Peroxidase Self-inactivation in Prostaglandin H Synthase-1 Pretreated with Cyclooxygenase Inhibitors or Substituted with Mangano Protoporphyrin IX*  

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Self-inactivation imposes an upper limit on bioactive prostanooid synthesis by prostaglandin H synthase (PGHS). Inactivation of PGHS peroxidase activity has been found to begin with Intermediate II, which contains a tyrosyl radical. The structure of this radical is altered by cyclooxygenase inhibitors, such as indomethacin and flurbiprofen, and by replacement of heme by mangano protoporphyrin IX (forming MnPGHS-1). Peroxidase self-inactivation in inhibitor-treated PGHS-1 and MnPGHS-1 was characterized by stopped-flow spectroscopic techniques and by chromatographic and mass spectrometric analysis of the metalloporphyrin. The rate of peroxidase inactivation was about 0.3 s⁻¹ in inhibitor-treated PGHS-1 and much slower in MnPGHS-1 (0.05 s⁻¹); as with PGHS-1 itself, the peroxidase inactivation rates were independent of peroxide concentration and structure, consistent with an inactivation process beginning with Intermediate II. The changes in metalloporphyrin absorbance spectra during inactivation of inhibitor-treated PGHS-1 were similar to those observed with PGHS-1 but were rather distinct in MnPGHS-1; the kinetics of the spectral transition from Intermediate II to the next species were comparable to the inactivation kinetics in each case. In contrast to the situation with PGHS-1 itself, significant amounts of heme degradation occurred during inactivation of inhibitor-treated PGHS-1, producing iron chlorin and heme-protein adduct species. Structural perturbations at the peroxidase site (MnPGHS-1) or at the cyclooxygenase site (inhibitor-treated PGHS-1) thus can influence markedly the kinetics and the chemistry of PGHS-1 peroxidase inactivation.

Prostaglandin H synthase (PGHS) catalyzes a key step in prostaglandin biosynthesis, the conversion of arachidonic acid to prostaglandin G₂/II₂ (1). PGHS undergoes irreversible self-inactivation during catalysis, thus limiting the overall number of turnovers (2–6). A recent mechanistic study of PGHS-1 peroxidase self-inactivation yielded two important findings: (a) the inactivation rate is independent of both peroxide and enzyme concentrations; and (b) a new spectral intermediate, Intermediate III, accumulates during the self-inactivation process after formation of Intermediate II and before the appearance of a terminal complex (7). Peroxidase inactivation thus does not occur by decomposition of Intermediate I (or Compound I), which contains a porphyrin radical. Instead, the branch point between peroxidase catalysis and irreversible self-inactivation is probably at Intermediate II, which contains a tyrosyl radical (8, 9). Factors that change the structure of Intermediate II might thus be expected to modify the self-inactivation mechanism.

Pretreatment of PGHS-1 with cyclooxygenase inhibitors, such as indomethacin, flurbiprofen, or aspirin, is known to alter the tyrosyl radical structure in Intermediate II (10, 11), and the altered radical fails to oxidize arachidonic acid to initiate cyclooxygenase activity (12, 13). A second approach to altering Intermediate II structure involves replacement of heme by mangano protoporphyrin IX (forming MnPGHS-1). The steady-state peroxidase activity of MnPGHS-1 is only ~4% of the iron enzyme because of very slow formation of Intermediate I (14), but essentially full cyclooxygenase activity is preserved (15–17). The peroxide-induced radical species in MnPGHS-1 displays EPR characteristics that are different from those of the iron enzyme, but the radical remains capable of oxidizing arachidonate to initiate the cyclooxygenase cycle (13). PGHS-1 treated with cyclooxygenase inhibitors and MnPGHS-1 thus provide useful systems to examine the relationship between reactive enzyme intermediates and peroxidase self-inactivation.

We have evaluated the peroxidase self-inactivation kinetics in MnPGHS-1 and in PGHS-1 pretreated with indomethacin or flurbiprofen. The inactivation mechanisms were similar to that of native PGHS-1, although key intermediates showed different heme structures, and the overall rates were much slower for MnPGHS-1. Thus, modification of the Intermediate II structure has a strong influence on the self-inactivation process in PGHS-1.

EXPERIMENTAL PROCEDURES

Materials—Hemin and MnPPIX were obtained from Porphyrin Products, Inc. (Logan, UT). Tween 20 and Tris(2-carboxyethyl)phosphine were from Pierce. Guaiacol, hydrogen peroxide, and indomethacin were from Sigma. Flurbiprofen was from Upjohn Company (Kalamazoo, MI). Arachidonic acid was from Nuchek Preps (Elysian, MN). Peracetic acid...
and 3-chloroperbenzoic acid were from Aldrich; ETOOH was from Poly- 
siences Inc. (Warrington, PA), and PPHP was from Cayman Chemical 
Co. (Ann Arbor, MI). 15-HPTE was prepared according to Graff et al. 
(18) and was purified by HPLC using a Dynamax Microsorb silicic acid 
column (4.6 × 250 mm, 5 μm). The other peroxides were quantified 
by area using Liquid Chrom, holoenzyme, and N,N,N′,N′-tetramethyl-
p-phenylenediamine but were not further purified. X-ray film was from 
Kodak (Rochester, NY).

