Characterization of the Catalase-Peroxidase KatG from *Burkholderia pseudomallei* by Mass Spectrometry*

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The electron density maps of the catalase-peroxidase from *Burkholderia pseudomallei* (BpKatG) presented two unusual covalent modifications. A covalent structure linked the active site Trp111 with Tyr238 and Tyr238 with Met264, and the heme was modified, likely by a perhydroxy group added to the vinyl group on ring I. Mass spectrometry analysis of tryptic digests of BpKatG revealed a cluster of ions at m/z 4525, consistent with the fusion of three peptides through Trp111, Tyr238, and Met264, and a cluster at m/z ~4525, consistent with the fusion of two peptides through Trp111 and Tyr238. MS/MS analysis of the major ions at m/z 4524 and 4540 confirmed the expected sequence and suggested that the multiple ions in the cluster were the result of multiple oxidation events and transfer of CH$_3$S to the tyrosine. Neither cluster of ions at m/z 4525 or 6585 was present in the spectrum of a tryptic digest of the W111F variant of BpKatG. The spectrum of the tryptic digest of native BpKatG also contained a major ion for a peptide in which Met264 had been converted to homoserine, consistent with the covalent bond between Tyr238 and Met264 being susceptible to hydrolysis, including the loss of the CH$_3$S from the methionine. Analysis of the tryptic digests of hydroperoxidas I (KatG) from *Escherichia coli* provided direct evidence for the covalent linkage between Trp111 and Tyr238 and indirect evidence for a covalent linkage between Tyr238 and Met264. Tryptic peptide analysis and N-terminal sequencing revealed that the N-terminal residue of BpKatG is Ser22.

The heme-containing catalase-peroxidases are bifunctional enzymes that degrade hydrogen peroxide either as a catalase (2H$_2$O$_2$ → 2H$_2$O + O$_2$) or as a peroxidase (H$_2$O$_2$ + 2AH → 2H$_2$O + 2A). The catalatic reaction, with a more rapid turnover rate, dominates over the peroxidatic reaction, and the in *vivo* peroxidatic substrate remains unidentified, suggesting that the main role of the enzyme is the removal of H$_2$O$_2$ preventing the formation of highly reactive and damaging breakdown products of H$_2$O$_2$. However, the enzyme has a close sequence resemblance to plant peroxidases (1, 2), and it remains a possibility that the peroxidatic reaction has a metabolic significance outside of degrading H$_2$O$_2$. Indeed, it is clear that the catalatic function evolved as an adaptation of the peroxidatic function because the simple change of a tryptophan to a phenylalanine in the distal heme pocket reduces catalatic activity by 1000-fold (of *Escherichia coli* HPI) and increases peroxidatic activity by 3-fold (3–5). Furthermore, the core structures of both the N- and C-terminal domains of the catalase-peroxidases from *Haloarcula marismortui* and *Burkholderia pseudomallei* closely resemble the structure of plant peroxidases (6, 7). Finally, the conversion of ionazid into its active antituberculosis form by KatG of *Mycobacterium tuberculosis* is clearly a result of the peroxidatic reaction using isonicotinic acid hydrazide (INH) as a substrate that must mimic the actual *in vivo* substrate.

The structures of the catalase-peroxidases from *H. marismortui* and *B. pseudomallei* have been reported (6, 7) and have revealed several features that are, so far, unique to this class of enzyme. Present in both structures is an unusual adduct or covalent linkage among the side chains of a tryptophan, a tyrosine, and a methionine (see Fig. 1). The likely mechanistic significance of the covalent structure is enhanced by the fact that the tryptophan lies in the active site and is essential for catalatic activity. A second feature, evident only in the structure of BpKatG, is a modification to the heme, likely a hydroperoxide group added to ring I of the heme in close proximity to the Trp-Tyr-Met adduct. This paper presents mass spectrometry evidence supportive of the existence of the Trp-Tyr-Met adduct originally deduced from the electron density maps of HmCIPx and BpKatG (6, 7).

