Autocatalytic Formation of a Covalent Link between Tryptophan 41 and the Heme in Ascorbate Peroxidase†

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ABSTRACT: Electronic spectroscopy, HPLC analyses, and mass spectrometry (MALDI-TOF and MS/MS) have been used to show that a covalent link from the heme to the distal Trp41 can occur on exposure of ascorbate peroxidase (APX) to H_2O_2 under noncatalytic conditions. Parallel analyses with the W41A variant and with APX reconstituted with deuteroheme clearly indicate that the covalent link does not form in the absence of either Trp41 or the heme vinyl groups. The presence of substrate also precludes formation of the link. Formation of a protein radical at Trp41 is implicated, in a reaction mechanism that is analogous to that proposed [Ghiladi, R. A., et al. (2005) *Biochemistry 44*, 15093−15105] for formation of a covalent Trp-Tyr-Met link in the closely related catalase peroxidase (KatG) enzymes. Collectively, the data suggest that radical formation at the distal tryptophan position is not an exclusive feature of the KatG enzymes and may be used more widely across other members of the class I heme peroxidase family.

The three most prominent members of the class I superfamily of heme peroxidases (*1*) are cytochrome *c* peroxidase (C*c*P),[1] ascorbate peroxidase (APX), and the bifunctional catalase−peroxidase (KatG) enzymes. These class I enzymes are distinguished from other peroxidases in the superfamily by the presence of a distal tryptophan residue, in place of the more usual phenylalanine residue, and a second active site tryptophan adjacent to the proximal histidine ligand (Figure 1). The catalase−peroxidases contain a unique structural feature, however, that distinguishes them from all other heme peroxidases: in all catalase−peroxidase enzymes for which crystal structures have appeared (*2*−*4*), a covalently bound Trp-Tyr-Met cross-link (involving Trp107, Tyr229, and Met255 in the *Mycobacterium tuberculosis* KatG enzyme, Figure 1a) has been observed. The formation of the Trp-Tyr-Met link is therefore believed to be a feature of all KatGs and is proposed (*5*, *6*) to be formed through an autocatalytic mechanism involving reaction with H_2O_2. Trp and Tyr residues are essential for complete formation of the link, but replacement of Met is tolerated if both Tyr and Trp are present (*5*−*7*). The functional role of this covalent link is not clearly established, but the fact that it is not observed in the monofunctional peroxidases (APX and C*c*P, Figure 1b,c) suggests that it might be connected with catalatic activity in KatGs. Mutagenesis data support this suggestion (*7*−*11*).

The proposed mechanism (*5*, *6*) of formation of the Trp-Tyr-Met link involves initial reaction with peroxide to form a Compound I intermediate (containing a ferryl heme and a porphyrin π-cation radical). This is followed by oxidation of both Trp107 and Tyr229 (numbering according to Figure 1a) by Compound I to form protein radicals at both residues. Radical cross-linking reactions then occur to form the Trp-Tyr link. A second cycle of reaction with H_2O_2 is then proposed for formation of the Tyr-Met link. A key feature is the proposed formation of a protein radical at the distal Trp residue, in a mechanism that can be considered as analogous to the oxidation of the proximal Trp191 residue in Compound I of C*c*P. At present, however, it is not clear whether other, related heme proteins are able to support oxidation of the distal Trp residue.

In this paper, we present the first direct evidence to demonstrate that covalent linking of Trp41 to the heme group in ascorbate peroxidase can occur on exposure of the enzyme to peroxide. A reaction mechanism involving formation of a protein radical at Trp41 is implicated to account for this observation. Together, the data suggest that radical formation at the distal tryptophan position is not an exclusive feature of the KatG enzymes and may be used more widely in other, related peroxidases.

EXPERIMENTAL PROCEDURES

Materials. L-Ascorbic acid (Aldrich Chemical Co.) and all buffers (Fisher) were of the highest analytical grade (99%+ purity) and were used without further purification. Sinapinic acid and α-cyano-4-hydroxycinnamic acid were purchased from Fluka. Water was purified by an Elga purelab

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[1] Abbreviations: APX, ascorbate peroxidase; rsAPX, recombinant soybean cytosolic ascorbate peroxidase; C*c*P, cytochrome *c* peroxidase; KatG, catalase−peroxidase; mtKatG, *Mycobacterium tuberculosis* catalase−peroxidase.