PGHS-1 apoenzyme was isolated from ram seminal vesicles as 
described previously (19). Holoenzyme was obtained by reconstitution of 
PGHS-1 apoenzyme with heme and subsequent removal of unbound 
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**RESULTS**

**Peroxidase Self-inactivation of Indomethacin- and 
Flurbiprofen-treated PGHS-1**

**Kinetics of Peroxidase Inactivation**—PGHS-1 pretreated 
with indomethacin or flurbiprofen was first reacted with hy-
droperoxide for different lengths of time, and the surviving 
peroxidase activity was quantified by reaction with excess 
guaiacol and H2O2, using the sequential mixing protocol de-
scribed under “Experimental Procedures.” The surviving per-
oxidase activity was found to decline with single-exponential 
kinetics at all initial PPHP and ETOOH levels (data not shown). 
The observed first-order rate constants for decay of peroxidase activity are shown in Fig. 1 as a function of initial 
peroxide concentration. The inactivation rates for indometha-
cin-treated PGHS-1 varied between 0.13 and 0.29 s−1 with an 
average value of 0.19 ± 0.06 s−1 (Fig. 1, A and B). Similar 
results were obtained for flurbiprofen-treated PGHS-1, but 
with a larger range, from 0.25 to 0.71 s−1; the average was 
0.37 ± 0.14 s−1 (Fig. 1, C and D). The data in Fig. 1 make it 
clear that the rate of peroxidase inactivation in both inhibitor-
treated PGHS-1 preparations was essentially independent of 
peroxide concentration with either ETOOH or PPHP. Varying 
the enzyme concentration from 0.1 to 2.4 μg/ml led to little 
change in the inactivation rate. In the case of flurbiprofen-
treated PGHS-1 reacted with ETOOH, there was a trend to-
ward higher decay rates with increased peroxide concentration 
for 0.36 and 0.9 μg/ml PGHS-1, but this trend is unlikely to be 
significant because it was not observed at either higher or 
lower enzyme concentrations (Fig. 1D).

**Heme Spectral Changes during Peroxidase Self-inactivation 
of Inhibitor-treated PGHS-1**—The Soret region spectra ac-
bquired by photodiode array detector during the reaction of indomethacin- 
and flurbiprofen-treated PGHS-1 with ETOOH were similar to those observed for native PGHS-1 (Fig. 2). Data 
from the first 100 ms reflect primarily the transient formation of Intermediate I and its subsequent conversion to Intermedi-
ate II. These spectral changes had rate constants of at least 200 s−1, much faster than the observed inactivation kinetics (Fig. 1). To simplify analysis of spectral changes during peroxidase 
inactivation, attention was focused on spectral changes after 
100 ms. Reconstructed spectra for the 0.1–1-s time range 
showed an isosbestic point at 403–404 nm and thus appeared 
to reflect a single transition between two species (Fig. 2. spec-
a. b, and c). Spectra obtained over the later 1–50-s period 
showed a simple decline in absorbance with little shift in wave-
length (Fig. 2, spectrum d). Data between 0.1 and 50 s were 
found to fit better to a linear three-species, two-step model than to 
models with more or fewer steps (not shown), and so rate 
constants for a two-step mechanism were evaluated. The rate 
constant for the first step was 0.50 s−1 for the native PGHS-1 
(Fig. 2A). Similar values were found for indomethacin- 
and flurbiprofen-treated PGHS-1, 0.28 and 0.58 s−1, respectively. 
These rate constants for the initial spectral transition in the 
0.1–1-s range were of the same order as the corresponding 
peroxidase inactivation rate constants for inhibitor-treated 
PGHS-1, 0.2 and 0.4 s−1 (Fig. 1). The fitted rate constant for
FIG. 1. Effects of peroxide concentration and structure on the rate of peroxidase self-inactivation in indomethacin-treated PGHS-1 (panels A and B) and flurbiprofen-treated PGHS-1 (panels C and D). Peroxidase inactivation kinetics were determined using the sequential stopped-flow method described under "Experimental Procedures" at various levels of PPHP (panels A and C) or EtOOH (panels B and D). Enzyme levels are indicated at the upper left of each panel.

