Detection of a Higher Oxidation State of Manganese-Prostaglandin Endoperoxide Synthase*

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Addition of arachidonic acid or 5-phenyl-4-pentenylhydroperoxide to manganese-prostaglandin endoperoxide synthase (Mn-PGH synthase) produced a species with an absorbance maximum at 418 nm. This maximum is distinct from those of resting enzyme (372 and 468 nm) or reduced enzyme (434 nm). The formation of the 418 nm-absorbing species was observed immediately after the addition of hydroperoxide to enzyme but only after a 10-s lag period following addition of arachidonate. Mn-PGH synthase exhibited a peroxidase activity that was 0.8% that of Fe-PGH synthase. Addition of peroxidase reducing substrates to the oxidized form of Mn-PGH synthase diminished the absorbance at 418 nm. In the case of N,N,N',N'-tetramethylphenylenediamine, reduction of the 418 nm-absorbing species was accompanied by an increase in absorbance at 610 nm due to the oxidized form of the amine. Thus, the spectral and chemical properties of the 418 nm-absorbing species are consistent with its existence as a higher oxidation state of Mn-PGH synthase. Kinetic analysis indicated that formation of the higher oxidation state proceeded or was coincident with oxygenation of the fatty acid substrate, eicosa-11,14-dienoic acid. The cyclooxygenase activity of Mn-PGH synthase was inhibited by the combination of glutathione and glutathione peroxidase at a glutathione peroxidase concentration 227-fold lower than the concentration that inhibited Fe-PGH synthase. The results suggest that Mn-PGH synthase forms a higher oxidation state following reaction with hydroperoxides added exogenously or generated endogenously from polyunsaturated fatty acid substrates. This higher oxidation state functions in the peroxidase catalytic cycle of Mn-PGH synthase, and its formation appears to be essential for activation of the cyclooxygenase catalytic cycle.

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PGH synthase catalyzes the first two steps in prostaglandin and thromboxane biosynthesis (1). Its cyclooxygenase activity catalyzes the bi-oxygenation of polyunsaturated fatty acids to hydroperoxy endoperoxides and its peroxidase activity catalyzes the reduction of the hydroperoxy intermediates to hydroxy endoperoxides (2-5). PGH synthase is widely distributed in mammalian tissues, and its levels are sensitive to modulation by a variety of growth factors and glucocorticoids (6). Although it was originally believed that a single gene exists for PGH synthase, recent evidence suggests the existence of a second PGH synthase gene, transcriptionally activated by viral transformation or growth factor treatment (7, 8).

The mechanism of action of PGH synthase has been the subject of much experimental investigation. The available evidence suggests removal of the 13-pro-S-hydrogen of arachidonic acid is the first step of the cyclooxygenase reaction (9, 10). There appears to be a requirement for activation of resting enzyme by hydroperoxy fatty acids because the combination of reduced glutathione and glutathione peroxidase inhibits cyclooxygenase activity (11-15). PGH synthase is a hemoprotein containing one Fe(III)-protoporphyrin IX per subunit; native enzyme is homodimer of 70-kDa subunits (16-18). Both cyclooxygenase and peroxidase activities require heme and higher oxidation states of the prosthetic group have been detected during peroxidase turnover (19-22). A plausible mechanism for hydroperoxide-dependent activation of the cyclooxygenase activity is formation of a peroxidase higher oxidation state. This implies a synergism between the two activities even though biochemical evidence suggests they are likely to occupy distinct regions of the protein (23, 24). Despite several lines of evidence linking peroxidase and cyclooxygenase activities in a functional sense, several different modified enzyme preparations have been reported that exhibit substantially reduced peroxidase activity but only slightly reduced cyclooxygenase activity. These include PGH synthase reconstituted with Mn(III)-protoporphyrin IX, trypsin-cleaved Fe-PGH synthase and site-directed mutants in which certain histidine residues are replaced by glutamine and alanine (25-28).