**EXPERIMENTAL PROCEDURES**

**Materials**—Standard chemicals and biochemicals were obtained from Sigma. Restriction endonucleases, polynucleotide kinase, DNA ligase, and the *Klenow* fragment of DNA polymerase were obtained from Invitrogen.

**Strains and Plasmids**—The plasmid pBG306 encoding BpKatG (8) and the plasmids pAH8 and pW105F encoding KatG (HPI) and its W105F variant from *E. coli* (3) were transformed into strain UM262 pro leu rpsL, hisD44 endA1 lacY1 katG2 katE12::Tn10 recA1 (8) for expression of the katG constructs and isolation of the mutant KatG proteins. Phagemids pKS* and pKS* from Stratagene Cloning Systems were used for mutagenesis, sequencing, and cloning. *E. coli* strains NM522 (supE thi lac-proAB)Δ sodA hisD44 endA1 lacY1 katG2 katE12::Tn10 (9), JM109 (recA1 supE44 endA1 hisD44 relA1 thy1 thi-lac-proAB)Δ sodA hisD44 endA1 lacY1 katG2 katE12::Tn10 (10), and CJ236

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(mut-1 ung-1 thi-1 relA/pGJ105 F′) (11) were used as hosts for the plasmids and for generation of single-stranded phage DNA using helper phage R408.

**Oligonucleotide-directed Mutagenesis**—The oligonucleotides CTGT-TACATCATGAAAATGGCATG (AAA encoding Lys in place of Arg<sup>108</sup>), CGCATGGCATTCTCACACGGCG (TTT encoding Phe in place of Trp<sup>111</sup>), and TCCGGCGGCTTGCAGTGAAC (CTG encoding Leu in place of Met<sup>264</sup>) were purchased from Invitrogen. They were used to mutagenize a 600-bp fragment from pBG306 generated by KanI-ClaI restriction following the Kunkel procedure (11), which was subsequently reincorporated into pBG306 to generate the mutagenized <i>hatG</i> gene. Sequence confirmation of all sequences was by the Sanger method (12) on double-stranded plasmid DNA generated in JM109. Subsequent expression and purification were carried out as described previously (3, 8). The catalase- and peroxidase-specific activities of the variants compared with the native BpKatG are summarized in Table I.

**Catalase, Peroxidase, Protein, and Spectral Determination**—Catalase activity was determined by the method of Rorh and Jensen (13) in a Gilson oxynograph equipped with a Clark electrode. One unit of catalase is defined as the amount that decomposes 1 μmol of H<sub>2</sub>O<sub>2</sub> in 1 min in a 60 mM H<sub>2</sub>O<sub>2</sub> solution at pH 7.0. Peroxidase activity was determined by the method of Rorh and Jensen (13) in a Gilson oxynograph equipped with a Clark electrode. One unit of catalase is defined as the amount that decomposes 1 μmol of H<sub>2</sub>O<sub>2</sub> in 1 min in a 60 mM H<sub>2</sub>O<sub>2</sub> solution at pH 7.0. Peroxidase activity was determined by the method of Smith et al. (15). One unit of peroxidase is defined as the amount that decomposes 1 μmol of 3-ethylbenzthiazolesulfonic acid in 1 min at 20°C. Absorption spectra were obtained using a Milton Roy MR3000 spectrophotometer. The samples were dissolved in 50 mM potassium phosphate, pH 7.0.

**N-terminal Sequencing**—The N-terminal sequence was determined by the Proteinics facility at the Institut de Biotecnologia i Biomedicina.

**Mass Spectrometry Analysis**—For mass spectrometry, protein was dialyzed into 5 mM ammonium acetate. The intact proteins were analyzed by electrospray ionization in an orthogonal time-of-flight mass spectrometer (16, 17). The declustering voltage was varied to assess the stability of the protein-heme complex. Digests of the proteins were prepared using tosylphenylalanyl chloromethyl ketone-treated trypsin and analyzed on the Manitoba/Sciex prototype MALDI QqTOF instrument (18). Initial analysis was done with equal volumes (0.5 μl) of digested protein and 2,5-dihydroxybenzoic acid (160 mg/ml in water: acetonitrile 3:1, 2% formic acid) spotted onto a custom target. Further separation was achieved by microliquid chromatography on the Agilent 1100 Series system. The samples (5 μl) were injected onto a 100-μm I.D. × 150-mm column (Vydac 218 TP C18, 5 μm) and eluted with a linear gradient of 1–80% acetonitrile (0.1% trifluoroacetic acid). Column effluent (4 μl/min) was collected at 1-min intervals by hand. Under the conditions used, the vast majority of tryptic fragments were eluted in 40 min. These were spotted onto the target as described above.