FIG. 2. Spectral changes during peroxidase inactivation of native PGHS-1 (panel A) and PGHS-1 treated with indomethacin (panel B) or flurbiprofen (panel C). 0.58 μM native PGHS-1, 1.58 μM indomethacin-treated PGHS-1, or 1.46 μM flurbiprofen-treated PGHS-1 was reacted with 32 μM EtOOH at 24 °C, and spectral changes were obtained from diode array stopped-flow measurements. Spectra were first analyzed by singular value decomposition between 0.1 and 1 s and between 1 and 50 s. Spectra of resting enzyme (a) are presented in solid lines for comparison. Fitting of the diode array data to a three-species, two-step linear mechanism was used to deconvolute spectra for individual reaction intermediates, with data from the initial 100 ms excluded from the fitting to minimize contamination from Intermediate I. Spectra are shown for resting enzyme (a), Intermediate II (b), Intermediate III (c), and reaction product at 50 s (d). Spectrum d in each panel was similar to the spectrum of the terminal complex after a 10-min reaction.
the second spectral step for the native PGHS-1 was 0.055 s⁻¹. This is similar to those obtained for the two inhibitor-treated samples (0.043 and 0.050 s⁻¹) and about an order of magnitude slower than the first step. The three spectral species deconvoluted from data acquired after 0.1 s are shown in Fig. 2 (spectra b, c, and d in both panels). The qualitatively different nature of spectral changes in the early phase (increasing above and decreasing below 403–404 nm) and in the later phase (decreasing both above and below the isosbestic point at 403–404 nm) (Fig. 2) confirms that spectra b and c reflect distinct intermediates even though their spectral line shapes are similar. The spectral changes observed during conversion from Intermediate II (spectrum b) to IIIa (spectrum c) for inhibitor-treated PGHS-1 (Fig. 2, B and C) differed only marginally in the position of the isosbestic point and the direction of amplitude changes on either side of the isosbestic point from those observed for the Intermediate II to III transition in native PGHS-1 (Fig. 2A). The presence of cyclooxygenase inhibitor thus did not markedly alter the transitions in heme optical spectrum during peroxidase self-inactivation.

Diode array stopped-flow data for reaction between indomethacin-treated PGHS-1 and PPHP gave results similar to those shown for reaction with EtOOH (data not shown), indicating that the spectral transitions observed are independent of the peroxide structure.

**Heme Structural Changes during Peroxidase Inactivation of Inhibitor-treated PGHS-1**—Flurbiprofen- and indomethacin-treated PGHS-1 reacted for various lengths of time with 10 eq of EtOOH. The chromatographic profile for the flurbiprofen-PGHS-1 control (mixing with buffer instead of EtOOH) in panel A showed a prominent A₂₂₀ peak corresponding to PGHS-1 protein (peak 3) and a prominent A₄₀₀ peak (peak 2) corresponding to native heme dissociated from the protein. For the sample reacted with EtOOH for 20 s shown in panel B, there was a ~50% decrease in the amount of free heme (peak 2) and an increase in two heme products (peaks 1 and 3). Peak 1 had a shorter retention time (~22 min) than heme itself (~24 min) and had little 220 nm absorbance, suggesting that it represented a heme derivative not associated with the protein. Peak 3 had significant absorbance at both 400 and 220 nm, indicating that it represented a heme adduct to the protein. Chromatographic profiles from a parallel experiment with indomethacin-treated PGHS-1 (Fig. 3, panels C and D) were very similar to those for flurbiprofen-treated PGHS-1.

The fact that peak 1 had a shorter retention time than intact heme, along with its Soret maximum at 398 nm (data not shown), was reminiscent of an iron chlorin product formed during reaction of myoglobin with H₂O₂ (23). Thus, a further comparison between peak 1 and heme chlorin product was conducted. Metmyoglobin was treated with hydrogen peroxide at pH 4.7 to generate the chlorin product (23) for analysis by HPLC. The retention time of the chlorin standard peak at about 22 min coincided with that for peak 1 from self-inactivated flurbiprofen-treated PGHS-1 (Fig. 4A), as confirmed by the increased peak height upon coinjection of the two samples (Fig. 4C).

LC-MS analysis was conducted to determine unambiguously the chemical structure of the peak 1 compound(s), as done previously for myoglobin inactivated by hydrogen peroxide (23). Parallel mass analyses of peak 1 HPLC fractions from myoglobin/H₂O₂ and flurbiprofen-treated PGHS-1/EtOOH samples are shown in Fig. 5. A dominant species, with an m/z
ratio of 632.2, is present in peroxide-treated samples derived from both myoglobin and flurbiprofen-treated PGHS (panels A and B in Fig. 5). This m/z 632 ion compound was shown previously to derive from a compound characterized by NMR as a hydroxychlorin (chemical structure shown in Fig. 5) (23). The major modified heme compound in peak 1 found in both flurbiprofen- and indomethacin-treated PGHS-1 after reaction with peroxide is thus a chlorin. There are other ions of lower intensity in the mass spectra of the chlorins which most likely represent impurities. The present results do not exclude the possibility that the heme product identified from PGHS is chromatographically indistinguishable isomer of the chlorin identified from myoglobin.