Several years ago, a novel mechanism for activation of cyclooxygenase was proposed in which a peroxidase higher oxidation state oxidizes a tyrosine residue at the cyclooxygenase active site.

1 The abbreviations used are: PGH synthase, prostaglandin H synthase; MeSO, dimethyl sulfoxide; EPR, electron paramagnetic resonance; Fe-PGHS, apoprostaglandin H synthase reconstituted with Fe(III)-protoporphyrin IX; GSH, reduced glutathione; HPLC, high-performance liquid chromatography; MnCCP, manganese cytochrome-c peroxidase; MnHRP, manganese horseradish peroxidase; Mn-PGHS, apoprostaglandin H synthase reconstituted with Mn(III)-protoporphyrin IX chloride; Mn-PPIX, manganese(III)-protoporphyrin IX; PPHP, 5-phenyl-4-pentenyl-1-hydroperoxide; TMPD, N,N,N',N'-tetramethyl-1,4-phenylenediamine.
ase active site (20, 29). The resulting tyrosyl radical acts as the oxidizing agent that removes the bis-aliphatic hydrogen from substrate fatty acid. Indeed, addition of arachidonic acid or hydroperoxides to Fe-PGH synthase at -12 °C followed by rapid freezing and EPR analysis allowed detection of a protein-derivated radical that was unequivocally identified as a tyrosyl radical (29). Power saturation experiments suggested the tyrosyl radical was located 7-12 Å from the metal center. More recent EPR experiments question the catalytic competence of the tyrosyl radical in the cyclooxygenase reaction and imply that its formation may reflect enzyme inactivation (30).

A key observation in the latter study was the absence of tyrosyl radicals following addition of arachidonic acid or hydroperoxides to Mn-PGH synthase, which exhibited a vigorous cyclooxygenase activity (30). A corollary of these observations is that formation of tyrosyl radicals by Fe-PGH synthase is mainly due to its peroxidase activity.

Mn-PGH synthase provides a unique opportunity for investigation of the cyclooxygenase activity without the complications of a highly active peroxidase activity to mask catalytic intermediates. Relatively few investigations of Mn-PGH synthase have been undertaken, possibly because of the difficulty in preparing apoenzyme preparations for reconstitution that would yield viable Fe-PGH synthase. We recently reported a straightforward procedure for apo-PGH synthase isolation that routinely yields greater than 99.5% apoprotein (31). In the present manuscript, we describe the use of this material for investigations of the reaction of Mn-PGH synthase with fatty acid and hydroperoxide substrates. We find that Mn-PGH synthase is converted to a spectroscopically detectable higher oxidation state(s) that appears to be an intermediate in a poorly efficient peroxidase activity.

The kinetics of formation of this species suggest it may be a catalytically competent intermediate that plays a role in cyclooxygenase catalysis.

EXPERIMENTAL PROCEDURES

Materials—Arachidonic acid and 11,14-eicosadionoic acid were obtained in >99% purity from Nu Chek Prep (Ely, MN). Tween-20 (Surfact-Amps 20) was obtained from Pierce. Manganese protoporphyrin IX chloride was purchased from Porphyrin Products (Logan, UT). Hematin, diethyldithiocarbamate (sodium salt), Sephadex G-200-120, Sephadex G-25-150, deoxycholate (sodium salt), EDTA (free acid), glutathione (reduced form, free acid), bovine serum albumin (98-99%, essentially fatty acid free), and guaiacol were all obtained from Sigma. The cyclooxygenase inhibitor SQ-29,535 was reconstituted in ice and kept frozen. Centricon 30 microconcentrators were obtained from Amicon (Danvers, MA). The nonindicating oxygen trap was an Oxy-Purge N from the Applied Science Division of Milton Roy Company (State College, PA). 5-Phenyl-1-pentenyli-hydroperoxide (PPHP) was synthesized as previously reported (32). Human plasma glutathione peroxidase was purified as described earlier (33). One unit is the amount required to reduce 1 µmol of 15(S)-hydroperoxy-5,8,11,13-eicosatetraenoic acid to 15(S)-hydroxy-5,8,11,13-eicosatetraenoic acid per min at 37 °C and pH 8.0 in the presence of 5 µM glutathione.

