Radical Formation at Tyr39 and Tyr153 Following Reaction of Yeast Cytochrome *c* Peroxidase with Hydrogen Peroxide†

Haoming Zhang,‡ Shouming He,§ and A. Grant Mauk*,‡

Departments of Biochemistry and Molecular Biology and of Chemistry, University of British Columbia, Vancouver, BC V6T 1Z3 Canada

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ABSTRACT: The formation of yeast cytochrome *c* peroxidase (C*c*P) compound I has been recognized for many years to be associated with formation of two protein-centered radicals. One of these radical sites is located at Trp191 and is directly involved in catalytic oxidation of ferrocytochrome *c* (Sivaraja, M., Goodin, D. B., Smith, M., Hoffman, B. M. (1989) *Science 245*, 738−740). The second radical has been proposed to arise from one or more tyrosyl residues of C*c*P. However, the tyrosyl residue (or residues) capable of forming this radical has not been identified, and the functional role of this radical remains poorly understood. In the present work, this issue has been addressed through the combined use of the spin-trapping reagent 2-methyl-2-nitrosopropane and peptide mapping by electrospray mass spectrometry to identify Tyr39 and Tyr153 as two tyrosyl residues that are capable of forming radical centers upon reaction of C*c*P with hydrogen peroxide. The implications of this observation to the catalytic mechanism of C*c*P are addressed with reference to the three-dimensional structure of C*c*P.

Yeast (*Saccharomyces cerevisiae*) cytochrome *c* peroxidase (C*c*P)[1] is a monomeric, heme-containing enzyme that catalyzes the oxidation of two molecules of ferrocytochrome *c* by hydrogen peroxide through a mechanism that involves initial reaction of the ferric "resting" enzyme with hydrogen peroxide to form an intermediate, referred to historically as compound ES. Compound ES is similar to the corresponding intermediate compound I formed during the catalytic reaction of other peroxidases with hydrogen peroxide in that it is oxidized by two equivalents relative to the resting (Fe(III)) enzyme. (This intermediate of C*c*P is referred to as compound I from this point forward.) One of the two sites of the enzyme that is oxidized in compound I is the heme iron atom, which exists as an oxyferryl group. The other site of oxidation is Trp191, which occurs as a π-cation radical in this form of the enzyme (*1*−*3*). Formation of a stable tryptophan radical intermediate distinguishes C*c*P from other heme peroxidases, the compound I intermediates of which involve formation of an oxyferryl heme iron center and a porphyrin-centered π-cation radical (*3*, *4*).

A number of studies, however, provide evidence that a second protein-centered radical may also form in addition to the Trp191 radical during oxidation of C*c*P by hydrogen peroxide. For example, studies of wild-type and variant forms of C*c*P (*5*, *6*) demonstrated the existence of a second radical with narrow EPR line shape and a *g* value of ∼2.0 that, in contrast to the EPR spectrum of the Trp191 radical, is detectable at 77 K. Subsequent studies have provided limited information regarding the residues responsible for the narrow EPR signal (*7*−*10*). Fishel et al. (*7*) observed that replacement of Tyr residues at positions 36, 42, 39, 229, and 236 does not affect the narrow EPR signal at $g \approx 2.0$. In addition, fluorescence spectroscopy and peptide mapping studies of the enzyme following spontaneous decay of compound I to the ferric form are consistent with oxidative modification of tyrosine residues (*8*−*10*), but the identities of the tyrosyl residues involved have not been determined. Finally, kinetic studies by Traylor and colleagues (*11*) of the reaction of dioxygen with Fe(II)−C*c*P were consistent with the formation of a previously undetected intermediate, referred to by these authors as compound I′, that is oxidized by one equivalent more than compound I, possibly through oxidation of an unidentified tyrosyl residue. Interestingly, the line shape of the narrow EPR signal observed for wild-type C*c*P is nearly identical to the spectrum ascribed to the well-characterized tyrosyl radicals of photosystem II (*12*, *13*), which further suggests that the narrow signal arises from a tyrosyl residue.

To understand better the electron-transfer chemistry of C*c*P and the structural control of oxidative intermediates of heme proteins in general, we have used a spin-trapping reagent and mass spectrometry to identify specific residues that are modified during the formation of compound I. In this work, tyrosyl radicals generated by reaction of wild-type C*c*P with hydrogen peroxide were labeled with 2-methyl-2-nitrosopropane (MNP), and LC−MS/MS was used to perform tryptic peptide mapping and sequence analysis of the MNP-labeled peptides and to identify two tyrosyl residues that form radical centers under these conditions. The implication of

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* Corresponding author. Phone: (604) 822-3179. Fax: (604) 822-6860. E-mail: mauk@interchange.ubc.ca.