LC-MS analysis of HPLC peak 3 from inhibitor-treated PGHS-1 was not successful. To confirm that the material eluting in peak 3 represented heme adducts to PGHS-1 protein, we utilized a chemiluminescence assay developed for detection of heme adducts to myoglobin (21). This method is specific for protein-heme adducts because native heme dissociates from the protein during electrophoresis and migrates at the dye front, whereas heme irreversibly bound to the protein gives a chemiluminescence signal. As shown in Fig. 6, luminescence indicative of protein-bound heme was found in both flurbiprofen- and indomethacin-treated PGHS-1 after reaction with peroxide but not in the controls, confirming that heme-protein adduct was formed during peroxidase inactivation.

The intensities of peaks 1 (modified heme), 2 (native heme), and 3 (heme-protein adduct) are shown as functions of the reaction time with peroxide for both indomethacin- and flurbiprofen-treated PGHS-1 (Fig. 7). The changes in peaks 1 and 2 approximated single-exponential kinetics, with rate constants of 0.20 s\(^{-1}\) and 0.40 s\(^{-1}\), respectively, for indomethacin-PGHS-1 (Fig. 7A) and 0.25 s\(^{-1}\) and 0.072 s\(^{-1}\), respectively, for flurbiprofen-PGHS-1 (Fig. 7B). The kinetics of changes in peak 3 (400 nm) were more complex, with an abrupt increase at the earliest time point compared with the control, but only slight further increase at later reaction times. peak 2 was the predominant A\(_{400}\) species in all HPLC profiles, accounting for at least half of the integrated absorbance. The peak 2 decay was slightly faster for indomethacin-treated PGHS-1 (0.40 s\(^{-1}\)) than for flurbiprofen-treated PGHS-1 (0.072 s\(^{-1}\)). The rate of peak 1 formation appeared similar in flurbiprofen-treated PGHS-1 (0.25 s\(^{-1}\)) and indomethacin-treated PGHS-1 (0.20 s\(^{-1}\)). The rates for the decreases in intact heme and the in-
the average value of 0.034 s$^{-1}$.

The slower rate constant for the second step was comparable to that of PGHS-1 (Fig. 8). Also, MnPGHS-1 retained 40% residual activity even after prolonged reaction. The peroxidase inactivation kinetics for MnPGHS-1 using 15-HPETE were similar to those obtained with PPHP (data not shown). The effects of peroxide concentration on MnPGHS-1 peroxidase inactivation kinetics were measured during reactions with 56 μM EtOOH and H$_2$O$_2$ are very poor substrates for the MnPGHS-1 peroxidase, so peroxidase inactivation in MnPGHS-1 was examined with either 15-HPETE or PPHP. Very little MnPGHS-1 peroxidase inactivation was observed in the first 10 s of incubation with PPHP; in contrast, PGHS-1 lost 90% of its peroxidase activity over this period (Fig. 8). The rate of MnPGHS-1 peroxidase inactivation was 0.048 s$^{-1}$, an order of magnitude slower than the value of 0.60 s$^{-1}$ determined for PGHS-1 under the same conditions (Fig. 8). Also, MnPGHS-1 retained ~40% residual activity even after prolonged reaction. The peroxidase inactivation kinetics for MnPGHS-1 using 15-HPETE were similar to those obtained with PPHP (data not shown). The effects of peroxide concentration on MnPGHS-1 peroxidase inactivation kinetics were examined with 15-HPETE and PPHP (Fig. 9). MnPGHS-1 activity declined in an exponential fashion in these reactions, with the rate constants ranging between 0.033 and 0.050 s$^{-1}$ for 15-HPETE (average 0.043 ± 0.006 s$^{-1}$) and 0.018 and 0.048 s$^{-1}$ for PPHP (average 0.034 ± 0.011 s$^{-1}$) (Fig. 8). This result indicates that the peroxidase inactivation in MnPGHS-1, although much slower than in PGHS-1, was still independent of peroxide concentration and structure. There was no obvious dependence of the peroxidase inactivation rate on the MnPGHS-1 enzyme concentration because similar outcomes were obtained at two quite different enzyme concentrations (Fig. 9).