PGH synthase was isolated from ram seminal vesicle microsomes as previously described and stored at -80 °C in 50 mM Tris-HCl (pH 8 at 4 °C) containing 300 µM diethyldithiocarbamate and 0.1% Tween-20 (34). Apo-PGH synthase was prepared via gel filtration procedure with minor variations (31). For each apo preparation, 15-18 mg of protein was 99.5-100% apo based on cyclooxygenase activity measured without and with the addition of excess hem. The specific activity of the apo-PGH synthase ranged from 50-87 kunits per mg of protein when assayed in 100 mM Tris-HCl (pH 8), 500 µM phenol, and 1 µM hematin at 37 °C. One unit is the amount of PGH synthase necessary to consume 1 nmol of O2/min.

A value of absorbance change by 0.020/min at 37 °C, which was assumed to be the linear portion of the curve. Controls were performed with no apoenzyme added to the cuvette; the rates calculated for these reactions were subtracted from each final rate of the enzymatic reaction before the peroxidase activities were compared.

Absorption Measurements—The instrument was used in the guaiacol peroxidase assay except that it was in the general UV-visible mode. Quartz cuvettes (3 ml) containing star stir bars were placed in each reaction. Manganese porphyrin (80 nM) was added to the cuvette with a magnetic stirrer held the cuvette. Air was blown on the cuvette to keep the humidity around the cuvette low. The baseline was first scaned with only buffer in the cuvette. The porphyrin stocks (500 µM in Me2SO) were at room temperature, and apoprotein, PPHP, and the Tris-HCl, 500 µM in Me2SO, were on ice through the entire experiment. Porphyrin (80 nM in Me2SO) and apo-PGH synthase were added to the cuvette containing 100 mM Tris-HCl, pH 8, at 10 °C, then 2 min elapsed before addition of 110 µl of 9 mM guaiacol (in Tris-HCl buffer). PPHP (10-40 mM) in Me2SO was added to the cuvette containing apo-PGH synthase. The absorbance of the reaction mixture was monitored at 436 nm in the kinetic mode. The first determination was at 0 s. Reactions were analyzed at 1-s intervals for 3 min; PPHP was added between 0 and 1.5 s and averaging the signal over the linear portion of the cuvette. Controls were performed with no apoenzyme added to the cuvette; the rates calculated for these reactions were subtracted from each final rate of the enzymatic reaction before the peroxidase activities were compared.

Reduction of Mn-PGH Synthase—A 2-ml solution of 1.5 µM Mn-PGH synthase in 90 mM Tris-HCl, pH 8, at ambient temperature, was placed inside a Thunberg cuvette with 1.5 cm path length. The cuvette remained on ice as it was made anaerobic by evacuating with a vacuum line, then flushing with argon that had been passed through an oxygen trap. This cycle was repeated five times to make anaerobic, then 1 ml of 100 mM Tris-HCl (pH 8), 0.5 mM phenol, 0.1 M sodium ascorbate, and 0.5 mM dithionite was added. The cuvette was flushed with 100% nitrogen and dithionite in the enzyme solution. The absorption spectrum was recorded at room temperature.

