Nitric Oxide Trapping of Tyrosyl Radicals Generated during Prostaglandin Endoperoxide Synthase Turnover

DETECTION OF THE RADICAL DERIVATIVE OF TYROSINE 385*

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Tyrosyl radicals have been detected during turnover of prostaglandin endoperoxide H synthase (PGHS), and they are speculated to participate in cyclooxygenase catalysis. Spectroscopic approaches to elucidate the identity of the radicals have not been definitive, so we have attempted to trap the radical(s) with nitric oxide (NO). NO quenched the EPR signal generated by reaction of purified ram seminal vesicle PGHS with arachidonic acid, suggesting that NO coupled with a tyrosyl radical to form inter alia nitrosocyclohexadienone. Subsequent formation of nitrotyrosine was detected by Western blotting of PGHS incubated with NO and arachidonic acid or organic hydroperoxides using an antibody against nitrotyrosine. Both arachidonic acid and NO were required to form nitrotyrosine, and tyrosine nitration was blocked by the PGHS inhibitor indomethacin. The presence of superoxide dismutase had no effect on nitration, indicating that peroxynitrite was not the nitrating agent. To identify which tyrosines were nitrated, PGHS was digested with trypsin, and the resulting peptides were separated by high pressure liquid chromatography and monitored with a diode array detector. A single peptide was detected that exhibited a spectrum consistent with the presence of nitrotyrosine. Consistent with Western blotting results, both NO and arachidonic acid were required to observe nitration of this peptide, and its formation was blocked by the PGHS inhibitor indomethacin. Peptide sequencing indicated that the modified residue was tyrosine 385, the source of the putative catalytically active tyrosyl radical.

Prostaglandin endoperoxide H synthase (PGHS),† a bifunctional heme enzyme, catalyzes the first two steps of prostaglandin and thromboxane biosynthesis. Its cyclooxygenase activity catalyzes the incorporation of two molecules of dioxygen into arachidonic acid to form prostaglandin endoperoxide G2, a hydroperoxy endoperoxide (Reaction 1). The peroxidase activity of PGHS then catalyzes the two-electron reduction of prostaglandin G2 to prostaglandin endoperoxide H2, a hydroxy endoperoxide (Reaction 2) (1–3).

REACTION 1

REACTION 2

The mechanism of prostaglandin synthesis by PGHS, particularly the relationship between the two activities of this enzyme, has been the subject of intense investigation. The peroxidase and cyclooxygenase activities of PGHS are separate and distinct (4). The two active sites are located on opposite sides of the protein and are separated by the heme prosthetic group. Nonsteroidal antiinflammatory drugs that bind in the cyclooxygenase active site do not inhibit the peroxidase (3). Despite this spatial separation the peroxidase and cyclooxygenase activities are functionally interrelated. Ligands to the distal heme binding site inhibit both activities (5–7). Scavenging of fatty acid hydroperoxides with glutathione peroxidase inhibits cyclooxygenase activity, and protein or heme modifications that reduce peroxidase activity induce a lag phase in cyclooxygenase activity that can be overcome by addition of hydroperoxide (8, 9). It is believed that peroxidase turnover activates the oxidizing agent responsible for cyclooxygenase turnover.

Ruf and co-workers (10, 11) first detected a tyrosyl radical by EPR spectroscopy upon addition of arachidonic acid or prostaglandin G2 to PGHS and proposed that the radical is the oxidant responsible for cyclooxygenase activity. They proposed that ferric PGHS is oxidized by a fatty acid hydroperoxide by two electrons to yield the oxoferryl porphyrin π cation radical intermediate, PGHS compound I (Reaction 3). Compound I oxidizes a tyrosine residue to a tyrosyl radical and is reduced to PGHS compound II (Reaction 4). The tyrosyl radical was postulated to abstract a hydrogen atom from arachidonic acid to initiate the cyclooxygenase cycle (11). Evidence supporting this mechanism was provided by the work of Tsai et al. (12), which demonstrated that under anaerobic conditions, the spectroscopically detectable PGHS-1 tyrosyl radical (formed by the reaction of PGHS-1 with ethyl hydroperoxide) oxidized arachidonic acid to a carbon-centered radical.
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\[ \text{YFe}^{\text{III}} + \text{ROOH} \rightarrow \text{[YFe}^{\text{IV}} = \text{O} \cdot \text{]} + \text{ROH} \]