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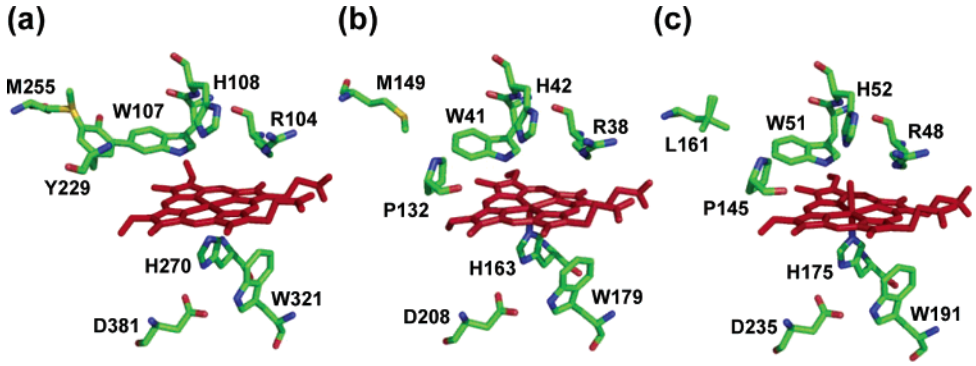


FIGURE 1: Comparison of active site structures of (a) mtKatG (PDB accession code 1SJ2), (b) rsAPX (1OAG), and (c) C*c*P (2CYP) showing the covalent links between residues W107, Y229, and M255 in mtKatG and the residues occupying the equivalent positions in rsAPX and C*c*P.

purification system, and all buffers were filtered (0.2 μm) prior to use. Hydrogen peroxide solutions were freshly prepared by dilution of a 30% (v/v) solution (BDH); exact concentrations were determined using the published absorption coefficient ($\epsilon_{240} = 39.4\ M^{-1}\ cm^{-1}$) (*12*). All molecular biology kits and enzymes were used according to manufacturer's protocols.

Mutagenesis, Protein Expression, and Purification. Site-directed mutagenesis on recombinant soybean cytosolic APX (rsAPX) was performed according to the QuikChange protocol (Stratagene Ltd., Cambridge, U.K.). The sequence of the W41A variant was confirmed by DNA sequencing as reported previously (*13*). Bacterial fermentation of cells and purification of rsAPX and W41A were carried out according to published procedures (*13*, *14*). Purified samples of rsAPX and W41A showed wavelength maxima at 407 (107), 525, and ≈630 nm and 405 (125), 525, and ≈630 nm, respectively, as reported previously (*13*, *15*). Enzyme concentrations for rsAPX and W41A were determined using absorption coefficients of $\epsilon_{407} = 107\ mM^{-1}\ cm^{-1}$ (*15*) and $\epsilon_{405} = 125\ mM^{-1}\ cm^{-1}$ (*13*), respectively. Cytochrome *c* peroxidase was expressed according to published procedures (*16*). The C*c*P expression construct was a kind gift from Professor Grant Mauk (University of British Columbia). Purified enzyme showed wavelength maxima at 409 (95), 504, and 529$^{(sh)}$ nm (150 mM potassium phosphate, pH 6). Enzyme concentrations for C*c*P were determined using an absorption coefficient of $\epsilon_{409} = 95\ mM^{-1}\ cm^{-1}$.

Electronic Absorption Spectroscopy. Spectra were collected using a Perkin-Elmer Lambda 35 or 40 spectrophotometer, linked to a PC workstation running UV-Winlab software. Pyridine−hemochromagen assays before and after reaction of the enzyme with H_2O_2 were carried out according to established protocols (*17*). The pyridine−hemochromagen experiment proceeds as follows: a solution of protein is mixed with a solution of pyridine in NaOH. Oxidized pyridine−hemochromagen, which is a soluble and stable compound, is formed rapidly. The spectrum between 600 and 500 nm is recorded just after addition of solid sodium dithionite, which yields the relatively unstable reduced pyridine−hemochromagen.

Steady-State Kinetics. Steady-state measurements (100 mM potassium phosphate, pH 7.0, 25 °C) for oxidation of ascorbate were carried out according to published protocols (*18*).

Reconstitution of rsAPX with Iron(III) Deuteroporphyrin IX Chloride. Apoenzyme was initially prepared by the method of Teale (*19*). Iron(III) deuteroporphyrin IX chloride (>97%; Porphyrin Products, Frontier Scientific Inc.) was dissolved in 0.1 M potassium hydroxide (1 mg/mL) which was added dropwise to the apoenzyme, with stirring on ice, until an Rz value of >2 was reached. The solution was incubated on ice for another 30 min. An additional amount of iron(III) deuteroporphyrin IX chloride solution was added, and the solution was further incubated on ice for another 15 min. This solution was dialyzed against potassium phosphate (20 mM, pH 7.0) overnight at 4 °C. The protein solution, which showed wavelength maxima at 402, 521, 557$^{(sh)}$, and 625 nm, was concentrated and exchanged in water. Any precipitated protein or excess heme was removed by centrifugation (13000 rpm, 5 min).