Metalloporphyrin Spectral Changes during Peroxidase Inactivation in MnPGHS-1—Reconstructed spectra obtained during 0.1–10 s of reaction between MnPGHS-1 and PPHP showed progressive increases in absorbance at 420 nm and decreases in absorbance at ~378 and ~472 nm, with isosbestic points at 392 and 447 nm (Fig. 10A). These spectral changes are similar to those observed previously during MnPGHS-1 peroxidase catalysis (14–17). Kinetic spectral data covering the first 50 s of reaction were optimally fitted by a three-species, two-step model (A → B → C), with rate constants of 0.5 s$^{-1}$ and 0.05 s$^{-1}$. The slower rate constant for the second step was comparable to the average value of 0.034 s$^{-1}$ observed for peroxidase inactivation in PPHP (Fig. 9). Deconvoluted spectra for the three spectral species are shown in Fig. 10B. The spectrum of the first species (X) was very similar to that of resting enzyme (compare spectra a and X in Fig. 10B). The spectrum of the second species (Y) was similar to that of MnPGHS-1 Intermediate II, which contains Mn$^{4+}$ = O and a radical (14). The spectrum of the third species (Z) showed the same general pattern as that of the second species, but with decreased intensity (Fig. 10B). The spectrum obtained after 10 min of reaction was similar to that of the second species, indicating that the third species was not stable (Fig. 10B). The very slow reversal of spectral characteristics after formation of the third species in MnPGHS-1 was not accompanied by a recovery of peroxidase activity (see above). Global fitting the data to a single step model, A → B, gave a rate constant 10 times faster than the observed decay rate. Global fitting to a four-species model, A → B → C → D, did not give a satisfactory fit either because the resulting rate constants for the first two steps were very similar, and the deconvoluted spectra for species B and C were essentially indistinguishable (data not shown).

Spectral changes during reaction of MnPGHS-1 with 15-HPETE resembled those seen during reaction with PPHP shown in Fig. 10 and yielded intermediates with very similar

**Peroxidase Inactivation in PGHS-1 Reconstituted with Mn-PPIX**

**Peroxidase Inactivation Kinetics—**EtOOH and H$_2$O$_2$ are very poor substrates for the MnPGHS-1 peroxidase, so peroxidase inactivation in MnPGHS-1 was examined with either 15-HPETE or PPHP. Very little MnPGHS-1 peroxidase inactivation was observed in the first 10 s of incubation with PPHP; in contrast, PGHS-1 lost 90% of its peroxidase activity over this period (Fig. 8). The rate of MnPGHS-1 peroxidase inactivation was 0.048 s$^{-1}$, an order of magnitude slower than the value of 0.60 s$^{-1}$ determined for PGHS-1 under the same conditions (Fig. 8). Also, MnPGHS-1 retained ~40% residual activity even after prolonged reaction. The peroxidase inactivation kinetics for MnPGHS-1 using 15-HPETE were similar to those obtained with PPHP (data not shown). The effects of peroxide concentration on MnPGHS-1 peroxidase inactivation kinetics were examined with 15-HPETE and PPHP (Fig. 9). MnPGHS-1 activity declined in an exponential fashion in these reactions, with the rate constants ranging between 0.033 and 0.050 s$^{-1}$ for 15-HPETE (average 0.043 ± 0.006 s$^{-1}$) and 0.018 and 0.048 s$^{-1}$ for PPHP (average 0.034 ± 0.011 s$^{-1}$) (Fig. 8). This result indicates that the peroxidase inactivation in MnPGHS-1, although much slower than in PGHS-1, was still independent of peroxide concentration and structure. There was no obvious dependence of the peroxidase inactivation rate on the MnPGHS-1 enzyme concentration because similar outcomes were obtained at two quite different enzyme concentrations (Fig. 9).

**Metalloporphyrin Spectral Changes during Peroxidase Inactivation in MnPGHS-1—Reconstructed** spectra obtained during 0.1–10 s of reaction between MnPGHS-1 and PPHP showed progressive increases in absorbance at 420 nm and decreases in absorbance at ~378 and ~472 nm, with isosbestic points at 392 and 447 nm (Fig. 10A). These spectral changes are similar to those observed previously during MnPGHS-1 peroxidase catalysis (14–17). Kinetic spectral data covering the first 50 s of reaction were optimally fitted by a three-species, two-step model (A → B → C), with rate constants of 0.5 s$^{-1}$ and 0.05 s$^{-1}$. The slower rate constant for the second step was comparable to the average value of 0.034 s$^{-1}$ observed for peroxidase inactivation in PPHP (Fig. 9). Deconvoluted spectra for the three spectral species are shown in Fig. 10B. The spectrum of the first species (X) was very similar to that of resting enzyme (compare spectra a and X in Fig. 10B). The spectrum of the second species (Y) was similar to that of MnPGHS-1 Intermediate II, which contains Mn$^{4+}$ = O and a radical (14). The spectrum of the third species (Z) showed the same general pattern as that of the second species, but with decreased intensity (Fig. 10B). The spectrum obtained after 10 min of reaction was similar to that of the second species, indicating that the third species was not stable (Fig. 10B). The very slow reversal of spectral characteristics after formation of the third species in MnPGHS-1 was not accompanied by a recovery of peroxidase activity (see above). Global fitting the data to a single step model, A → B, gave a rate constant 10 times faster than the observed decay rate. Global fitting to a four-species model, A → B → C → D, did not give a satisfactory fit either because the resulting rate constants for the first two steps were very similar, and the deconvoluted spectra for species B and C were essentially indistinguishable (data not shown).