**REACTION 3**

\[ \text{[YFe}^{\text{IV}} = \text{O} \cdot \text{]} \rightarrow \text{[YFe}^{\text{IV}} = \text{O} + \text{H} \]

**REACTION 4**

Identification of the catalytically active tyrosyl radical has been problematical. Smith and co-workers (13, 14) showed that mutation of tyrosine 385 to phenylalanine abolishes cyclooxygenase but not peroxidase activity. Tyrosine 385 is present in the cyclooxygenase active site in between the heme group and the putative arachidonic acid binding site. However, reaction of the Y385F mutant with peroxide produces a tyrosyl radical that is qualitatively similar by EPR spectroscopy to the radical produced from wild-type enzyme (14, 15). Either the spectrally detectable radical in wild-type enzyme is not derived from tyrosine 385 or a different tyrosine is oxidized in the Y385F mutant. Thus, to this point there is no conclusive evidence that tyrosine 385 is oxidized to a radical during PGHS turnover.

The apparent limitation of site-directed mutagenesis in combination with EPR for identification of PGHS tyrosyl radicals prompted us to use a different approach to solve this problem. The reaction of tyrosyl radicals with nitric oxide (NO) to form nitrosocyclohexadienone has been demonstrated in other enzymes such as ribonucleotide reductase (16, 17) and photosystem II (18, 19). It has been shown both in these enzymatic systems as well as with simple phenols that the reaction of NO with phenoxyl radicals to form nitrosocyclohexadienones is reversible (16–22). Recently, it has been shown that NO quenches the PGHS-2 tyrosyl radical signal, but the reaction is irreversible due to the eventual formation of nitrotyrosine (23). We have observed that NO quenches the tyrosyl radical signal formed by reaction of PGHS-1 with arachidonic acid. The subsequent oxidation of nitrosocyclohexadienone to nitrotyrosine (presumably by the peroxidase activity of PGHS) provides a potential marker for identification of the modified tyrosine. Thus, using this method in conjunction with peptide mapping techniques, PGHS tyrosyl radicals generated during arachidonic acid oxidation can be identified.

**EXPERIMENTAL PROCEDURES**

**Materials**—Arachidonic acid was purchased from Nu-Chek Prep, Inc. (Elysian, MN), 1,1-Tosylamido-2-phenylethylchloromethyl ketone (TPCK)-treated trypsin (type XIII) from bovine pancreas, superoxide dismutase from bovine erythrocytes, tetramethanone, and hematin were purchased from Sigma. Diethylamine NONOate (DEA/NO) was purchased from Alexis Biochemicals (San Diego, CA). 3-Chloroperoxybenzoic acid was purchased from Aldrich. PGHS from rat seminal vesicles was purified as described previously (24). Apo-PGHS was prepared from purified holo-PGHS as described previously (25). Its cyclooxygenase activity was determined by monitoring \( O_2 \) consumption using a Gilson 6/5 oxygraph equipped with a Clark-type electrode maintained at 37 °C (Gilson Medical Electronics, Inc., Middleton, WI). The specific activity of the apo-PGHS used in these studies was \( \sim 17 \mu \text{mol of arachidonic acid per mg protein per min} \).

**EPR Spectroscopy**—PGHS samples were prepared by reconstitution of apo-PGHS with 1 mol of heme per mol of protein. The resulting stock solution was diluted to a final concentration of 50 \( \mu \text{M} \) in 200 \( \mu \text{M} \) of 100 mM potassium-phosphate buffer, pH 8.0, containing 25% glycerol. Samples were stored on dry ice until immediately before use. For each experiment, the sample was thawed to \( \sim 12^\circ \text{C} \) and placed into 3-mm quartz EPR tubes. Arachidonic acid was added to a final concentration of 1 mM in a volume of 20 \( \mu \text{l} \) from a stock solution dissolved in ethanol. When used, the NO generators, Proli/NO or DEA/NO, were dissolved in cold 10 mM NaOH and were allowed to decompose in the PGHS-1 solution for one half-life (2 s in the case of Proli/NO and 180 s in the case of DEA/NO) before the addition of the arachidonic acid. The samples were frozen in liquid nitrogen 5 s after the addition of arachidonic acid and were transferred to a quartz finger tip Dewar that was inserted into a TMM, cavity of a Bruker ESP 300 EPR spectrometer. Spectra were acquired using the following instrument settings: modulation amplitude, \( 1 \\text{G} \); microwave power, 2 mW; microwave frequency, 9.49 GHz; modulation frequency, 100 kHz.