High-Performance Liquid Chromatography. HPLC analyses of protein samples before and after reaction with H_2O_2 were conducted on a Varian ProStar HPLC system with an analytical Vydac C4 reverse-phase HPLC column under computer control. Solvents were as follows: A = 0.1% (w/v) trifluoroacetic acid (TFA) in 95% H_2O and 5% acetonitrile; B = 0.1% (w/v) TFA in 80% acetonitrile and 20% H_2O. The column was preequilibrated with 42% buffer B, and protein/heme separation was achieved using the following elution gradient: 42% B for 5 min, 42−55% B for 25 min, 55−100% B for 10 min, and 100% B for 5 min. Chromatograms were monitored at 215 nm for protein-containing fractions and 398 nm for heme-containing fractions.

For separation and collection of fragments, an Agilent Series 1100 binary pump HPLC system was used, fitted with a diode array detector. For collection of the hydroxylated heme species, protein samples, after reaction with H_2O_2, were injected onto a Phenomenex JUPITER 5u C4 300A column (250 × 2 mm); fractions eluting at 4 min were collected, freeze-dried, and resuspended in 200 μL of H_2O to be used for MALDI-TOF mass spectrometry.

Samples for trypsin digestion were treated with 50:1 (w/w) enzyme:trypsin in 0.4 M NH_4HCO_3, pH 8, at 37 °C for 18 h. The digestion was stopped by addition of TFA (1 μL). The digested mixture was then injected onto a reverse-phase C8 column (P. E. BrownLee Columns Aquapore Octyl, RP-300 C8 7 μm, 220 × 2.1 mm). Solvents were as follows: A = 0.1% (w/v) TFA in 95% H_2O and 5% acetonitrile; B =

0.1% (w/v) TFA in 80% acetonitrile and 20% H_2O. Separation of peptide fragments was achieved using the following elution gradient: $t = 0$ min, B = 2%; $t = 2$ min, B = 2%; $t = 45$ min, B = 75%; $t = 50$ min, B = 100%; $t = 55$ min, B = 100%. Peptide fractions showing both heme and protein absorbances were collected and then evaporated to dryness and dissolved in H_2O.

Mass Spectrometry. Protein samples for MALDI-TOF mass spectrometry were exhaustively exchanged into 0.4 M NH_4HCO_3, pH 8, using a Millipore Centricon (Centrifugal Filter Devices) ultrafiltration device (molecular mass cutoff 10 kDa) and by centrifugation at 3000*g*. The sample was then diluted 1:1 or 1:50 (depending on sample concentration) with matrix (sinapinic acid, 10 mg/mL, and 0.1% TFA in 1:1 acetonitrile/H_2O). Analysis was carried out as follows: 1 μL of the protein/matrix mixture was spotted onto a MALDI target plate using the drying droplet method. The MALDI-TOF mass spectrometer was calibrated in the range 2000−45000 Da using horse heart myoglobin. Spectra were an average of 1000 shots.

Peptide samples (1 μL) were mixed with matrix (α-cyano-4-hydroxycinnamic acid, 5 mg/mL, and 0.1% TFA in 1:1 acetonitrile/H_2O) for MALDI-TOF mass spectrometry. For these experiments, the MALDI-TOF mass spectrometer was calibrated in the range 500−6000 Da with a peptide mass calibration kit (Sigma; used according to manufacturer's instructions). Spectra of the HPLC-purified peptide fragments were collected in the same mass range using an average of 250 laser shots. All MALDI-TOF spectra were analyzed using Data Viewer software (Applied Biosystems).

MS/MS Analyses. HPLC-purified samples of peptide fragments prepared as above were resuspended in water (100 μL). The solution was then mixed in a 1:1 ratio with acetonitrile/0.2% formic acid and infused from a syringe pump (Harvard Apparatus Ltd., Edenbridge, Kent, U.K.) at a flow rate of 0.5 μL/min to the MicroIonspray source of a 4000 Q-Trap mass spectrometer (Applied Biosystems, Warrington, U.K.). The sample was sprayed directly from a fused-silica PicoTip (New Objective Inc., Woburn, MA) maintained at a potential of 2800 V in the positive ion mode. Tandem mass spectrometry was carried out for ions of interest. Spectra of fragment ions were interpreted manually.