‡ Department of Biochemistry and Molecular Biology.

§ Department of Chemistry.

[1] Abbreviations: C*c*P, cytochrome *c* peroxidase; ENDOR, electron nuclear double resonance; EPR, electron paramagnetic resonance; ESI-MS, electrospray ionization mass spectrometry; LC−MS, liquid chromatography−mass spectrometry; MS, mass spectrometry or mass spectrometric; MNP, 2-methyl-2-nitrosopropane.

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this result for the catalytic mechanism of C*c*P is discussed with reference to the three-dimensional structure of the enzyme.

MATERIALS AND METHODS

Sample Preparation. Recombinant wild-type C*c*P was expressed in *Escherichia coli* as described previously (*14*), except that the BL21(DE3)-Codon-Plus strain was used as the host for improved yield (Stratagene). MNP was purchased from Sigma and used without further purification. TPCK-inhibited trypsin (Worthington) was purchased from Cooper Biomedical and dissolved in 0.1 M HCl (1 mg/mL).

The reaction with MNP was initiated by adding H_2O_2 (5−10-fold molar excess) to a solution of C*c*P (0.2−0.5 mM) in the presence of MNP (18 mM) in potassium phosphate buffer (0.1 M, pH 6.0). The reaction was allowed to proceed for 3 min in the dark prior to other measurements. As the MNP label has been reported to be unstable under the conditions required for peptide mapping and treatment of the MNP-labeled peptide with ascorbic acid has been found to prevent this instability (15), excess ascorbic acid was added to the reaction mixture prior to proteolytic digestion and mass spectrometric measurements.

Proteolytic Digestion and ESI-MS Analysis of MNP-Labeled CcP. All MS analyses were performed with a PE-Sciex API 300 triple-quadrupole mass spectrometer (PE-Sciex, Thornhill, Ontario, Canada). To map the modified polypeptides, MNP-labeled C*c*P was first denatured by exposure to low pH (2−3), and heme was removed by extraction with 2-butanol, as described by Yonetani (*16*). For tryptic digestion, apo-C*c*P (∼0.1 mg) was incubated with 1:50 TPCK-inhibited trypsin (2 μg) at 25 °C for 12 h (in 150 μL of 50 mM Tris buffer, pH 8.1). The digested sample was stored frozen (−80 °C) until used. For MS analysis, the tryptic peptides were charged onto a C-18 reverse-phase HPLC column (3.9 × 150 mm) equilibrated with solvent A (0.05% trifluoroacetic acid, 2% acetonitrile in water), and the column was developed with a gradient of 0−60% solvent B (0.045% trifluoroacetic acid, 90% acetonitrile in water) at a flow rate of 0.5 mL/min. The eluent was divided with a postcolumn splitter to permit collection of 90% of the eluate in a fraction collector for subsequent MS/MS analysis and introduction of the remainder of the eluate directly into the mass spectrometer. The quadrupole mass analyzer was scanned over an *m*/*z* of 100−2300 amu.

Identification of Modified Residues by MS/MS. The modified peptides were identified by comparing the total ion chromatogram (TIC) and mass spectrum of the MNP-labeled C*c*P to those of the unlabeled enzyme. In analyses of these samples, the first quadrupole was set to admit only the fragment with the desired *m*/*z* ratio, and fragmentation of the admitted peptide was induced by collision with nitrogen in the second quadrupole. The collision-induced fragments were detected in the third quadrupole, and the sequences of the modified peptides were deduced from the resulting peptide fragments.

EPR Spectroscopy. EPR spectra were measured at room temperature with a Bruker model EPR300E spectrometer equipped with a Hewlett-Packard model 5352 frequency counter. Protein samples (∼50 μL) were loaded into a capillary EPR tube that was placed into a standard quartz EPR tube, and spectra were recorded with the following instrumental parameters unless specified otherwise: microwave power, 20 mW; frequency, 9.8 GHz; modulation frequency, 100 kHz; and modulation amplitude, 1 G.