Glutathione Peroxidase Inhibition Assay (Cyclooxygenase)—Fe-PGH synthase was the initial M-PGH synthase (40% hole) with specific activity of 35 µmol of O2/mg/min. Mn-PGH synthase was apo-PGH synthase reconstituted with 2 µg of Mn-PPIX chloride in situ. Reconstituted Mn-PGH synthase had a specific activity of 27 µmol of O2/mg/min. Peroxidase constant and substrate constant were inverted so that the Mn-PGH synthase was incubated with varying amounts of human plasma glutathione peroxidase. The reaction mixture contained 100 mM Tris-HCl (pH 8), 0.2 mM phenol, 0.5 mM guaiacol, 0.5 mM Mn-PGH synthase, and 1.25 µM porphyrin in 1 ml Tris-HCl buffer. Each reaction mixture was incubated for 2 min at 37 °C in the oxygen cuvette before
Mn-PGH Synthase Higher Oxidation State

RESULTS

Mn-PGH synthase displayed absorbance maxima at 372, 468, and 554 nm. Upon incubation with arachidonate at either 10 °C or ambient temperature, a new maximum appeared at 418 nm (Fig. 1). The appearance of the 418-nm absorbance was accompanied by a decrease in the 372-nm absorbance of Mn-PGH synthase, a decrease and shift in the absorbance at 468-466 nm, and a shift of the 554-nm maximum to 548 nm. When Mn-PGH synthase was preincubated with the cyclooxygenase inhibitor indomethacin, arachidonic acid-dependent formation of the 418-nm absorbance was prevented. Formation of the 418-nm peak was also prevented by heat inactivation of Mn-PGH synthase before addition of arachidonate.

Addition of the organic hydroperoxide PPHP to Mn-PGH synthase produced changes in the Soret absorbances of Mn-PGH synthase identical to those produced by arachidonate (Fig. 2). This suggests the formation of the 418-nm peak was due to a hydroperoxide-dependent reaction at the metal center of Mn-PGH synthase. To determine if these spectral changes were due to reduction, Mn-PGH synthase was reduced with sodium dithionite under anaerobic conditions. The resulting species exhibited an absorbance maximum at 434 nm (Fig. 2).

The results of these experiments imply that Mn-PGH synthase reacts with exogenous or endogenously generated hydroperoxides to produce higher oxidation states of its metal center. Such higher oxidation states are known to be intermediates of peroxidase catalytic cycles (35). Their detection with Mn-PGH synthase is surprising because the manganese enzyme is believed to be devoid of peroxidase activity (25). Therefore, the peroxidase activity of Mn-PGH synthase was measured with guaiacol as reducing substrate and PPHP as hydroperoxide substrate (Table I). The rate of guaiacol oxidation by Mn-PGH synthase was 0.8% of the rate exhibited by Fe-PGH synthase. Guaiacol peroxidase activity by Mn-PGH synthase was 45 times greater than the activity of apo-PGH synthase plus protoporphyrin IX and 36 times greater than the apparent activity of Mn(III)-PPIX. Thus, the peroxidase activity of Mn-PGH synthase was not an artifact due to small amounts of Fe-PGH synthase in the apoprotein or to a nonspecific peroxidase activity of Mn-PPIX. HPLC analysis of the products of PPHP metabolism by Mn-PGH synthase in the presence of p-methoxythioanisole as reducing substrate revealed the formation of 5-phenyl-4-pentenylalcohol and 5-phenyl-4-pentenylaldehyde.

The spectra displayed in Figs. 1 and 2 appear to represent steady-state mixtures of resting enzyme and an oxidized enzyme. Therefore, difference spectroscopy was employed to study the kinetics of appearance and disappearance of individual peaks. A typical difference spectrum recorded after addition of arachidonic acid to Mn-PGH synthase is presented in Fig. 3A. A peak was apparent at 418 nm and troughs at 374 and 472 nm. A similar difference spectrum was recorded following addition of PPHP to Mn-PGH synthase (Fig. 3B).