RESULTS

Reaction of rsAPX with Hydrogen Peroxide and Characterization of Products. Reaction of rsAPX with 6 equiv of H_2O_2 resulted in the initial formation of a Compound I intermediate, as observed previously (*18*) (data not shown). This is followed by immediate conversion of this Compound I to a more stable Compound II species (Figure 2). Compound II then decays over a period of ≈8 h to a final product that has a spectrum that is similar but not identical to that of rsAPX (Figure 2). The activity of rsAPX against ascorbic acid was monitored before and after reaction with H_2O_2 under the conditions described above. Values for k_{cat} after reaction with H_2O_2 were essentially unchanged from those of the wild-type enzyme (*18*).

A pyridine−hemochromagen assay was carried out on rsAPX both before and after reaction with H_2O_2. For rsAPX before treatment with H_2O_2, the spectrum of the reduced pyridine−hemochromagen complex obtained in this way showed a maximum at 556 nm (Figure 3). In this experiment, complete extraction of the heme from the protein is observed, and the spectrum of the hemochromagen complex is consistent with a noncovalently bound heme structure, in which neither heme vinyl group is modified (*17*). When the same experiment was carried out with rsAPX after treatment with H_2O_2, the peak was shifted to 553 nm (Figure 3). These spectroscopic changes are diagnostic of the covalent attachment to one of the two vinyl groups on the heme (*20*).

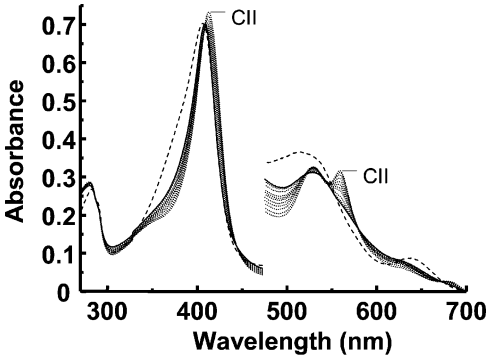


FIGURE 2: Selected electronic spectra collected during the reaction of ferric rsAPX with 6 equiv of H_2O_2. Conversion of rsAPX (dashed line) to the initial Compound II intermediate (indicated as CII on the figure) is observed, followed by conversion back to a ferric-like species (solid line). Intermediate spectra between Compound II and ferric-like are shown as dotted lines. The total reaction time was 8 h. The visible region has been multiplied by a factor of 5. Sample conditions: enzyme, 5 μM; hydrogen peroxide, 30 μM; 0.1 M potassium phosphate, pH 7.0; 25 °C.

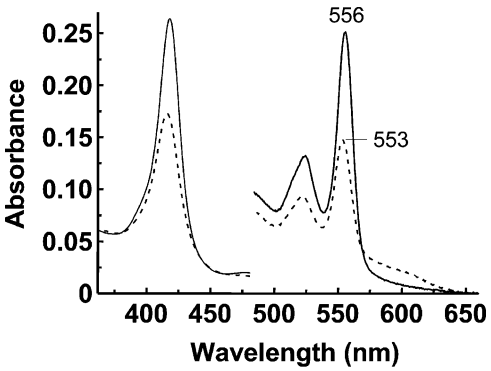


FIGURE 3: Spectra of the reduced pyridine−hemochromagen complexes of rsAPX before (solid line) and after (dashed line) reaction with H_2O_2.

HPLC Analyses. HPLC analysis of the product of the reaction of rsAPX with H_2O_2 (prepared as above) showed that the protein (monitored at 215 nm) and a significant proportion of the heme (monitored at 398 nm) coelute at 24 min (Figure 4b); this is in direct contrast to the HPLC profile of rsAPX which has not been treated with H_2O_2, in which the heme (11 min) and the protein (24 min) do not coelute (Figure 4a). Coelution of the heme and the protein fragments is a clear indication of covalent heme attachment and has been used previously to identify covalently linked heme in various other heme proteins (*14*, *21*−*25*). In separate experiments (data not shown), a commercial sample of hemin eluted at 11 min, confirming the assignment for free heme above. For the sample of rsAPX treated with H_2O_2, there is a second peak that elutes earlier (4 min) than free heme

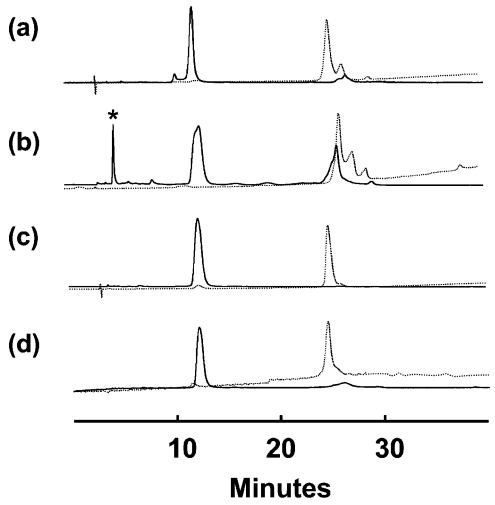


FIGURE 4: HPLC analyses of rsAPX and W41A before and after reaction with H_2O_2 monitored at 398 nm (solid line) and 215 nm (dotted line). (a) rsAPX before reaction with H_2O_2; (b) rsAPX after reaction with H_2O_2; (c) W41A before reaction with H_2O_2; (d) W41A after reaction with H_2O_2.