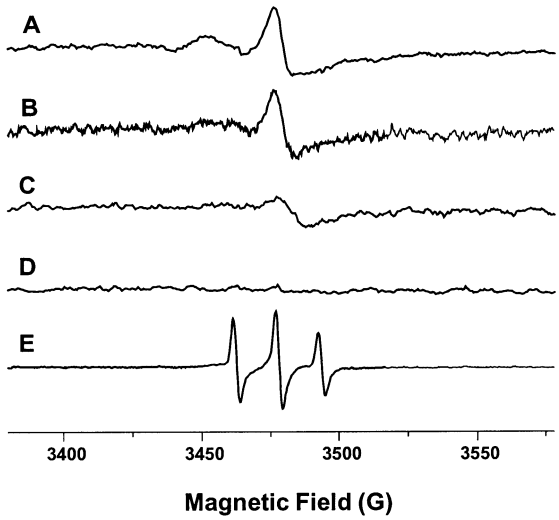

FIGURE 1: EPR spectra (ambient temperature) of C*c*P before and after reaction with hydrogen peroxide and MNP. (A) H_2O_2 (2.5 mM) was added to C*c*P (0.5 mM) in the presence of MNP (18 mM). (B) A after dialysis against 1000 volumes of 0.1 M phosphate buffer (pH 6.0) at 4 °C for 2 h. (C) Same as A but without MNP. (D) H_2O_2 (2.5 mM) was mixed with MNP (18 mM). (E) A after proteolytic digestion by Pronase (2 mg/mL). Spectra were collected within 3 min after addition of H_2O_2. Instrument parameters were as follows: microwave power, 20 mW; frequency, 9.8 GHz; modulation frequency, 100 kHz; and modulation amplitude, 1 G.

RESULTS

EPR Spectroscopy. EPR spectra (298 K) of C*c*P were obtained following reaction with 5-fold excess of hydrogen peroxide in the presence and absence of MNP (Figure 1). As shown, no signal was detected when MNP was mixed with 5-fold excess of H_2O_2 (Figure 1D), while addition of H_2O_2 to the enzyme generated a signal at $g = 2.0$ (Figure 1C). Addition of 5-fold excess of H_2O_2 to the enzyme in the presence of MNP gave rise to a broad EPR signal typical of a highly immobilized nitroxide (Figure 1A). Dialysis (molecular weight cutoff = 10 000) of the reaction mixture did not eliminate the nitroxide signal, indicating that MNP is bound to the protein. Proteolytic digestion of this sample with Pronase resulted in an isotropic three-line signal that is consistent with a rapidly tumbling nitroxide species (Figure 1E). The hyperfine coupling of 14.5 G determined from the three-line signal is indicative of coupling with a nitrogen nucleus (*17*−*20*). These EPR results were consistent with formation of an MNP radical adduct under the conditions used in this study.

Sequence Analysis by LC−MS/MS. To identify the modified peptides, the MNP-labeled apo-C*c*P was subjected to tryptic hydrolysis, and the resulting peptides were analyzed by LC−MS/MS. The total ion chromatograms of tryptic digests of C*c*P prepared for enzyme reacted with H_2O_2 in the absence and presence of MNP are shown in Figure 2A and B, respectively. As shown, the total ion chromatograms of these two hydrolysates are quite similar to each other. Nevertheless, the two peaks with retention times of 22 and 31 min (marked with asterisks) are significant because they