The time courses of the spectral changes in the Soret region associated with addition of arachidonate or PPHP to Mn-PGH synthase at 10 °C are summarized in Fig. 4. A 10-s lag time existed between the addition of arachidonate to Mn-PGH synthase and the onset of spectral changes. The maximal increase in absorbance at 418 nm was observed after approximately 70 s of reaction at 10 °C. As the 418-nm peak increased, the troughs at 374 and 472 nm became deeper. Arachidonate concentrations from 1.5 to 100 μM produced similar time courses (data not shown). Addition of PPHP to Mn-PGH synthase resulted in the immediate appearance of the absorbance at 418 nm (Fig. 4B). The maximum absorbance at 418 nm was attained within 20 s. In the absence of reducing agents, the species produced by addition of arachi-

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**Table I**

| Catalyst       | Activity | % of Fe-PGHS |
|----------------|----------|--------------|
| Apo-PGHS + PPIX| 0.00055  | 0.00002      |
| Mn-PPIX        | 0.0007   | 0.0002       |
| PPHP           | 0.023    | 0.002        |
| Mn-PGHS        | 0.026    | 0.001        |
| Fe-PGHS        | 3.1 ± 0.7| 100          |

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5 G. R. Reddy, unpublished results.
The effect of peroxidase-reducing substrates on the 418 nm-absorbing species was assessed. After a 40-s reaction between 1.5 µM Mn-PGH synthase and an excess of arachidonate (100 µM), addition of 5 µM hydroquinone immediately decreased the absorbance at 418 nm (Fig. 5). With the arachidonate concentration of 100 µM, the 418-nm maximum slowly regenerated after being quenched by 5 µM hydroquinone. When a 12-fold higher concentration of hydroquinone was used, the 418-nm peak did not reappear. The two troughs in the difference spectra of the steady-state Mn-PGH synthase mixture (376 and 472 nm) increased in absorbance as the 418-nm absorption decreased following addition of hydroquinone. Another PGH synthase-reducing substrate, TMPD, also reduced the 418-nm absorbance of Mn-PGHS. Using difference spectra, absorbance changes were simultaneously monitored at 610 nm to detect oxidized TMPD and at 418 nm for oxidized Mn-PGH synthase. With each TMPD addition, oxidized TMPD was detected as the 418-nm maximum diminished, directly linking oxidation of a peroxidase-reducing substrate with reduction of Mn-PGH synthase's higher oxidation state (Fig. 6).

It was of interest to compare the time course of appearance of the 418-nm peak to the time course of cyclooxygenase activity. The rapidity of the spectral changes necessitated the use of a continuous assay for cyclooxygenase activity, preferably spectrophotometric. The major product of arachidonic acid oxygenation by Mn-PGH synthase is prostaglandin G2, which does not possess a chromophore (25). Several hydroxy fatty acids are also produced but they are minor products, and the extent of their formation depends upon the reaction conditions. However, Fe-PGH synthase oxygenates eicosanoid acid 11,14-dienoic acid to 11-hydroperoxyeicosa-12,14-dienoic acid, which exhibits an intense UV absorbance at 236 nm (Fig. 4, A). The 418-nm peak was detectable (albeit with low intensity) up to 2 h after arachidonate addition to Mn-PGH synthase at 10 °C.

The absorbance at 520 nm at the same time point. B, as in A, except 10 mM PPHP (in MeOH) was added to initiate the reaction. Solid squares, 418 nm; open squares, 374 nm; triangles, 472 nm.

![Figure 3](image-url)  
**Fig. 3.** Difference spectra from the reaction of Mn-PGH synthase with arachidonate (A) or PPHP (B). See Figs. 1 and 2 for reaction conditions. A is the difference spectrum constructed from Fig. 1. B is the difference spectrum of Fig. 2 and the corresponding Mn-PGH synthase spectrum.

![Figure 4](image-url)  
**Fig. 4.** Changes in the Soret absorbances of Mn-PGH synthase upon reaction of 1.5 µM Mn-PGHS and 50 µM substrate. A, apo-PGH synthase and Mn-PPIX were combined in a final volume of 2 ml as described under “Experimental Procedures.” Addition of a freshly prepared solution of 10 mM sodium arachidonate (in 10% MeOH) to the cuvette occurred 1-2 s after the first spectrum was scanned. Absorbance changes were determined from difference spectra. The absorbance change at the indicated wavelength is relative to the absorbance at 520 nm at the same time point. B, as in A, except 10 mM PPHP (in MeOH) was added to initiate the reaction. Solid squares, 418 nm; open squares, 374 nm; diamonds, 472 nm.

donate or PPHP to Mn-PGH synthase persisted. The 418-nm peak was detectable (albeit with low intensity) up to 2 h after arachidonate addition to Mn-PGH synthase at 10 °C.