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**Fig. 7. Kinetics of derivatized heme formation in inhibitor-treated PGHS-1.** Integrated areas of A$_{max}$ for peak 1 (filled triangles), peak 2 (filled circles), and peak 3 (open triangles) are shown as a function of reaction time with EtOOH for indomethacin-treated PGHS-1 (panel A) and flurbiprofen-treated PGHS-1 (panel B). The values represent averages of two data sets and are normalized to the area of the corresponding A$_{280}$ protein peak. Fitted curves are shown as solid lines for peak 2, dashed lines for peak 1, and dash-dot lines for peak 3.

**Fig. 8. Comparison of peroxidase self-inactivation kinetics in MnPGHS-1 and PGHS-1.** Decay of peroxidase activity of 1.0 μM PGHS-1 (open circles) and 1.8 μM MnPGHS-1 (filled circles) was measured during reactions with 56 μM PPHP. Solid lines represent single-exponential fits to the data; fitted rate constants are given next to the curves.
deconvoluted spectra characteristics (data not shown), indicating that the spectral changes during self-inactivation were not sensitive to the peroxide structure.

Metalloporphyrin Structural Changes during Inactivation of MnPGHS-1 Peroxidase—The chromatographic profile of control MnPGHS-1 (Fig. 11A) showed a major 400 nm absorbing peak (peak 2), corresponding to free MnPPIX, and a major 220 nm absorbing peak (peak 3), corresponding to PGHS-1 protein. The chromatographic profile for MnPGHS-1 reacted with PPHP for 20 s (Fig. 11B) and had three conspicuous 400 nm peaks. The dominant peak (peak 2) had a retention time (~24 min) corresponding to free MnPPIX. The retention time of peak 3, ~28 min, coincided with the A_{220} peak, indicating that peak 3 represents protein-bound porphyrin. Peak 1, with a retention time of ~22 min, was composed of two partially overlapping peaks, 1A and 1B. The intensity of peak 2 at the first time point was considerably lower than the control, but the peak 3 intensity changed little with time (Fig. 11, C and D). The intensities of peaks 1A and 1B indicated that these species are likely to represent only a small fraction of the metalloporphyrin even at 60 s of reaction, when the majority of the peroxidase is inactivated. Thus, although the peak 1A and 1B species accumulated during the time self-inactivation occurs, their formation is probably not the cause of self-inactivation.

**DISCUSSION**

Cyclooxygenase Inhibitors and Peroxidase Inactivation—Self-inactivation of PGHS has long been thought to reflect collateral damage to the protein or the heme prosthetic group in side reactions of oxidized intermediates generated during peroxidase catalysis (2, 24). Previous kinetic and spectroscopic evidence (7) indicated that the process of peroxidase inactivation in PGHS-1 originates with Intermediate II, a species with two oxidizing equivalents: ferryl heme in the peroxidase site and a tyrosyl radical at Tyr-385 in the cyclooxygenase site (Scheme 1) (8, 10, 25). As depicted in Fig. 12, cyclooxygenase inhibitors, such as indomethacin and flurbiprofen, bind in the cyclooxygenase channel of PGHS-1, putting the inhibitor about 9 Å from the heme in the peroxidase site (26). Inhibitor binding does not significantly perturb the heme electronic absorbance.
spectrum (10) or the kinetics of the initial steps in peroxidase catalysis (14). Cyclooxygenase inhibitors thus seem unlikely to influence directly any side reactions of the ferryl heme oxidant during inactivation. However, bound flurbiprofen does approach within 4 Å of Tyr-385 (26), the location of the tyrosyl radical, which is a key oxidant in cyclooxygenase catalysis (8, 12). Earlier studies using site-directed mutagenesis and EPR characterization (12, 13, 25, 27) and our recent ENDOR analysis (28) strongly indicate that the peroxide-induced tyrosyl radical of inhibitor-treated PGHS-1 is probably not located at Tyr-385. This “alternative” tyrosyl radical is not competent for cyclooxygenase catalysis (12). Such repositioning of the second oxidizing equivalent in Intermediate II by inhibitor clearly has the potential to alter oxidant-driven reactions during inactivation.

In other studies, cyclooxygenase inhibitors increased the resistance of PGHS-1 peroxidase activity to denaturation by heat or pH extremes (29) and blocked proteolytic attack at the distant Arg-277 residue (30–32). As depicted in Fig. 12, considerable distance separates the positions of bound inhibitor and Arg-277 in PGHS-1. This indicates that cyclooxygenase site ligands can have long range effects on the structural dynamics of the protein. Such structural changes could conceiv-
ably impact the self-inactivation process. It was thus not surprising that the presence of indomethacin or flurbiprofen was observed to alter events during peroxidase inactivation, although the alterations turned out to be perhaps more subtle than might have been anticipated.