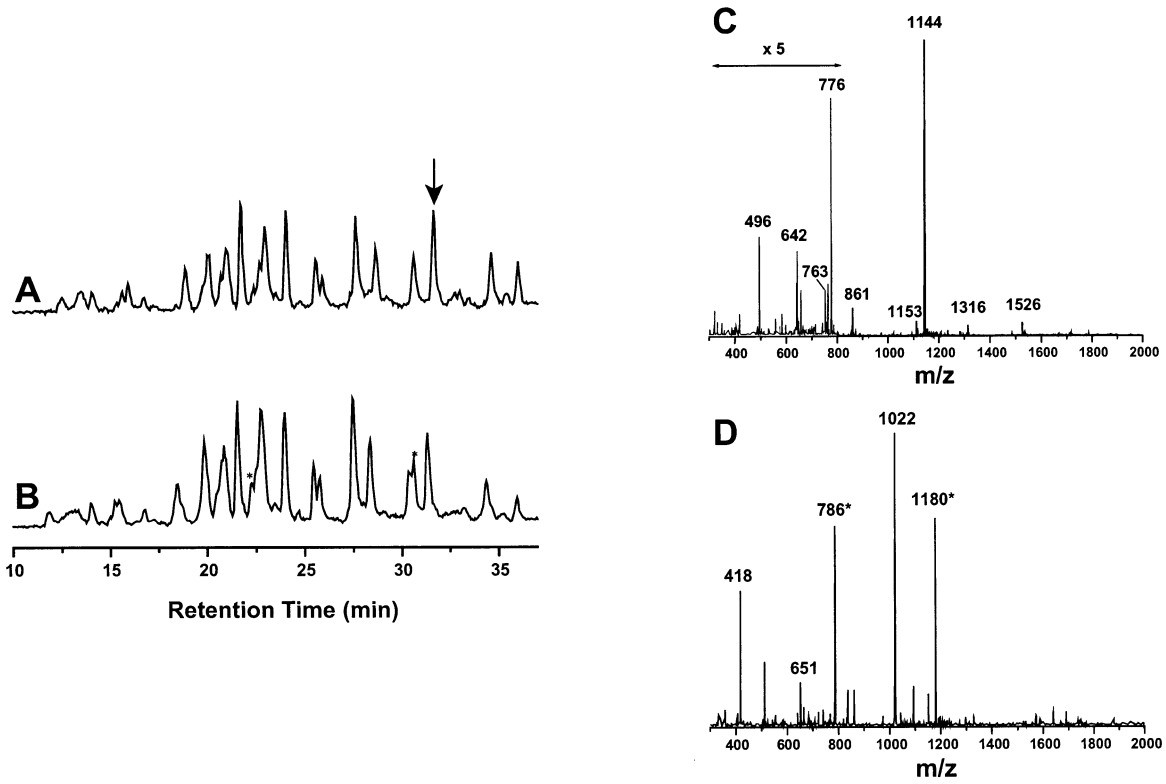

FIGURE 2: MS analysis of tryptic peptides derived from C*c*P reacted with H_2O_2 in the presence and absence of MNP. Apo enzymes were prepared and then subjected to tryptic hydrolysis prior to MS analysis (see Materials and Methods). (A) TIC profile of the tryptic hydrolysate of apo-C*c*P reacted with H_2O_2 in the absence of MNP. (B) TIC profile of tryptic hydrolysate of apo-C*c*P reacted with H_2O_2 in the presence of MNP. (C) ESI-MS of the peptide indicated with an arrow in (A). (D) ESI-MS of the peptide marked with an asterisk in (B) (retention time 31 min).

represent molecular ions with *m*/*z* values that are not observed in the mass spectra of C*c*P reacted with H_2O_2 in the absence of MNP (i.e., the control sample). As shown in Figure 2C, the doubly charged species with *m*/*z* 1144 amu corresponds to the tryptic peptide LREDDEYDNYIGYG-PVLVR (residue 30−48) that has an anticipated mass of 2288 Da. For the MNP-labeled enzyme, the doubly charged species with *m*/*z* 1180 amu corresponds to a peptide with a mass of 2360 Da (Figure 2D). This mass is 72 Da greater than that observed for the unlabeled peptide LREDDEYD-NYIGYGPVLVR. Notably, a change in mass of +71 Da has been observed previously following the reaction of MNP with a Tyr radical under similar circumstances in the case of metmyoglobin (*15*). The corresponding triply charged species are also observed with *m*/*z* 763 and 786 amu, respectively. The sequence of this peptide (residues 30−48) includes three tyrosyl residues at positions 36, 39, and 42. Therefore, this fragment is a likely site of tyrosyl radical formation.

To identify the residue within this peptide that has been modified, the sequences of both the control and MNP-labeled peptides were analyzed by MS/MS. The triply charged ions with *m*/*z* 763 and 786 amu were used to sequence the control and MNP-labeled peptides, respectively. The fragment patterns of the ions with *m*/*z* 763 and 786 amu and the deduced peptide sequences are shown in Figure 3. The sequence map obtained for the ion with *m*/*z* 763 amu matches perfectly with the theoretical sequence of the peptide corresponding to residues 30−48, as shown in Figure 3C (labeled as Y ions and B ions). The peptide sequence deduced for the ion with *m*/*z* 786 amu obtained from the MNP-reacted enzyme is also shown in Figure 3C (labeled as MNP^{+++}). The theoretical fragment pattern expected for the peptide LRED-DEYDNYIGYGPVLVR accounts for most of the fragment ions observed in this analysis. As shown, all Y ions to the *N*-terminal side of Ile40 have exactly the masses predicted by the peptide sequence, indicating that Tyr42 is not modified. However, fragmentation of the peptide at Tyr39 results in an increased mass of 15 Da, as previously observed (*15*), and no further mass increase is observed to the *C*-terminal side of Tyr36. This observation is also true if the peptide sequences are analyzed using the doubly charged molecular ions with *m*/*z* 1180 amu (labeled as MNP^{++} in Figure 3C). The additional mass of 15 Da was not observed during identical sequence analysis of the corresponding peptide obtained from the control C*c*P samples. Thus, we can identify Tyr39 unambiguously as the site of modification by MNP and rule out modification of Tyr36 and Tyr42 in the spin-trap experiment.