**Nitration of PGHS-1 with NO and Arachidonic Acid**—Due to its rapid decomposition at neutral pH, DEA/NO was chosen as a source of NO. Stock solutions of DEA/NO were prepared using 10 mM NaOH. At this pH, the DEA/NO is stable for several weeks. Because of the relatively slow decomposition of DEA/NO at pH 8.0, Centricron-10 concentrators (Amicon) were used to change the apo-PGHS-1 buffer from 80 mM Tris (pH 8.0) to phosphate buffer (pH 7.2). PGHS-1 (1.1 mg in 250–400 \( \mu \text{l} \)) was placed on the concentrator, and the volume was increased to 2.0 ml using 100 mM phosphate buffer, pH 7.2. Following a 90-min centrifugation (4000 \( \times \) \( g \)), a 1.7 ml phosphate buffer was added, and the samples were centrifuged for an additional 60–90 min.

Apo-PGHS-1 was reconstituted by adding heme (1 mol/mol PGHS subunit) and incubating at room temperature for 5 min. The PGHS-1-iododemecain complex was generated by adding indomethacin (1.2 mol/mol PGHS subunit) to reconstituted PGHS-1 and incubating at room temperature for 25 min. Generation of NO was accomplished by adding 500 \( \mu \text{mol} \) of DEA/NO to PGHS-1. Iodomethacin complex and incubating at room temperature for 10 min. 10 eq of arachidonic acid was then added and allowed to react for 3 min at room temperature. The entire reaction volume (400 \( \mu \text{l} \)) was then placed on a Bio-Rad 10DG gel filtration column to separate PGHS from the other reagents. The column was equilibrated with 100 mM Tris buffer (pH 8.0) and 0.1% Tween 20.

**Nitration of PGHS-1 with CPA and NO**—The procedure was exactly the same as that described for arachidonic acid above except that 10 eq of CPA was used in place of arachidonic acid. 3 min after addition of CPA, the reaction mixture was passed over a 10DG desalting column as described above. The concentration of the CPA stock solution was determined by spectrophotometrically monitoring the oxidation of ferric horseradish peroxidase to horseradish peroxidase compound I according to the method of Bakovic and Dunford (26).

**Nitration of PGHS-1 with Tetraniromethane**—Nitration of PGHS-1 with tetraniromethane was carried out in 80 mM Tris, pH 8.0, 0.1% Tween 20. Thus, no buffer change was necessary. The reaction of tetraniromethane with PGHS-1 or PGHS/indomethacin was carried out by adding tetraniromethane (1 mM final concentration, 1.7% ethanol) to enzyme at room temperature for 5 min. Following reaction, PGHS-1 was separated from unreacted tetraniromethane and other small molecules by passing the mixture through a 10DG gel filtration column (Bio-Rad) as described above.

**Detection of Nitrotyrosine by Western Blot Analysis**—Frozen aliquots (20 \( \mu \text{l} \)) were thawed quickly and heated with Laemmli reducing sample buffer at 95 °C for 3 min. Samples (1.25 \( \mu \text{g} \)) were run on 10% denaturing polyacrylamide gel electrophoresis for 45 min at 180 V then transferred onto nitrocellulose membranes for 2 h at 70 V. The blot was probed overnight with 2 \( \mu \text{g} \) of rabbit anti-nitrotyrosine, polyclonal IgG (Upstate Biotechnology) and 1 h with donkey anti-rabbit horseradish peroxidase (Amersham Pharmacia Biotech) as the secondary antibody. Detection was carried out for 10 s with the ECL Western blotting system (Amersham).