As with the native enzyme, the peroxidase inactivation rate in the inhibitor-PGHS-1 complex was independent of peroxide concentration and structure (Fig. 1). This is likely because of the much shorter lifetime of Intermediate I (Compound I) than that of Intermediate II, as found for native PGHS-1. This result also indicates a similar overall inactivation process in PGHS-1 and inhibitor-treated PGHS-1, originating from an enzyme intermediate after Intermediate I, probably Intermediate II. The rate constant for peroxidase inactivation was not changed significantly when cyclooxygenase inhibitors were bound, going from $0.32 \pm 0.09 \, \text{s}^{-1}$ for PGHS-1 itself (7) to $0.19 \pm 0.06 \, \text{s}^{-1}$ for the indomethacin complex, and $0.37 \pm 0.14 \, \text{s}^{-1}$ for the flurbiprofen complex (Fig. 1). As with native PGHS-1, the heme spectral changes during inactivation of the inhibitor complexes were consistent with a two-step process beginning with Intermediate II. The rate constant for the first, faster step in the spectral changes, $0.2-1.0 \, \text{s}^{-1}$, was similar to the average rates for loss of peroxidase activity, $0.19-0.37 \, \text{s}^{-1}$, consistent with a linkage between conversion of Intermediate II to Intermediate III and loss of peroxidase activity.

Even though cyclooxygenase inhibitor did not alter the general features of peroxidase inactivation as indicated by the similar heme spectroscopic changes during the self-inactivation process (Fig. 2), some events at the peroxidase site during inactivation were clearly perturbed when inhibitor was present. The most prominent changes are the increased levels of chlorin (peak 1) and heme-protein adduct (peak 3) generated when inhibitor is present during self-inactivation of PGHS-1 (compare Fig. 3 with Fig. 5 of Ref. 7). The results of LC-MS analysis of peak 1 (Figs. 4 and 5) and the luminescence assay of peak 3 (Fig. 6) confirm that these species are derived from the PGHS-1 heme. The increased formation of these heme derivatives indicates that the redox reactions at the heme site during inactivation are quite different when inhibitor is bound at the cyclooxygenase site. Our data suggest that there are at least three different processes that occur during peroxidase inactivation in inhibitor-treated PGHS-1: 1) chemical alteration of the protein, with heme left intact (peak 2); 2) chemical modification of heme without attachment to protein (peak 1); and 3) covalent attachment of heme to the protein (peak 3). The first process is predominant for the peroxide-inactivation in native PGHS-1 (7), whereas the latter two processes are prominent with inhibitor bound. This result implies that in inhibitor-treated PGHS-1 the oxidized heme intermediate(s) have a higher propensity to convert to the chlorin. It may be that the redox coupling between the porphyrin cation radical and Tyr-385 in native PGHS-1 is tighter than that between the porphyrin cation radical and the alternative tyrosine in inhibitor-treated PGHS-1. Thus, the porphyrin $\pi$ cation radical efficiently oxidizes Tyr-385 to produce the tyrosyl radical in native PGHS-1, whereas in the inhibitor-treated enzyme, the cation radical instead tends to convert to a chlorin by intramolecular charge transfer (23).

Chromatographic analyses of reaction-inactivated PGHS samples were monitored at 400 nm. Heme oxidation products are expected to have significant (though perhaps reduced) absorbance at 400 nm unless the pyrrole structure is degraded. Peroxide inactivation of heme proteins usually does not lead to complete destruction of the pyrrole structure (23, 33). It thus seems likely that most heme products from the PGHS reactions were detected, provided they eluted from the HPLC column.

About half of the heme absorbance was lost during inactivation of inhibitor-treated PGHS-1, and the kinetics of the heme loss ($0.1-0.4 \, \text{s}^{-1}$; Fig. 7) were similar to those for loss of peroxidase activity ($0.19-0.37 \, \text{s}^{-1}$; Fig. 1 and Scheme 1). The increases in the chlorin and heme-protein adduct peaks accounted for about half of the decrease in the heme peak (Fig. 7). However, the extinction coefficients of modified heme products are known to be lower than that of intact heme (33). Thus, the sizes of peaks 1 (chlorin) and 3 (heme-protein adduct) in Figs. 3 and 7 are likely to underestimate the amounts of modified heme, and the two modified heme species observed may well account for the majority of the decrease in intact heme (peak 2).

It is particularly interesting that inhibitor binding both increases the formation of heme-protein adduct (Figs. 3 and 7) and probably shifts the location of the tyrosyl radical in Intermediate II from Tyr-385 to another tyrosine residue (10–13, 25, 27, 28). Characterization of the site of heme attachment in the protein adduct formed in inhibitor-treated PGHS-1 may provide clues for identifying the alternative tyrosine and may help explain why a radical in that position would be disposed to chemical disruption of the heme.