The second modified peptide was determined in a similar manner to be comprised of residues 131−155, VDTPEDT-TPDNGRLPDADKDADYVR. As can be seen, this peptide contains one tyrosyl residue at position 153. The triply charged molecular ion with *m*/*z* 949.5 amu obtained from the MNP-labeled enzyme has a mass 70 amu greater than that of the corresponding triply charged molecular ion (*m*/*z* = 926.4 amu) observed in the corresponding spectrum obtained from the control enzyme (Figure 4A,B). Breakage of the peptide at Tyr153 results in a mass increase of 15 amu that is observed for all subsequent Y ions. In the control experiment, corresponding analysis of the same peptide obtained in the absence of MNP produced the results

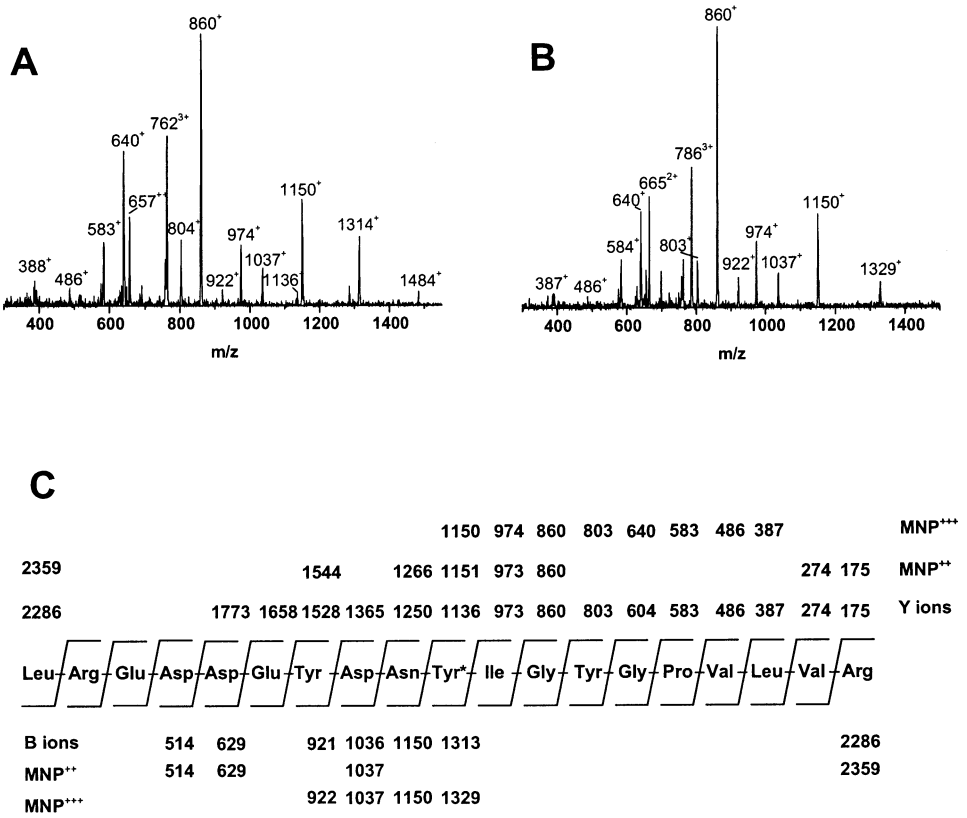

FIGURE 3: ESI-MS/MS of the modified polypeptide comprising C*c*P residues 30−48. (A) Fragment patterns of the triply charged peak at *m*/*z* 763 amu from the control enzyme. (B) Fragment patterns of the triply charged peak at *m*/*z* 786 amu from the MNP-labeled C*c*P. (C) Mass map deduced for the peptide of residues 30−48. Y ions and B ions are the theoretical masses for the *C*-terminal and *N*-terminal parts of the cleaved polypeptide, respectively. MNP^{++} and MNP^{+++} indicate the masses deduced from the ESI-MS/MS using doubly charged and triply charged *m*/*z* peaks, respectively.