(labeled with an asterisk in Figure 4b). This is indicative of a hydroxylated heme product (*21*), and a similar assignment is proposed here. MALDI-TOF analysis of this heme fragment confirmed this assignment: a mass of 650 Da was observed (data not shown), which is consistent with a doubly hydroxylated heme species. Nonspecific hydroxylation of heme groups has been reported in other peroxidase systems (*23*, *26*).

In separate experiments (data not shown) with rsAPX under the same conditions as those used above for Figure 4b but in the presence of a large excess of ascorbate, it was shown that no HPLC peak corresponding to protein-bound heme was observed at ≈24 min. This indicates that under turnover conditions no formation of covalently bound heme occurs.

Mass Spectrometric Analyses. Mass spectrometry data are consistent with the HPLC analyses above and provided further evidence for partial covalent attachment of the heme to the protein. MALDI-TOF analysis of rsAPX before treatment with H_2O_2 (Figure 5a) showed a mass of 28312.0 Da, which corresponds closely to the predicted mass (28318.9 Da) of the apoprotein and is consistent with noncovalent attachment of the heme to the protein in rsAPX. After treatment with H_2O_2, two peaks were observed in the MALDI-TOF spectrum (Figure 5b). The first is at 28320.6 Da, which is consistent with the mass of the apoprotein (as above). The second peak is at 28954.3 Da, which corresponds to an increase in mass of 634 Da over the apoprotein, and is consistent with covalent attachment of the heme (616 Da) to the protein. The additional mass of 18 amu is assigned as arising from hydroxylation of the fragment.

To establish the nature of the heme−protein covalent link, tryptic digestion of the product of the reaction of rsAPX with H_2O_2 was carried out, and HPLC was used to isolate heme-containing peptide fragments (i.e., showing both heme and protein absorbances) from the resulting peptide mixture (data not shown).[2] The mass of 1863.9 Da observed for this fragment (Figure 5c) is 16 Da higher than the calculated mass of 1848 Da expected for the $L^{39}A\mathbf{W^{41}}HSAGTFDK^{49}$ peptide fragment containing heme covalently bound to Trp41.

MS/MS mass spectrometry was used to obtain sequence information for this heme-containing fragment. In this case, a *y*-ion fragmentation series allowed amino acids to be identified sequentially from His42 through to Thr46 with the remaining C-terminal mass (409.1 Da) consistent with residues Phe47 through to Lys49, which corresponds to the sequence HSAGTFDK (Figure 6). The remaining N-terminal mass, together with the absence of fragment ions for the amino acids (LAW^{41}), indicates that the heme forms a covalent cross-link with this part of the peptide. Since Leu and Ala are unlikely to be able to form a covalent cross-link to the heme, the data are interpreted as being consistent with the formation of a covalent link from the heme to Trp41.

Experiments with Iron(III) Deuteroporphyrin. To provide further information on the nature of the covalent attachment, a sample of apo-rsAPX was reconstituted with iron(III) deuteroporphyrin, in which hydrogen atoms replace the 2- and 4-vinyl groups, and subsequently reacted with H_2O_2 as above. No peak corresponding to covalently bound heme was observed by HPLC, because heme was released from the protein and eluted at 5 min both before (Figure S1a in the Supporting information) and after (Figure S1b in the Supporting Information) reaction with H_2O_2. A commercial sample of deuteroheme was also shown to elute at 5 min (data not shown).

Examination of the W41A Variant. Further experiments were carried out with the W41A variant, which has been previously shown (*13*) to be catalytically competent for reaction with H_2O_2 and substrate oxidation. Hence, the W41A variant was reacted with H_2O_2 under the same conditions as those used for rsAPX (*vide supra*), and the corresponding HPLC and MALDI-TOF analyses were carried out. HPLC analysis prior to treatment with H_2O_2 (Figure 4c) showed that the heme and the protein elute separately as for rsAPX under the same conditions. However, on treatment with H_2O_2 no change in the HPLC elution profile was observed, indicating that no covalent attachment of the heme to the protein had occurred in this case (Figure 4d). In addition, and in contrast to rsAPX, there is no evidence for formation of a hydroxylated heme species in the HPLC analyses.