![Figure 5](image-url)  
**Fig. 5.** Effect of a peroxidase-reducing substrate on the reaction of 1.5 µM Mn-PGH synthase and 100 µM 20:4. Conditions as in Fig. 4A, except 5 µM hydroquinone (2 µl of 5 mM hydroquinone in 100 mM Tris-HCl, pH 8, at 4 °C) was added to the 2-ml reaction volume at 40 s as indicated by the arrow. Solid squares, 418 nm; open squares, 376 nm; triangles, 472 nm.

![Figure 6](image-url)  
**Fig. 6.** Effect of TMPD on reactions of arachidonate with 1.5 µM Mn-PGH synthase. Mn-PGH synthase (1.5 µM) was placed in 10 mM Tris-HCl, pH 8, at 4 °C. A total of 6 µM sodium arachidonate (from a 10 mM stock in 10% MeOH) was added in 1.5 µM aliquots from 0 to 200 s, then a cyclooxygenase inhibitor (3 µM SQ 29535) was added at 500 s. TMPD (in 3% MeOH) was added in 0.375 µM aliquots at the times indicated by the arrows. The final reaction volume was 2 ml. Solid squares, 418 nm; open squares, 610 nm.
~24,000) (36). Thus, by performing difference spectroscopy over the range of 200–550 nm, it was possible to monitor simultaneously the time course of substrate oxygenation and prosthetic group oxidation in the same incubation mixture. Fig. 7 presents the results of a typical experiment. The absorbance maximum of the oxidized enzyme increased rapidly and reached a steady-state level within 10–20 s. Thus, the lag phase in the production of the 418-nm peak was much shorter following addition of eicosadienoic acid than arachidonic acid. The concentration of the higher oxidation state decreased only slightly from 20 to 120 s. Substrate oxygenation, as judged by absorbance at 236 nm, proceeded in a linear fashion for approximately 40 s then began to slow. The oxygenation of eicosadienoic acid represented stoichiometric substrate conversion and as a result, the 236-nm absorbance continued to slowly grow even as the steady-state levels of the 418-nm absorbance declined. In these experiments, an absorbance maximum at 286 nm was also detected, and the rate of its formation paralleled that of the 236-nm peak (Fig. 7). The 286-nm absorbing species is probably a keto fatty acid produced by decomposition of the hydroperoxy fatty acid intermediate. The absorbance at 286 nm could be seen not only in the difference spectra, but also in the absorbance spectra at all time points.

These kinetic experiments are consistent with the possibility that the higher oxidation state(s) of Mn-PGH synthase plays an important role in cyclooxygenase turnover. In the case of Fe-PGH synthase, support for the importance of oxidized forms of the enzyme in cyclooxygenase catalysis is provided by the observation that the combination of glutathione (GSH) and glutathione peroxidase (GSH peroxidase) inhibit cyclooxygenase activity (11). This inhibition is proposed to be due to lowering of the level of hydroperoxide activators of enzyme activity (13). Therefore, we conducted experiments to determine the sensitivity of Mn-PGH synthase to GSH/GSH peroxidase. The GSH peroxidase used for these experiments was the human plasma enzyme purified in these laboratories (33). Fig. 8 compares the concentration dependence of cyclooxygenase inhibition of Mn-PGH synthase and Fe-PGH synthase. It is evident that the manganese enzyme is much more sensitive to inhibition than the iron enzyme. Under the conditions of these experiments, 50% inhibition of cyclooxygenase activity was detected at GSH peroxidase levels of 0.28 and 63.7 units for equal amounts of the manganese and iron enzymes, respectively. Thus, the manganese enzyme is approximately 227-fold more sensitive to inhibition than the iron enzyme. As observed with the iron enzyme, Mn-PGH synthase is not inhibited by GSH or GSH peroxidase alone, or GSH in the presence of heat-inactivated GSH peroxidase.