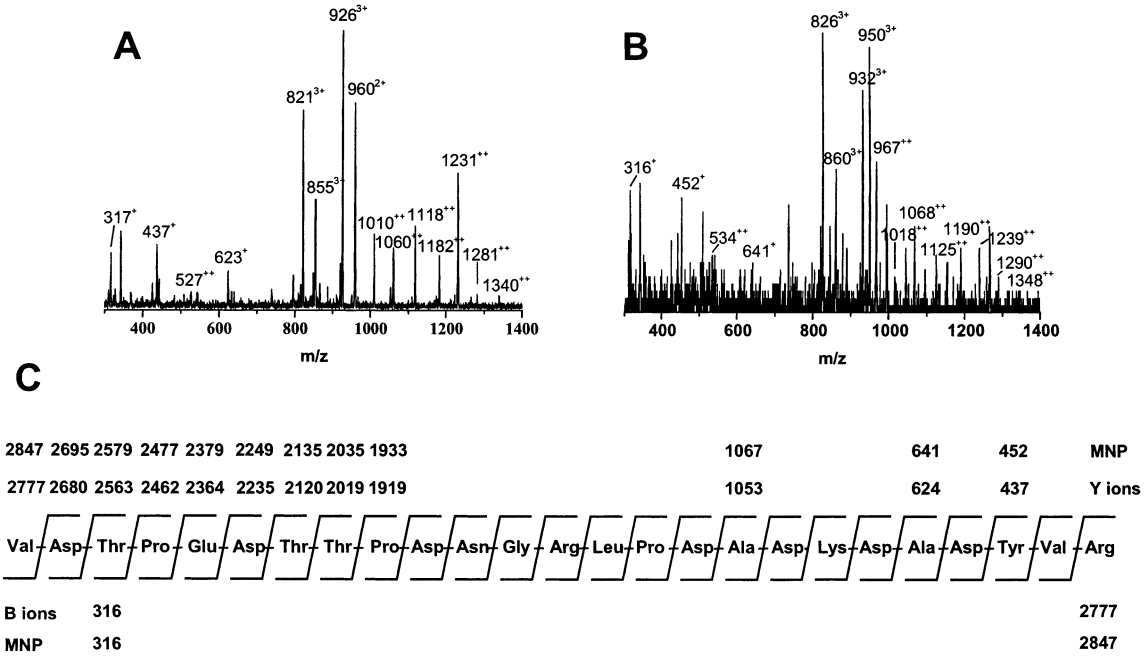

FIGURE 4: ESI-MS/MS of the modified polypeptide comprising C*c*P residues 131−155. (A) Fragment patterns of the triply charged peak at *m*/*z* 926.4 amu from the control enzyme. (B) Fragment patterns of the triply charged peak at *m*/*z* 949.5 amu from the MNP-labeled C*c*P. (C) Mass map deduced for the peptide of residues 131−155.

anticipated from the known sequence of the enzyme. The present result demonstrates that Tyr153 is the only residue in this peptide that has been modified by MNP (Figure 4C). Further examination of the TIC profiles and the mass spectra of the MNP-labeled C*c*P reveals no additional modified residues.

DISCUSSION

In previous studies, tyrosyl and tryptophanyl radicals have been identified in peroxide-treated heme proteins through the use of spin-trapping reagents. Examples of proteins studied in this manner include metmyoglobin (*15*, *17*, *18*), cytochrome *c* (*19*), lignin peroxidase (*21*), prostaglandin H synthase (*22*), cytochrome *c* oxidase (*20*), myeloperoxidase, and lactoperoxidase (*23*, *24*). A recent study of protein-derived tyrosyl radical in the reaction of cytochrome *c* and hydrogen peroxide by MNP/MNP-d_9 labeling, HPLC, and MS showed that MNP adduct is formed at the C3/C5 positions of the tyrosine phenyl ring (*25*). The present results demonstrate that Tyr39 and Tyr153 are modified by MNP during the reaction of yeast C*c*P with hydrogen peroxide, consistent with the known efficiency of MNP in trapping for carbon-centered free radicals. In this work, oxidation of Tyr39 and Tyr153 appears to occur in parallel with formation of compound I rather than resulting from spontaneous (endogenous) reduction of compound I. C*c*P compound I is known to be remarkably stable and to exhibit a half-life as long as several hours (7 ± 1 h) (*26*). In contrast, C*c*P modification by MNP following reaction of the enzyme with peroxide is very fast, insofar as detection of the immobilized nitroxide signal is observed immediately following addition of H_2O_2. Within the reaction time employed in our experiments (<60 s), decay of compound I should be negligible.

The conclusion that tyrosyl residue oxidation occurs in parallel with compound I formation rather than as a result of compound I decay may appear initially to run counter to the literature concerning the reaction of C*c*P with hydrogen peroxide. However, it is important to note the range of reaction conditions used in various studies. In the present work, a 5-fold molar excess of hydrogen peroxide was used. Conceivably, the results of this study could have been different if a stoichiometric amount or a 10-fold molar excess of peroxide had been employed or if the reaction with MNP had been initiated by addition of the trapping agent some time after mixing C*c*P and peroxide. Although we have not attempted a rigorous determination of the abundance of Tyr radical formation relative to heme iron and Trp191 oxidation, we estimate that only 20−25% of the enzyme present in the reaction mixture undergoes oxidation of these more peripheral residues under the conditions of our experiment. Presumably, the probability of oxidizing these newly identified Tyr residues is a function of peroxide concentration. Furthermore, both the identity and abundance of tyrosyl radicals formed in this reaction may be influenced significantly by amino acid substitutions elsewhere in the protein that in some way influence the relative reactivity of these or other Tyr residues or the stabilities of their oxidized products.