**Results**

**Table I**

| Variant | Catalase units/mg | Peroxidase units/mg |
|---------|------------------|---------------------|
| BpKatG  | 1010             | 1.1                 |
| R108K   | 90               | 0.55                |
| W111F   | 1.3              | 0.53                |
| M264L   | 2.4              | 1.3                 |

**Fig. 1.** Schematic showing the key residues in the heme-containing active site of BpKatG. The perhydroxy group on the vinyl ring of ring I of the heme and the covalent bonds linking Trp<sup>111</sup> to Tyr<sup>238</sup> and Tyr<sup>238</sup> to Met<sup>264</sup> are indicated. The classic triad of active site residues on the distal side of the heme in peroxidases includes Arg<sup>108</sup>, Trp<sup>111</sup>, and His<sup>112</sup>. The dashed lines indicate possible hydrogen bonds. The figure was prepared using SETOR (19).

**Fig. 2.** Sequences of the three peptides containing, respectively, Trp<sup>111</sup>, Tyr<sup>238</sup>, and Met<sup>264</sup> that are covalently linked in BpKatG (A) and Trp<sup>105</sup>, Tyr<sup>226</sup>, and Met<sup>226</sup> in HPI (B). The dashed lines indicate possible covalent links, and the sizes of the peptides are shown on the right along with the sizes of the peptides that would result from Trp-Tyr and Trp-Tyr-Met covalent linkages.
Table II

| Expected ion [M + H+] | Observed in BpKatG | Observed in W111F | Residues | Sequence | Confirmed by MS/MS |
|-----------------------|--------------------|-------------------|----------|----------|-------------------|
| 1140.53               | No                 | 1140.53           | 109-118  | MAFHSAGTYR| Yes               |
| 1179.54               | No                 | 109-118           |          | MAWHSAGTYR| Yes               |
| 1459.65               | 1459.65            | 27-40             |          | CPEHQAGNGTSNR| Yes               |
| 2062.06               | No                 | 1-18              |          | MGPSDAGPPRRVGHQQRR| Yes               |
| 2091.98               | 2091.99            | 264-283           |          | XAMNDEETVIAIGGHTFGK| Yes               |
| 3316.66               | 3316.62            | 224-255           |          | XAMNDEETVIAIGGHTFGK| Yes               |
| 3332.66               |                    |                   |          | QLENPLAAVQMGLIYVNPEGPDGPNPDVAAAR| Yes               |
| 4493.18               | 4493*              |                   | Trp-Tyr adduct of 109-118 and 224-255 | No        |
| 4509*                 | No                 |                   | One oxidation of Trp-Tyr adduct       | No        |
| 4525*                 | No                 |                   | Two oxidation of Trp-Tyr adduct       | Yes       |
| 4543*                 | No                 |                   | Three oxidations and/or CH₃-S added to Trp-Tyr adduct | Yes |
| 4555*                 | No                 |                   | Unknown                                   | No        |
| 4573*                 | No                 |                   | Mixed ion of oxidized and modified Trp-Tyr adduct     | No        |
| 4589*                 | No                 |                   | Mixed ion of oxidized and modified Trp-Tyr adduct     | No        |
| 6583.17               | No                 |                   | Trp-Tyr-Met adduct of 109-118, 224-255, and 264-283 | No        |

* Average m/z of the cluster is presented. Difficulty in identifying the real monoisotopic ion in a mixture of ions precludes the accurate measurement of masses. The identification of oxidized forms of the fragments is complicated by the fact that every ion produces two daughter ions, from loss of water (–18) and loss of NH₃ (–17), giving rise to three overlapping series of ions differing by +16, –17, and –18. The situation is further complicated by the possibility that CH₃-S may also be present, producing a +46 series.