MALDI-TOF analyses (Figure S2 in the Supporting Information) confirmed these observations since essentially identical molecular masses are observed for W41A before (28207.1 Da) and after (28202.5 Da) treatment with H_2O_2. Both of these values are in good agreement with the calculated mass for the apo-W41A variant (28203.7 Da).[3]

Examination of CcP. In order to examine whether C*c*P exhibits similar behavior to rsAPX when treated with hydrogen peroxide, HPLC analysis was carried out before (Figure S1c in the Supporting Information) and after (Figure S1d in the Supporting Information) treatment of C*c*P with hydrogen peroxide under the same conditions as those used for rsAPX above. No evidence for formation of a covalent link between the heme and the protein was observed in this case.

[2] There were three peaks with significant absorbance at 398 nm. The MALDI-TOF mass spectrum of the product eluting at 35.1 min gave a mass of 616 Da, indicating that this HPLC peak corresponds to free heme species. MALDI-TOF mass spectrometry of the peptide fragments eluting at 26.4 and 27.5 min both gave spectra with identical masses of 1863.9 Da, indicating that both HPLC peaks are assigned to a single peptide (Figure 5c).

[3] A minor peak at 27488.2 Da is also observed, which may arise from nonspecific cleavage of the protein.

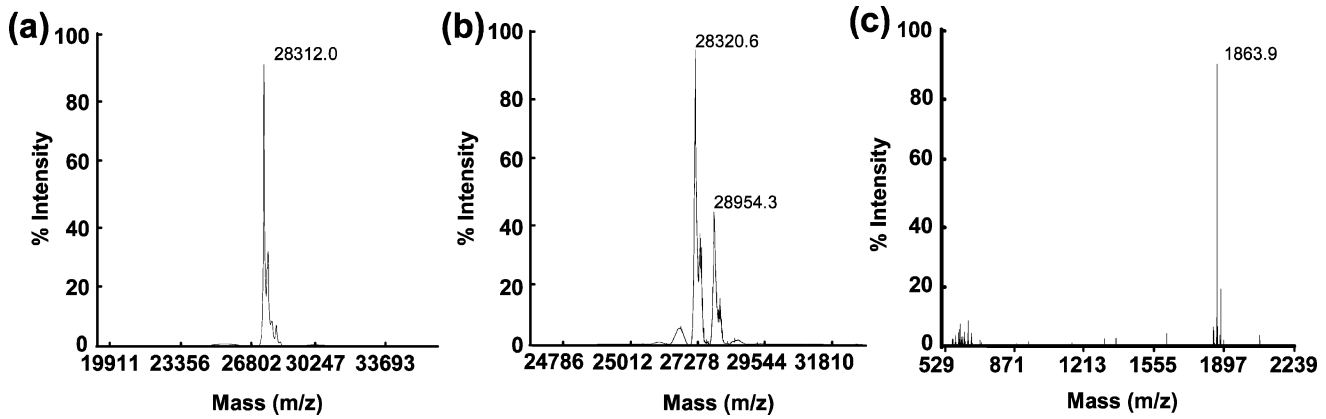


FIGURE 5: MALDI-TOF mass spectrum of rsAPX before (a) and after (b) reaction with 6 equiv of H_2O_2. (c) MALDI-TOF mass spectrum of the HPLC-purified heme-containing peptide fragment obtained after reaction of rsAPX with H_2O_2.

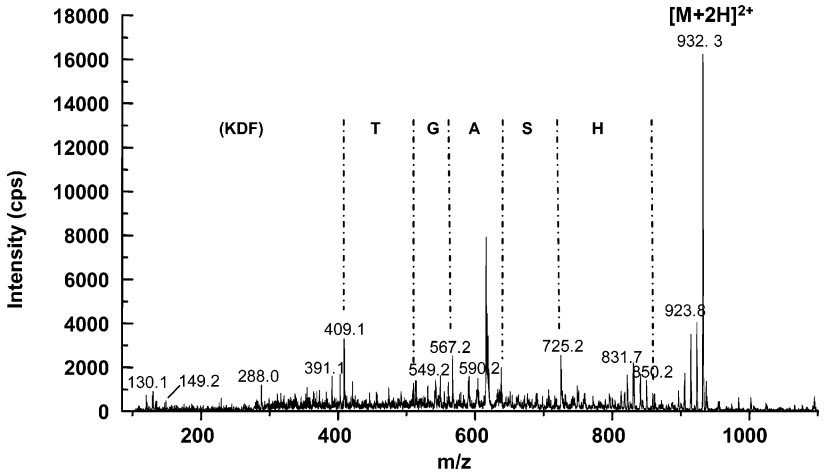


FIGURE 6: MS/MS spectrum of the HPLC-purified heme-containing peptide fragment showing the doubly charged precursor ion (932.56 Da). The annotated peptide sequence corresponds to the *y*-ion fragment series.