Of the 294 amino acid residues present in yeast C*c*P, 14 are tyrosyl residues. This large number of tyrosyl residues raises the question of why Tyr39 and Tyr153 should be unusually susceptible to oxidation by hydrogen peroxide relative to the other 12. From the crystal structure of wild-type C*c*P (*27*−*28*), eight of the tyrosyl residues exhibit at least some degree of solvent accessibility (i.e., Tyr residues 16, 36, 39, 42, 67, 153, 229, and 236). While these residues might be expected to be capable of direct reaction with peroxide from bulk solvent without the need to invoke more complex electron-transfer pathways, these residues are apparently not modified in this manner. Mason and colleagues have found, for example, that although tyrosyl residues of cytochrome *c* oxidase can be oxidized to tyrosyl radicals by reaction of the protein with hydrogen peroxide, formation of these radicals does not occur during similar reaction of the cyanide-inhibited enzyme (*20*). While we have found that cyanide does not fully inhibit compound I formation and peroxide-induced tyrosyl residue oxidation in C*c*P, we do find that reaction of apo-C*c*P with hydrogen peroxide under conditions similar to those used in the current study fails to produce any EPR-detectable Tyr radicals (data not shown). Thus, formation of tyrosyl radicals in native C*c*P reported here appears to be mediated by the heme group. It is not immediately apparent why only Tyr39 and Tyr153 were observed to be modified by MNP in our experiments. For now, it is probably most prudent to consider that any of these Tyr residues have the potential to be oxidized and that the distribution of Tyr radical formation may vary with reaction conditions (pH, temperature, ionic strength, hydrogen peroxide concentration) and may be influenced by seemingly unrelated amino acid substitutions. Any condition or modification that alters the reactivity of a Tyr residue to reaction with peroxide or the stability or reactivity of a tyrosyl radical has the potential, in principle, to influence the results of such analyses significantly.

To understand the structural determinants for modification of tyrosyl residues by oxidative intermediates, Ischiropoulos and co-workers (*29*) conducted a comparative study of nitration of tyrosyl residues in three different proteins having similar sizes but different three-dimensional structures. These authors concluded that the tyrosyl residues that are more likely to be modified by peroxynitrite have three structural characteristics: (a) the aromatic ring is exposed to protein surface or to solvent phase; (b) tyrosine is located on a loop together with a turn-inducing amino acid residue such as glycine or proline within -5 to $+5$ of the tyrosyl residue; and (c) more importantly, the target tyrosyl residue is within a few angstroms of negatively charged amino acid residues such as aspartate and glutamate. To evaluate the applicability of this model to C*c*P, we inspected the three-dimensional structure of the enzyme and found that Tyr39 conforms perfectly to this model. The entire aromatic ring of Tyr39 is exposed on the surface of the protein, and Tyr39 is located on a loop between two helices, together with a turn-inducing glycine residue (G41). Moreover, Tyr39 is located in a highly negatively charged environment with D37, E35, D34, and D33 nearby. This consistency suggests that, to some extent, the chemistry of nitration of tyrosyl residues bears similarity with that of MNP reaction with tyrosyl residues. These structural factors facilitate formation of tyrosyl radicals, probably by stabilizing the charge on tyrosyl residues.

Tyr39 and Tyr153 are both ~14 Å away from Trp191, and a mechanism or pathway for electron transfer between either tyrosyl residue and the Trp191 is not immediately apparent. However, both Tyr39 and Tyr153 are in relative proximity to the heme prosthetic group (Figure 5). For example, Tyr39 exhibits connectivity with the heme propionate through Asp37, Asn38, and His181. In particular, H181 bridges a heme propionate and residues 37−39 through two hydrogen-bonding interactions. The propionate, His181, and residues 37−39 are all in van der Waals contact and could constitute a channel for electron transfer. Alternatively,

