide molecules involved in the oxidation (B) and reduction (D) are shown in potential hydrogenbonded conformations.

peroxidases presents a considerable deviation from the monofunctional catalases (35) which utilize a single His— Asn pair for both stages. This conclusion does beg the question, if the indole ring of HPI is so important to the catalatic process, why does CCP not exhibit catalatic activity? Most likely, the answer will lie in small structural differences between the active sites of CCP and HPI, and this question will only be answered when the crystal structure of HPI becomes available.

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