**Tryptic Digestion of PGHS-1 for Peptide Mapping**—Following the desalting procedure, PGHS-1 prepared under a variety of reaction conditions was proteolytically cleaved using TPCK-treated trypsin. To obtain predominantly peptide a (a nitrated PGHS peptide with \( \sim 75 \text{ min retention time} \)), a PGHS-1 to trypsin ratio of 10:1 (w/w) was used. The digestion was allowed to incubate for 18 h at 37 °C. To obtain predominantly peptide b (a longer nitrated PGHS peptide with \( \sim 81 \text{ min retention time} \)), a PGHS-1 to trypsin ratio of 10:1 (w/w) was used with an incubation of 1.8 h at 37 °C. Digestion was stopped by the addition of 20 \( \mu \text{l} \) of glacial acetic acid per ml sample. Each sample was then centrifuged for 5 min, and the resulting peptides were separated by HPLC.

**Separation of PGHS-1 Peptides by HPLC**—PGHS-1 peptides were separated by reversed-phase HPLC using a Zorbax SBC-18 reversed-phase column (3.0 × 250-mm) (MAC-MOD Analytical, Inc., Chadds Ford, PA) at 0.4 ml/min. Buffer A was water, 0.1% trifluoroacetic acid, and buffer B was 80% acetonitrile, 0.1% trifluoroacetic acid. Peptides were separated using the following nonlinear gradient: 0–50% buffer B, 0–75 min; 50–75% buffer B 75–100 min; 75–100% buffer B, 100–115 min. Elution of peptides was monitored using a diode array detector.
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RESULTS

The reaction of PGHS-1 with arachidonic acid resulted in the formation of a tyrosyl radical as detected by EPR spectroscopy (Fig. 1A). The detected radical has a line width of 26 G, corresponding to the narrow singlet spectrum previously reported (27). The radical intermediate persisted for over 1 min at either −12 or 0 °C (data not shown). No tyrosyl radical was detected when the reaction was carried out in the presence of 1 mM proli/NO (Fig. 1B). Similarly, no tyrosyl radical was detected prior to the addition of arachidonic acid (Fig. 1C). These data are consistent with results obtained previously with ribonucleotide reductase (17) and photosystem II (18, 19) in which protein tyrosyl radicals reacted with NO to form nitrosocyclohexadiene and/or nitrite esters. These results are also consistent with those recently obtained by Gunther et al. (23) using PGHS-2. However, unlike the reaction of PGHS-2 with arachidonic acid in the presence of NO, no iminoxyl radical intermediate was detected when PGHS-1 was used in place of PGHS-2. Thus, no evidence was provided by EPR to suggest the subsequent oxidation of nitrosocyclohexadiene to iminoxyl radical.

To determine whether nitrotyrosine was formed as a result of the reaction of PGHS-1 with arachidonic acid in the presence of NO, we utilized antinitrotyrosine antibodies in Western blot analysis to probe for nitration of PGHS-1 (Fig. 2). Antinitrotyrosine antibody was observed to bind PGHS following its reaction with arachidonic acid in the presence of NO (lane 3). Both arachidonic acid and NO were required to detect nitrotyrosine (lanes 1 and 2). Superoxide dismutase did not inhibit nitration (lane 4), indicating that peroxynitrite was not the species responsible for the nitration of tyrosine. Much higher concentrations of superoxide dismutase (800 μM/62,500 units/ml) inhibited nitration to a certain extent, but this was due to a nonspecific effect of elevated protein concentration. A similar extent of inhibition was observed with an equivalent concentration of glutathione transferase in the absence of glutathione. Turnover of PGHS was also required to observe nitration of the protein. When PGHS-1 was treated with the inhibitor indomethacin before the addition of DEA/NO and arachidonic acid, no tyrosine nitration was observed (lane 5). The order of addition of arachidonic acid and NO was also important. If arachidonic acid was added 3 min before DEA/NO, no nitration was observed (data not shown). The blot shown in Fig. 2 was also probed with anti-PGHS-1 antibody. As expected, PGHS was detected in all 5 lanes at equal concentrations (data not shown).