Table III

| N | Expected ion | m/z 4524 | m/z 4540 |
|---|--------------|----------|----------|
| y ions |          |         |         |
| y22 | 4493.18     | 4524.15  | 30.97    |
| y29 | 4123.00     | 4169.69  | 46.69    |
| y22 | 3429.62     | 3459.67  | 30.05    |
| y20 | 3241.56     | 3269.56  | 28.00    |
| y18 | 3015.39     | 3060.21  | 44.82    |
| y15 | 1462.69     | 1462.64  | 0.05     |
| y13 | 1236.60     | 1236.57  | 0.03     |
| y10 | 967.50      | 967.48   | 0.02     |
| y6  | 584.35      | 584.35   | 0.00     |
| b ions |         |         |         |
| b15 | 2863.90     | 45.95    |
| b17 | 3031.50     | 3061.51  | 30.01    |
| b19 | 3257.59     | 3287.54  | 29.95    |
| b22 | 3526.70     | 3556.66  | 29.96    |
| b24 | 3897.76     | 3928.58  | 29.82    |
| b26 | 3969.54     | 3999.81  | 29.97    |
| b31 | 4319.07     | 4350.21  | 44.99    |
| b32 | 4475.17     | 4506.09  | 30.92    |

Met264 that was broken during mass spectrometry analysis and suggests that the remainder of the cleaved methionine should be present in one of the ions. The expected ion for the Met264-containing peptide at m/z 2092 is present in the spectra of both BpKatG and the W111F variant but is accompanied by an unexpected ion at m/z 2062 only in the BpKatG spectrum (Fig. 5, A and B). MS/MS analysis of the ions at m/z 2062 and 2092 (Fig. 5, C and D, and Table IV) reveals the common sequence XAMNDEETVIAIGGHTFGK, in which X has the correct mass for Met in the ion at m/z 2092 but has a mass 30 Da smaller in the ion at m/z 2062. For the fragmentation of the ion at m/z 2062, all of the b series ions, containing the N-terminal residue, are 30 Da smaller than the expected mass, whereas all of the y series ions, with the exception of y32, are in agreement with the expected m/z values (Table IV). Assigning X as homoserine, which would arise from hydrolysis of the Tyr-Met covalent bond (Fig. 4) and which has a mass 30 Da less than that of Met, explains the mass differential between the ions at m/z 2062 and m/z 2092. In addition, this represents further indirect evidence for the covalent link between Tyr238 and Met264.
ion in catalase-peroxidases. This was explored in an analysis of the trypic digest of HPI (KatG) from *E. coli* and its W105F variant (Table V). All three of the fragments containing, respectively, Trp<sup>105</sup> (m/z 1149), Met<sup>252</sup> (m/z 2532), and Tyr<sup>226</sup> (m/z 3206) are present, with their identities confirmed by MS/MS analysis. Although this suggests that the adduct may not be present in HPI, a cluster of ions, also separated by −16 Da, is evident near m/z 4350 (Fig. 6A) in the digest of native HPI but not in the digest of the W105F variant (Fig. 6B). Analysis of the predominant ion by MS/MS reveals a fragmentation pattern consistent with the presence of the Trp-Tyr covalent structure (Fig. 6C). The location of the modifications, either from the addition of CH₃-S (+47 less one proton for +46) or oxidation (+48 for three oxygen atoms less 2 protons for +46), could be localized to the hybrid fragment bounded by ions y<sub>25</sub> and y<sub>16</sub> (Fig. 6C and Table VI). Unfortunately, ions at m/z 6885, indicative of the triple adduct, and m/z 2504, indicative of Met<sup>252</sup> modified to homoserine, were not identified, making the only evidence for the Tyr-Met portion of the adduct in HPI the possible presence of CH₃-S in the ion at m/z 4351.