**Metalloporphyrin Substitution and Peroxidase Inactivation—**MnPGHS-1 peroxidase was dramatically more resistant than PGHS-1 peroxidase to inactivation by substrate (Figs. 1, 8, and 9). This slower inactivation in MnPGHS-1 parallels the earlier observation of a slower destruction of catalytic activity during reconstitution of apoenzyme with MnPPIX instead of heme (34). The clear parallels between the slower spectral changes and the slower peroxidase inactivation upon substitution of the metalloporphyrin suggest that the spectral changes reflect, directly or indirectly, the damaging events leading to loss of activity. MnPPIX, like heme, protects PGHS-1 from proteolytic attack at Arg-277 (30, 35), a residue some distance from the porphyrin site (Fig. 12), indicating that the general structural effects caused by heme binding are mimicked by MnPPIX binding, so such structural changes are unlikely to account for the slower inactivation in MnPGHS-1. The slower formation of Intermediate I in MnPGHS-1 than PGHS-1 also cannot be the reason for the slower inactivation because the rate of inactivation was not dependent on peroxide concentration (Fig. 9), whereas the rate of Intermediate I formation is obviously dependent on the peroxide level (14). MnPGHS-1 does have cyclooxygenase activity comparable to that of PGHS-1 (34), and the MnPGHS-1 radical has been shown to be chemically competent for cyclooxygenase catalysis (13), making it likely that formation of the catalytic radical at Tyr-385 is not impaired by metalloporphyrin substitution. Differences between PGHS-1 and MnPGHS-1 are present in both the metalloporphyrin and the amino acid radical parts of Intermediate II (10, 13, 17). Both the oxyferryl and the Mn$^{4+}=\text{O}$ moieties are strong oxidizing agents, but the half-filled d orbitals of the latter may reduce its chemical reactivity with neighboring oxidizable groups on the protein. In addition, the EPR of the initial peroxide-induced radical in MnPGHS-1 is quite distinct from the corresponding radical in PGHS-1 (10, 13, 17), leaving no doubt that the structure of the radical in MnPGHS-1 is greatly influenced by the character of the metalloporphyrin. In addition, the maximal radical accumulation in MnPGHS-1 is about one-fourth that in PGHS-1 with either 15-HPETE or arachidonate as substrate (10, 17). With metalloporphyrin substitution thus affecting both the peroxidase site redox characteristics and the cyclooxygenase site radical structure and radical intensity, it is possible that the dramatically slower rate of peroxidase inactivation in MnPGHS-1 (Figs. 1, 8 and 9) is caused by changes at the peroxidase site or the cyclooxygenase site, or both.
MnPghs-1 samples incubated for even a short time with peroxide lost about half the content of intact MnPPIX, and accumulation of modified MnPPIX species accounted for only about 10% of the A_{380} lost from the parent compound (Fig. 11), indicating that MnPPIX was converted primarily to a species not detected chromatographically. This loss of MnPPIX might be explained by rapid bleaching by peroxide of MnPPIX dissociating from the MnPghs-1 preparation. PGHs-1 apoenzyme exhibits lower affinity for MnPPIX than for heme (36). Because of this, the MnPghs-1 preparations were not treated with DEAE-cellulose to remove loosely bound metalloporphyrin, in contrast to the routine during reconstitution with heme. Some loosely bound MnPPIX would be able to dissociate from the protein, remaining chemically intact (and thus detected in peak 2) unless destroyed by exposure to peroxide in the rapid quench apparatus. The inactivation process in MnPghs-1 thus appears similar to the process in PGHs-1 in that it is accompanied by relatively little generation of recognizable modified metalloporphyrin or metalloporphyrin-protein adduct (Fig. 11 and Ref. 7).

Overall, there are many similarities among the processes of peroxidase inactivation in PGHs-1, inhibitor-treated PGHs-1, and MnPGHs-1, as summarized in Scheme 1. In each case, the loss of activity appears to originate with the equivalent of Intermediate II, rather than with Intermediate I. In each case, the spectral changes suggest a two-step process, with the conversion of Intermediate II to the next spectral species being kinetically associated with loss of activity. Modification of the peroxidase site by metalloporphyrin substitution leads to dramatic changes in the rates of the inactivation process and the associated spectral changes but has little effect on production of modified metalloporphyrin or metalloporphyrin-protein adduct. In contrast, modification of the cyclooxygenase site with inhibitor has little effect on the inactivation kinetics but increases the damage to the heme and formation of heme-protein adduct. Further characterization of the nature of the damage to the protein during peroxidase inactivation in PGHs-1 and MnPGHs-1 and to the heme and protein in inhibitor-treated PGHs-1 should be useful in aiding our understanding the chemical mechanism of PGHs-1 self-inactivation.

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Peroxidase Self-inactivation in Prostaglandin H Synthase-1 Pretreated with Cyclooxygenase Inhibitors or Substituted with Mangano Protoporphyrin IX

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