**DISCUSSION**

Approximately 10 s after arachidonate addition to Mn-PGH synthase, a species that absorbs at 418 nm is detectable as a part of a steady-state mixture of resting enzyme and oxidized Mn-PGH synthase (Figs. 1 and 4). The immediate appearance of this species following the addition of a hydroperoxide substrate to Mn-PGH synthase suggests it is a higher oxidation state (Figs. 2 and 4). Compounds known to be peroxidase-reducing substrates for Fe-PGH synthase not only quench the 418 nm-absorbing species of Mn-PGH synthase, but are simultaneously oxidized by it (Figs. 5 and 6 and Table I) (37).

When PPHP is used with guaiacol in a peroxidase assay, Mn-PGH synthase exhibits low but reproducible peroxidase activity that does not result from trace amounts of Fe-PGH synthase in the apo preparation or from Mn-PPIX alone (Table I). Accurate comparison of the peroxidase activities of Fe-PGH synthase and Mn-PGH synthase is difficult because the reaction with Fe-PGH synthase proceeds extremely rapidly. When PPHP and guaiacol are the substrates, the initial rate of guaiacol oxidation by Fe-PGH synthase declines within 5 s at 10 °C. In contrast, under identical assay conditions, the rate of guaiacol oxidation by Mn-PGH synthase remains linear for at least 80 s. The persistence of the Mn-PGH synthase peroxidase activity compared to the iron enzyme is also characteristic of their respective cyclooxygenase activities. Upon reconstitution of apo-PGH synthase with Mn-PPIX chloride, cyclooxygenase activity typically proceeds three times longer and consumes three times more oxygen than apo-PGH synthase reconstituted with hematin even though the initial velocity of oxygen uptake by Mn-PGH synthase is lower than that of Fe-PGH synthase.

Manganese derivatives of other peroxidase enzymes form higher oxidation states upon addition of hydrogen peroxide (38–40). Characterization of these peroxide compounds by electronic absorption and electron paramagnetic resonance spectroscopies suggests that the higher oxidation state of manganese horseradish peroxidase (MnHRP) contains manganese (IV), whereas the higher oxidation state of manganese cytochrome-c peroxidase (MnCCP) contains Mn(V) (38–40). Electronic absorption spectra of both the resting enzymes and the higher oxidation states of MnHRP and MnCCP are similar to the spectra of Mn-PGH synthase, although there are some differences in the absorbance maxima in the 500–700-nm region. Upon addition of hydrogen peroxide, the 564-nm maximum of MnHRP disappears and maxima at 509 and 605 nm are evident (40). In contrast, the MnCCP maximum at 568 nm shifts only slightly to 555 nm upon formation of
its higher oxidation state (38, 39). Both of these MnCCP-derived maxima display small shoulders at slightly longer wavelengths. Mn-PGH synthase exhibits a maximum at 554 nm that shifts to 545 nm upon addition of arachidonate or PPHP (Fig. 1). However, there appears to be considerable nonspecific binding of Mn-PPIX to PGH synthase at the stoichiometry of Mn-PGH synthase used in these experiments, which tends to obscure the peroxide-induced spectral changes in the visible region. Strieder et al.3 have concluded from similar experiments that the oxidation state of the metal in the higher oxidation state of Mn-PGH synthase is Mn(IV).

Reactions with PPHP corroborate the initial formation of a Mn(V) oxidation state in Mn-PGH synthase. To produce 5-phenyl-4-pentenyl alcohol, two-electron reduction of PPHP is required. This suggests the enzyme is oxidized by two electrons; it seems likely that both electrons were provided by the metal. Since the spectrally detectable higher oxidation state is Mn(IV), the initially formed Mn(V) must be rapidly reduced by one electron.