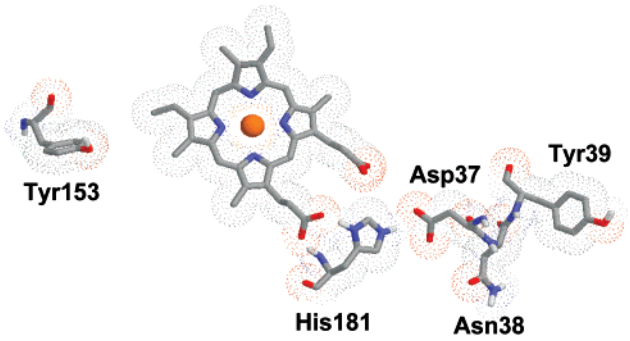

FIGURE 5: Location of the radical-forming tyrosyl residues of C*c*P identified in the current study and their positions relative to the heme prosthetic group.

electron transfer may occur through the helix-loop formed by residues 39−52. Notably, identification of Tyr39 as the radical site is consistent with peptide mapping analysis of a C*c*P dimer that forms after reaction of the enzyme with 10-fold molar excess of peroxide for 24 h (*8*). From this analysis, Spangler and Erman demonstrated that a tryptic peptide comprised of residues 32−48 is modified during dimer formation and probably participates in formation of an intermolecular dityrosine cross-link (*8*). A recent study of redox-active amino acids of C*c*P confirmed that sequence 36−42 of C*c*P is the prime site for protein cross-link during the reaction of C*c*P with excess hydrogen peroxide (*30*). The sequence 36−42 contains three tyrosyl residues (Tyr36, Tyr39, and Tyr42). Substitution of all three tyrosyl residues with phenyalanine was found to prevent protein cross-linking in the presence of excess hydrogen peroxide. However, neither of these studies identified which of the three tyrosyl residues present in this peptide was involved in this reaction.

Oxidation of Tyr153 was not observed in any previous study, although Ivancich et al. (*31*) suggested that Tyr153 may be a good candidate to form tyrosyl radical, based on its location in the crystal structure. This tyrosyl residue does not conform to the model of Ischiropoulos and co-workers for modification of tyrosines, as Tyr153 is located in a helical structure and a relatively neutral environment. However, Tyr153 is only ~5.5 Å from the vinyl group of pyrrole ring A, as shown in Figure 5. EPR and ENDOR studies have shown, however, that the phenol group and Cβ carbon of tyrosyl residues can exhibit considerable mobility (*32*, *33*). Slight rotation of the Cβ carbon toward the heme can bring the phenolic side chain of Tyr153 into van der Waals contact with the vinyl carbon of the pyrrole ring A, which could constitute an efficient route of electron transfer. Regardless of the exact electron-transfer pathway, the present results suggest that compound I probably dissipates the excess oxidizing equivalents through the porphyrin ring rather than through the Trp191 cation radical. This mechanistic preference is reinforced by the observation that compound I derivatives of most heme peroxidases (e.g., horseradish peroxidase, ascorbate peroxidase, and manganese peroxidase) involve porphyrin π-cation radicals rather than amino acid-centered radicals. Conceivably, in the presence of excess oxidant, a transient porphyrin-centered radical may form in response to the oxidative stress and channel the excess oxidizing equivalents to Tyr residues. While such a transient porphyrin-centered radical species has not been experimentally observed for the wild-type protein, this species has been observed for the Trp191Phe variant of C*c*P (*34*).

Tyr236 has been suggested previously to be the site of the second radical center in C*c*P compound I on the basis of studies of aminothiozole oxidation by the Trp191Gly variant of C*c*P (*10*). Specifically, a modified Tyr236 was identified by peptide mapping and Edman degradation of the variant enzyme following catalysis of aminothiozole oxidation. As discussed above, however, it is quite likely that the reactivity of tyrosyl residues in C*c*P can be influenced significantly by details of the reaction conditions and by modification of the enzyme through mutagenesis and other means. A recent study by multifrequency high-field EPR dismisses Tyr236 as the tyrosyl radical site in wild-type C*c*P (*31*). Thus, Tyr236 oxidation during oxidation of aminothiozole by the Trp191Gly variant is unique to that system and not observed in the current work.

In summary, we have demonstrated the efficacy and efficiency of LC−MS/MS as an analytical approach for identification of protein radical sites stabilized by spin-trapping agents. More specifically, we have identified Tyr39 and Tyr153 as efficient sites of tyrosyl radical formation in peroxide-treated C*c*P, as detected with the spin-trap agent MNP. From the three-dimensional structure of C*c*P, we propose that the porphyrin ring is the primary conduit of electron transfer from these tyrosyl residues rather than Trp191 cation radical. As not all tyrosyl residues are modified by MNP in C*c*P, formation of a tyrosyl radical is thus a selective process in which an oxidizing equivalent is transferred either to tyrosyl residues that are in contact with the heme edge or to those that are structurally favored.

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