DISCUSSION

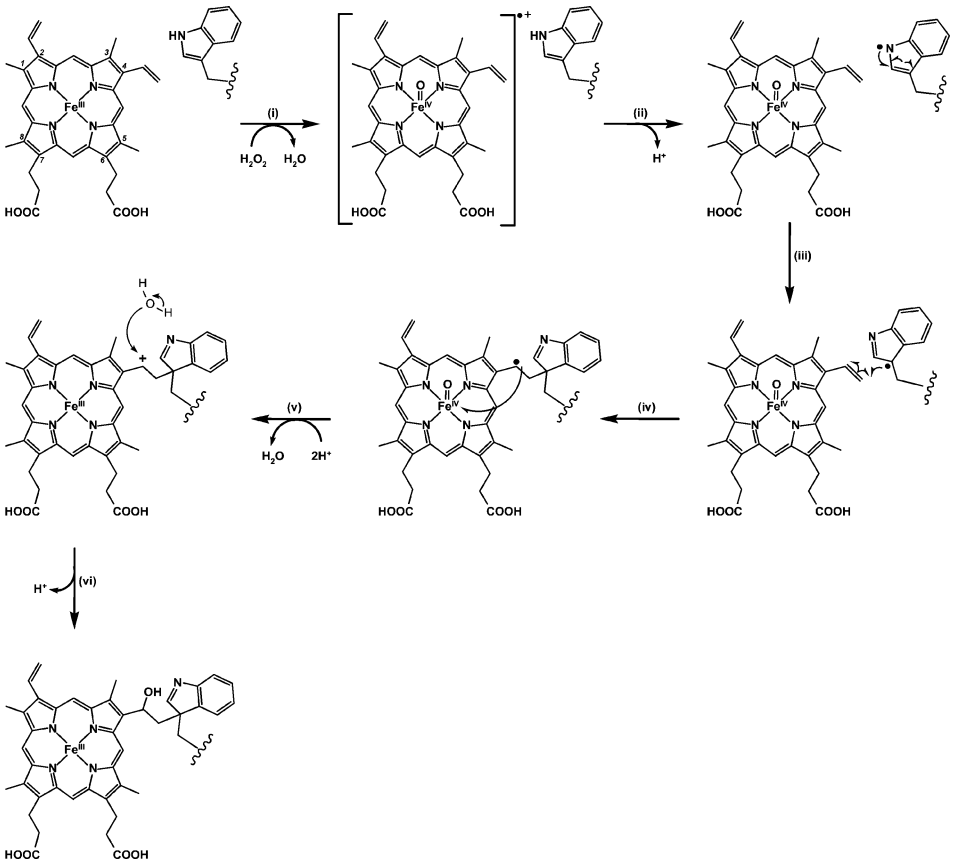
In the mammalian heme peroxidases, it is known that different types of covalent link from protein amino acids to the heme group are used (*27*). Although covalently bound heme groups are not observed in other peroxidases, there is an increasing amount of evidence to show that these links can be engineered into an existing peroxidase framework if the correct residue is inserted at the correct site and if the peroxidase in question is competent for reaction with H_2O_2 (see, for example, refs *14* and *23*). This represents a substantial change in the way we think about these modified peroxidases: collectively, what it tells us is that there is no intrinsic reason why any peroxidase, or any heme protein for that matter, cannot form a particular covalent link under the right conditions. *In extremis*, this might even mean that some proteins may need to be poised in an environment that specifically "switches off" these covalent links. Collectively, and in view of the very similar active site structures of the class I peroxidase enzymes, these ideas indicated to us that the same principle may apply to the covalent Trp-Tyr-Met link in KatG (at least in the class I enzymes). More specifically, the mechanisms that lead to formation of the Trp-Tyr-Met link in KatG might also be accessible under certain conditions in other class I peroxidases.

The mechanism of formation of the Trp-Tyr-Met link in KatG is proposed to involve initial formation of both Trp and Tyr radicals through a normal oxidative peroxidase mechanism (*5*, *6*). Although both C*c*P and APX contain a distal Trp residue in an essentially identical structural environment to that observed in KatG (Figure 1), the Trp-Tyr-Met link is not observed in either of these enzymes because the active site Tyr residue is missing in APX (Figure 1b) and because both Tyr and Met are missing in C*c*P (Figure 1c). In this paper, we have presented evidence from electronic spectroscopy, HPLC analyses, and mass spectrometry to show that under noncatalytic conditions (*i.e.* in the absence of substrate) and over long time scales a covalent link from the heme to the Trp41 can be formed in APX. This link does not form in the presence of substrate (ascorbate). Experiments with the W41A variant and with deuteroheme clearly indicate that the covalent link does not form in the absence of either Trp41 or the heme vinyl groups.