Higher oxidation states of Mn-PGH synthase appear to play a role in cyclooxygenase catalysis. By monitoring absorbance changes at the 200-500 nm region during the reaction of Mn-PGH synthase with eicosadienoic acid, it is possible to determine simultaneously the kinetics of formation of the higher oxidation state and the oxygenation of the cyclooxygenase substrate. The data in Fig. 7 suggest that formation of oxidized enzyme precedes fatty acid oxygenation which is what one would expect for a catalytically competent intermediate. Presumably, formation of the higher oxidation state results from reaction of Mn-PGH synthase with hydroperoxide impurities in the eicosadienoic acid.

It is important to note that the time courses in Fig. 7 represent different types of chemical events. The formation of the higher oxidation state represents the approach to and maintenance of a steady state, which contains some fraction of the resting enzyme. In contrast, the increase in absorbance at 235 nm represents conversion of substrate molecules to products and is an integrated response. Using a molar absorptivity of 24,000 M⁻¹ cm⁻¹, one calculates that after 120 s, 45 μM product is formed (41). Thus, 30 molecules of substrate are oxidized per molecule of enzyme (assuming all the enzyme is active). The time course for oxygenation of the first substrate molecules is much faster than the time course for accumulation of 235 nm-absorbing material in Fig. 7 and is probably coincident with the time course of formation of the enzyme higher oxidation state.

The likelihood that the higher oxidation state plays a role in fatty acid oxygenation is also suggested by the effect of GSH/GSH peroxidase on the cyclooxygenase activity of Mn-PGH synthase. Plasma GSH peroxidase is a potent inhibitor of the oxygenation of arachidonate by Mn-PGH synthase. Direct comparison of the same apo-PGH synthase preparation reconstituted with either manganese or iron indicates that the manganese enzyme is 227-fold more sensitive than the iron enzyme to inhibition by GSH/GSH peroxidase. The difference in sensitivity to GSH peroxidase is similar to the difference in peroxidase activities of the manganese and iron enzymes (123-fold). Kulmacz (42) has reported a 5-fold higher peroxide concentration required for activation of Mn-PGH synthase as opposed to Fe-PGH synthase. The 227-fold difference in GSH peroxidase sensitivity of the two preparations suggests there are also significant differences in rate coefficients for reaction of hydroperoxide with the metal center. This is borne out by the difference in peroxidase

3 Strieder, S., Schaible, K., Scherer, H.-J., Dietz, R., and Ruf, H. H. (1992) J. Biol. Chem. 267, 13870-13878.
to control the stereochemistry of oxygenation by binding the fatty acid in the conformation displayed in Scheme I. Such a scheme requires multiple fatty acid binding sites on the protein and translocation of the fatty acid from a site where it is oxidized to a site where it is the oxidizing agent. It also does not explain why there are differences in $Vin_{max}$ between fully activated Mn-PGH synthase and Fe-PGH synthase.

Regardless of the precise role of the oxidized metal in the cyclooxygenase reaction, the present results demonstrate the utility of Mn-PGH synthase for mechanistic studies. Its higher oxidation state exhibits spectral properties distinct from resting enzyme [Mn(III)] or reduced enzyme [Mn(II)]. This is not the case for Fe-PGH synthase because resting enzyme and its peroxidase compound I have the same $\lambda_{max}$ but different molar absorptivities (19). Mn-PGH synthase is two orders of magnitude more sensitive to inhibition by GSH/GSH peroxidase than Fe-PGH synthase. This not only provides insight into the mechanism of the cyclooxygenase reaction but should be exploitable in kinetic studies of the higher oxidation state. Finally, the application of rapid scan diode array spectroscopy to the analysis of the reaction of Mn-PGH synthase with polyunsaturated fatty acids provides an opportunity rare in enzymology: the ability to simultaneously monitor substrate conversion and enzyme intermediates in a single reaction vessel.

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