**Identification of the N Terminus of BpKatG**—The N-terminal 34 residues predicted by the DNA sequence were not evident in the electron density maps of BpKatG (7), raising the question of whether they were present and disordered or absent as a result of N-terminal processing. The trypic fragments corresponding to residues 1–9 and 19–26, including possible partial digest fragments, are absent from the spectrum, whereas the fragment corresponding to residues 27–40 is present, and its sequence is corroborated by MS/MS analysis (Table II). From these data, it can be concluded that the N-terminal sequence is truncated between residues 19 and 26, and this was confirmed by N-terminal sequencing to be at<sub>22</sub>SNEA. Truncation to Ser<sub>22</sub> could have resulted from post-translational proteolysis or from translation initiation at Met<sup>21</sup> followed by removal of the terminal formylmethionine. There is a strong Shine-Dalgarno sequence AGGAG upstream of the ATG codon for Met<sup>21</sup>, and comparison with the much weaker ribosome-binding region upstream of the codon for Met<sup>21</sup> suggests that Met<sup>21</sup> may be the preferred start site for translation of the BpKatG mRNA in *E. coli*.

**TABLE IV**

| N | Expected ion | Observed | Difference | Fig. 5C | Expected ion | Observed | Difference | Fig. 5D |
|---|--------------|---------|------------|--------|--------------|---------|------------|--------|
| y<sub>20</sub> | 592.91 | 592.91 | 0.00 | 592.92 | 0.01 | 592.92 | 0.01 |
| y<sub>19</sub> | 435.83 | 435.83 | 0.00 | 435.83 | 0.00 | 435.83 | 0.00 |
| y<sub>18</sub> | 378.75 | 378.75 | 0.00 | 378.75 | 0.00 | 378.75 | 0.00 |
| y<sub>17</sub> | 321.67 | 321.67 | 0.00 | 321.67 | 0.00 | 321.67 | 0.00 |

**MS Characterization of the Trp-Tyr-Met Adduct in HPI**—The presence of the covalent adduct in catalase-peroxidases from two such disparate sources as the archaeabacterium *H. marismortui* and the Gram-negative bacterium *B. pseudomallei* suggested that it may be a feature common to all catalase-peroxi-
DISCUSSION

Mass spectrometry evidence for the Trp<sup>111</sup>-Tyr<sup>238</sup>-Met<sup>264</sup> adduct in the tryptic digests of BpKatG corroborates the conclusions drawn from electron density maps. Large ions consistent with three peptides linked by Trp<sup>111</sup>-Tyr<sup>238</sup>-Met<sup>264</sup> and two peptides linked by Trp<sup>111</sup>-Tyr<sup>238</sup> are evident in the spectrum of BpKatG but not in the spectrum of the W111F variant. The covalent bonds in the adduct are sensitive to breakdown under mass spectrometry conditions with the Tyr-Met bond being more labile, presenting indirect evidence for its existence in the breakdown products, including CH<sub>3</sub>-S attached to Tyr<sup>238</sup> and homoserine. In fact, the existence of ions differing only in having methionine or homoserine suggests that the breakdown of the Tyr-Met link may proceed by more than one pathway. The complete absence of any Trp<sup>111</sup>-containing peptide indicates that breakdown of the Trp-Tyr bond, although less prevalent, produces a Trp-containing breakdown product that has not been identified. The tryptic digest of HPI presents direct evidence for the Trp-Tyr linkage and indirect evidence for the Tyr-Met linkage.