To obtain more information regarding the number and identity of PGHS tyrosines nitrated by arachidonic acid/NO, we turned to peptide mapping using the unique absorption characteristics of nitrotyrosine as a marker for modified peptides. Tryptic digestion of PGHS-1 prepared by reaction with arachidonic acid/NO produced many peptides as evidenced by the HPLC elution profile observed at 230 nm (Fig. 3B). Relatively few peaks were detected at 360 nm (Fig. 3A). Moreover, many of the peaks detected at 360 nm did not have spectra resembling nitrotyrosine. Only two peptides (peptide a and peptide b in Fig. 3A) had spectra consistent with nitrotyrosine at acidic pH. A typical spectrum of peptide a is shown in Fig. 3 (inset). Peptide a was consistently observed under these reaction conditions. Peptide b on the other hand was not consistently detected using these trypsin digestion conditions. The other peptides with absorbance at 360 nm, particularly those eluting after peak b, did not have an absorption maximum at 360 nm, but instead showed a maximum at 400 nm. These data sug-
gested that nitration of PGHS-1 by arachidonic acid/NO occurred predominantly on a single peptide, peptide a.

Similar results were obtained when arachidonic acid was replaced by the organic hydroperoxide CPBA (data not shown). Other modifications of the arachidonic acid/NO protocol were also examined. For example, following the addition of arachidonic acid, another 500 nmol of DEA/NO was added followed by an additional 20 eq of arachidonic acid added in two aliquots. In another protocol, phenol (10 enzyme eq) was present as a reducing substrate during the multiple additions of DEA/NO and arachidonic acid. These slight modifications did not affect either the location or extent of PGHS nitration. Consistent with the Western blotting results, both arachidonic acid and NO were required to observe the nitrotyrosine-containing peptide a (Fig. 4). In the absence of DEA/NO, no nitrotyrosine was observed (Fig. 4B). Similarly, when arachidonic acid was withheld from the reaction very little of this nitrated peptide was detected (Fig. 4C). It is also important to point out that reversing the order of addition (i.e. arachidonic acid added 3 min before DEA/NO) prevented the appearance of peptide a (data not shown). We observed that in the absence of a peroxidase-reducing substrate, the added amount of arachidonic acid (10 eq) was sufficient to cause irreversible inactivation of PGHS-1. These data suggest that irreversibly inactivated protein is unable to carry out the nitration reaction.

It has been shown previously that nitration of three PGHS tyrosines by tetranitromethane (Tyr-355, Tyr-385, and Tyr-417) can be blocked by indomethacin (13). Our results with Western blotting showed that tyrosine nitration of PGHS with arachidonic acid and NO was also sensitive to inhibition by indomethacin. Thus, we determined that indomethacin may provide valuable insight to the identity of the nitrated tyrosine observed in peptide a (see Figs. 3A and 4A). We observed that the reaction of PGHS with arachidonic acid/NO or tetranitromethane resulted in nitration of what appeared to be the same peptide (Fig. 5, A and C). Indeed, when either tetranitromethane or arachidonic acid/NO were used as nitrating agents, indomethacin was able to block the formation of nitrated peptide a (Fig. 5, B and D). To confirm that peak a observed in Fig. 5, A and C, corresponded to the same peptide, the peptide was collected from each separation. These fractions were then re-
The HPLC elution profile for cycle 7 as observed at 268 nm is shown in panel A, and the profile for cycle 8 is shown in panel B. Panel C shows the spectra corresponding to the peaks indicated in panels A and B. The phenylthiohydantoin derivatives of nitrotyrosine and histidine are indicated by nit-Y and H, respectively. Diphenylthiourea is shown as dptu, and the expected retention time for the phenylthiohydantoin derivative of tyrosine is indicated by Y.
after cycle 7. These data confirm that tyrosine 385 of PGHS-1 is nitrated during the oxidation of arachidonic acid in the presence of NO.

**DISCUSSION**

Ruf and co-workers (11) proposed 10 years ago that a tyrosyl radical was required for prostaglandin synthesis by PGHS, and since that time, Smith and others (13, 14) have shown that tyrosine 385 is essential to the cyclooxygenase activity of PGHS. In light of the ideal position of tyrosine 385 as shown by PGHS crystal structures, many have believed that tyrosine 385 must be the source of the tyrosyl radical observed by EPR and the radical responsible for hydrogen abstraction from arachidonic acid. However, the use of site-directed mutagenesis in conjunction with EPR has failed to produce clear data to support this proposal.