A possible mechanism for formation of the covalently bound heme species that embodies all of the available data is presented in Scheme 1. In this mechanism, initial formation of a Compound I intermediate (step i)[4] is followed by oxidation and deprotonation of Trp41 (step ii) and addition of the Trp41 radical across the 4-vinyl group of the heme

[4] We assume that formation of the link is not dependent on the exact nature of the oxidant and that any other oxidant that is capable of oxidizing the heme to Compound I (for example, peracetic acid) would also lead to a covalently bound heme.

Scheme 1: Proposed Mechanism for Formation of a Covalent Link between Trp41 and the Heme Group in Ascorbate Peroxidase[a]



[a] Steps i−vi are described in the text.

(steps iii[5] and iv). Subsequent reduction of the ferryl group and release of H_2O (step v) lead to formation of a carbocation. Nucleophilic addition of H_2O as the final step (step vi) gives a heme structure that is consistent with the mass spectrometry data. The shortest distance from the C^{β} on the 4-vinyl group to the C^{γ} of Trp41 is 4.8 Å. We note that hydroxylation of a covalently bound heme fragment has also been observed previously (*14*). Further evidence in support of this mechanism comes from previous work in which formation of a protein-based radical, proposed to be a Trp radical, on reaction of APX with H_2O_2 in the absence of substrate has been reported (*28*).

The data for C*c*P are curious because no evidence for formation of an analogous link to Trp51 was observed. C*c*P is unique among the class I peroxidases in that it utilizes a protein amino acid, Trp191, during its catalytic cycle. Hence, the enzyme diverts oxidizing equivalents to Trp191 as the immediate product of H_2O_2 oxidation without formation of a porphyrin π-cation radical. Diversion of the radical to Trp191 would preclude formation of a radical at Trp51 according to the mechanism proposed in Scheme 1. Interestingly, there is separate evidence that formation of a radical on Trp51 in C*c*P may occur under certain conditions (*29*). Hence, Poulos and co-workers have shown that peroxide-dependent formation of a Trp51-Tyr52 link occurs in the H52Y variant of C*c*P and the formation of this link is proposed to involve oxidation of Trp51 to Trp$^{\bullet}$ and oxidation of Tyr52 to Tyr$^{\bullet}$ in a mechanism that is analogous to that proposed for KatG (*5*, *6*) (i.e., without formation of the expected Compound I species, with a radical on Trp191).

In summary, these data add a new dimension to our wider understanding of the differences between the KatG enzymes and the monofunctional peroxidase enzymes. The data indicate that, under noncatalytic conditions, there is no intrinsic barrier to formation of a radical at Trp41 in APX analogous to that thought to be used in the KatGs. The direct implication is that formation of a covalent Trp-Tyr-Met link in APX is limited not by the inherent reactivity of the enzyme but by the absence of a tyrosine residue adjacent to Trp41.

A separate question arises, however: how do the KatG enzymes protect their heme groups from modification by Trp? Clearly, the presence of the relevant amino acids close to the distal Trp is essential, but other, more subtle, effects are likely to also be important. For example, very slight differences in the precise orientation of key amino acids may well be critical in defining whether a particular covalent link is formed. As a case in point we note that a covalent link between an engineered methionine residue at position 160 (S160M variant) and the heme, analogous to those found in the mammalian peroxidases, can be incorporated into the APX framework but that cytochrome *c* peroxidase, which already contains a Met residue at this position (Met172), does not form the same covalent heme-Met link under any accessible conditions.

[5] We have proposed a radical on C^{γ} on the basis that this will be more stable (tertiary radical) and because a radical on this carbon retains the aromatic ring structure of Trp41 compared to the other resonance forms.

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SUPPORTING INFORMATION AVAILABLE

HPLC analysis of rsAPX reconstituted with iron(III) deuteroporphyrin before (Figure S1a) and after (Figure S1b) reaction with H_2O_2 and of C*c*P before (Figure S1c) and after (Figure S1d) reaction with H_2O_2 and MALDI-TOF mass spectrum of W41A before (Figure S2a) and after (Figure S2b) reaction with 6 equiv of H_2O_2. This material is available free of charge via the Internet at http://pubs.acs.org.

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