The core structure of the individual N- and C-terminal domains of catalase-peroxidases is very similar to the core structure of plant peroxidases, suggesting that the enzyme is a peroxidase that has adopted an efficient catalatic activity during evolution. A small number of clues for how this adaptation took place are provided by the structure of the active site. The active site Trp<sup>111</sup> is clearly not required for the generation of Cpd I (Cpd I <sup>Fe<sup>III</sup></sup>)<sub>2</sub> (Por<sup>−</sup>-Fe<sup>III</sup>)<sub>2</sub>-H<sub>2</sub>O<sub>2</sub>), which is unaffected by removal of the indole ring, but is required for the second stage reduction of compound I (Cpd I<sup>Fe<sup>III</sup></sup>)<sub>2</sub>-H<sub>2</sub>O<sub>2</sub> → Cpd I<sup>Fe<sup>II</sup></sup>(Por<sup>−</sup>-Fe<sup>II</sup>)<sub>2</sub> + H<sub>2</sub>O), which is unaffected by removal of the indole ring, but is required for the second stage reduction of compound I (Cpd I<sup>Fe<sup>II</sup></sup>(Por<sup>−</sup>-Fe<sup>II</sup>)<sub>2</sub>)<sub>2</sub>-H<sub>2</sub>O<sub>2</sub> → Enz (Por<sup>−</sup>-Fe<sup>III</sup>)<sub>2</sub>+H<sub>2</sub>O + O<sub>2</sub>), which is reduced to 1% of native levels by removal of the indole ring. However, the distal Trp is not the sole determinant, because peroxidases with Trp in the equivalent position, such as cytochrome c peroxidase and ascorbate peroxidase, do not exhibit significant catalatic activity. Furthermore, the two other key active site residues in peroxidases, the arginine and histidine equivalent to Arg<sup>108</sup> and His<sup>112</sup> of BpKatG, are spatially oriented much the same as in BpKatG. Indeed, the root mean square deviation of the Co of 133 residues in conserved α-helical segments, including the three active site residues, is just 0.97 Å comparing BpKatG and cytochrome c peroxidase (7).
The most significant differences between the catalase-peroxidases and the peroxidases, aside from sheer size, lie in the unusual post-translational modifications in the catalase-peroxidases, the Trp-Tyr-Met adduct, and the modified heme, and it is reasonable to consider how these features might make the catalase reaction possible.

The covalently linked residues would form a very rigid structure that would fix the position of the indole nitrogen of the essential Trp relative to the heme iron and imidazole ring of the essential His. Such precise positioning with little effect on peroxidatic activity (Table I), and mutation of the equivalent of Tyr238 in Synecocystis KatG has a similar effect (20). The covalent linkages may also affect the electronic environment of the indole, enhancing its ability to bind $\text{H}_2\text{O}_2$ for reduction of compound I. Indeed, mutation of Met264, which would prevent formation of at least part and possibly all of the covalent structure, significantly reduces catalatic activity, with little effect on peroxidatic activity (Table I), and mutation of the equivalent of Tyr238 in Synecocystis KatG has a similar effect (20). The covalent linkages may also affect the electronic environment of the indole, enhancing its ability to bind $\text{H}_2\text{O}_2$ for reduction of compound I. In addition the adduct creates an obvious route for electron delocalization of the radical from the heme of compound I, a process recently demonstrated in $M$. tuberculosis KatG (21).

From the standpoint of the peroxidatic reaction, electron tunneling from a peroxidatic substrate on the surface (7) to the heme for reduction of compound I or compound II may also be facilitated by the adduct.

A number of tryptic fragments, in particular those near the catalytic center, were present as clusters of ions differing by $\sim$16 Da. This is suggestive of multiple oxidations, but the multiplicity of peaks poses a problem for this simple interpretation. The spectrum of the cluster of ions at $m/z$ 4525 contains seven ions, although there are only three obvious sites of oxidation in the fragment, Trp$^{111}$, Met$^{129}$, and Met$^{234}$. Even allowing for up to two oxygens on each Met and one on the Trp, only five ions should be present. Furthermore, the only site of oxidation that was obvious in the electron density maps was the indole ring of Trp$^{111}$ (7), and any other oxidations must be randomly distributed at an occupancy too low to be clearly evident. This makes it very likely that the additional, more intense ions, 46 Da and larger than the base ion, contain the $\text{CH}_3\text{S}$ portion of Met$^{234}$ transferred to Tyr$^{238}$. Thus, at least two overlapping series of ions are responsible for the cluster at $m/z$ $\sim$4525.

It is tempting to speculate about the reaction mechanism responsible for the Trp-Tyr-Met covalent structure, and both free radical and ionic mechanisms can be presented that may be initiated by oxidation of the most reactive group, Met$^{264}$. However, structural analysis of variants lacking the three involved residues and other nearby residues are required to determine what residues are necessary and to see whether partial adducts can be formed will provide a much firmer basis for such speculation.

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