Consistent with other tyrosyl radical-containing proteins, the PGHS-1 tyrosyl radical detected during arachidonic acid oxidation is quenched by NO. As with these other proteins, a likely intermediate in the reaction is nitrosocyclohexadienone or a nitrite ester. Our Western blotting and peptide mapping studies both indicate that the nitrosocyclohexadienone adduct is oxidized to form nitrotyrosine. Furthermore, peptide mapping and subsequent amino acid sequence analysis showed that only one tyrosine, tyrosine 385, is nitrated during arachidonic acid oxidation in the presence of NO. Taken together these data provide the first direct evidence to show that tyrosine 385 is oxidized to a free radical intermediate during PGHS catalysis.

An alternative mechanism to explain the nitration of PGHS-1 in the presence of NO is coupling to a tyrosyl radical to nitrogen dioxide, (NO₂) the product of NO oxidation by molecular oxygen. This reaction would produce nitrotyrosine directly without the intermediacy of a nitrosocyclohexadienone intermediate.

Although NO appears to trap all of the spectriscopically detectable tyrosyl radicals as evidenced by their disappearance from the EPR spectrum, recent investigations have shown that NO does not appear to significantly inhibit the rate of arachidonic acid oxidation by PGHS (28, 29). This suggests that under the conditions employed in the present experiments, NO does not compete effectively with arachidonic acid for the tyrosine 385 radical. Thus, many molecules of arachidonic acid are oxidized on each enzyme molecule before the catalytically active tyrosine 385 radical is trapped by NO. Recent investigations into the effect of NO on arachidonic acid oxidation by PGHS would seem to support such a proposal. It is important to recognize that NO is an efficient reducing substrate for the PGHS peroxidase (29) and as such should greatly stimulate cyclooxygenase activity (2). Contrary to this expectation, NO (added as DEA/NO) has little effect on the initial rate and inhibits by one-third the extent of arachidonic acid oxygenation when compared with a reaction that contains no peroxidase-reducing substrate (29). Moreover, when compared with a reaction containing the peroxidase-reducing substrate aminopyrine, NO inhibits the extent of arachidonic acid oxygenation by ~65% (29). It is possible that the unique inability of NO to stimulate arachidonic acid oxidation commensurate with its efficiency as a reducing substrate stems from its role in nitration of tyrosine 385.

Considerable controversy has surrounded the question of whether the tyrosyl radicals detected spectriscopically during PGHS turnover represent catalytic intermediates or inactivated enzyme (14, 15, 30–32). Our finding that NO quenches the tyrosyl radical(s) and produces nitrotyrosine at tyrosine 385 is consistent with the hypothesis that the EPR signal detected during turnover arises from a catalytic intermediate. However, this interpretation must be considered tentative. Our identification of tyrosine 385 as a trapped radical requires that its tyrosyl radical react with NO and that the nitrosocyclohexadienone intermediate be further oxidized to nitrotyrosine. The coupling of NO to protein tyrosyl radicals appears to be completely reversible. Therefore, if the nitrosocyclohexadienone-trapping product from a particular tyrosine was not further oxidized to nitrotyrosine, it would decompose to tyrosine and NO and not be detected by peptide mapping (Reactions 5 and 6).

Further, any nitrite ester trapping product would not oxidize to nitrotyrosine and would not be detected. Thus, although the present study establishes that tyrosine 385 is oxidized to a radical intermediate during arachidonic acid oxygenation, it does not answer the question of whether the radical signals detected by EPR spectroscopy represent catalytic intermediates or inactivated enzyme.

In summary, the nitration of PGHS tyrosine 385 by reaction with arachidonic acid or organic hydroperoxide in the presence of NO appears to result from the reaction of NO with a tyrosyl radical. This provides solid evidence that tyrosine 385 is oxidized to a free radical intermediate during arachidonic acid oxidation. This is consistent with the proposed requirement for a tyrosyl radical in the cyclooxygenase catalytic cycle and previous investigations that have shown tyrosine 385 to be essential to the cyclooxygenase activity of PGHS. The nitration of only one tyrosine is consistent with a role for the tyrosyl radical of tyrosine 385 in cyclooxygenase catalysis but does not prove that tyrosine 385 is the only tyrosine oxidized to a radical intermediate during turnover of the wild-type PGHS. The detection of tyrosyl radicals during the turnover of the PGHS mutant Y385F indicates that other tyrosines can be oxidized in mutant and possibly wild-type enzymes. It will be interesting to determine whether the methodology developed in the present report is useful for the identification of tyrosyl radicals generated from site-directed mutants of PGHS.

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