Studies on the Reduction of Endogenously Generated Prostaglandin G₂ by Prostaglandin H Synthase*

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Prostaglandin H synthase oxidizes arachidonic acid to prostaglandin G₂ (PGG₂) via its cyclooxygenase activity and reduces PGG₂ to prostaglandin H₂ by its peroxidase activity. The purpose of this study was to determine if endogenously generated PGG₂ is the preferred substrate for the peroxidase compared with exogenous PGG₂. Arachidonic acid and varying concentrations of exogenous PGG₂ were incubated with ram seminal vesicle microsomes or purified prostaglandin H synthase in the presence of the reducing cosubstrate, aminopyrine. The formation of the aminopyrine cation free radical (AP⁺) served as an index of peroxide reduction. The simultaneous addition of PGG₂ with arachidonic acid did not alter cyclooxygenase activity of ram seminal vesicle microsomes or the formation of the AP⁺. This suggests that the formation of AP⁺, catalyzed by the peroxidase, was supported by endogenous endoperoxide formed from arachidonic acid oxidation rather than by the reduction of exogenous PGG₂. In addition to the AP⁺ assay, the reduction of exogenous versus endogenous PGG₂ was studied by using [5,6,8,9,11,12,14,15-3H]arachidonic acid and unlabeled PGG₂ as substrates, with gas chromatography-mass spectrometry techniques to measure the amount of reduction of endogenous versus exogenous PGG₂. Two distinct results were observed. With ram seminal vesicle microsomes, little reduction of exogenous PGG₂ was observed even under conditions in which all of the endogenous PGG₂ was reduced. In contrast, studies with purified prostaglandin H synthase showed complete reduction of both exogenous and endogenous PGG₂ using similar experimental conditions. Our findings indicate that PGG₂ formed by the oxidation of arachidonic acid by prostaglandin H synthase in microsomal membranes is reduced preferentially by prostaglandin H synthase.

Prostaglandins are formed from arachidonic acid by the bifunctional enzyme prostaglandin H synthase. The initial step of this process is a peroxidation of arachidonic acid at carbon 11 and the subsequent formation of the endoperoxide

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EXPERIMENTAL PROCEDURES

Materials—[5,6,8,9,11,12,14,15-3H]Arachidonic acid (100 Ci/mmol) and 15-[5,6,8,9,11,12,14,15-3H]HETE (150 Ci/mmol) were obtained from Du Pont-New England Nuclear. Arachidonic acid (d- AA), 98% pure, was from Nu-Chek Prep, Elysian, MN, [5,6,8,9,11,12,14,15-3H]HETE, hydroxyeicosatetraenoic acid (HE TE), and [3,3,4,4-3H]PGF₂α were from Cayman Chemicals, Ann Arbor, MI; and PGG₂ (99% pure) and PGH₂ were from Calbiochem. Stock solutions of PGG₂ and PGH₂ plus arachidonic acid were prepared by evaporating the organic solvents from the commercial preparation of PGG₂ and reconstituting in ethanol or arachidonic acid dissolved in ethanol. The purity of the PGG₂ was checked periodically by thin layer chromatography and found to remain at approximately 95%. Phenol was from Fisher Scientific, and aminopyrine was obtained from Sigma. All chemicals were reagent grade or better.

Enzyme Preparations—Ram seminal vesicles (RSV) were obtained from Dr. L. J. Marnett, Vanderbilt University, and stored at −70 °C before use. RSV microsomes were prepared by essentially the method of Marnett and Wilcox (9) except that the microsomes were washed three times to reduce the amount of available endogenous cosubstrate for the peroxidase. For some experiments the microsomal protein was solubilized with 1% Tween 20. Protein concentrations were deter-

The abbreviations used are: PG, prostaglandin; d,-arachidonic acid, [5,6,8,9,11,12,14,15-3H]arachidonic acid; d-PGE₂, [5,6,8,11,12,14,15-3H]PGE₂; d-PGF₂α, [3,3,4,4-3H]PGF₂α; AP⁺, aminopyrine cation radical; RSV, ram seminal vesicles; 15-HP-PGE₂, 15-hydroperoxy-PGE₂; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; HPLC, high pressure liquid chromatography; GC, gas chromatography; MS, mass spectrometry.
Coupling of Cyclooxygenase with Peroxidase

Results

Metabolism of Arachidonic Acid by Prostaglandin H Synthase

Arachidonic acid is oxidized by the cyclooxygenase activity of prostaglandin H synthase to the unstable endoperoxide prostaglandin G2. PGG2 is reduced to prostaglandin H2 by the peroxidase activity of prostaglandin H synthase, and PGH2 then decomposes intramolecularly to prostaglandin E2. When the peroxidase activity is limited, the PGG2, which is not reduced to PGH2, is decomposed to the hydroperoxide 15-hydroperoxy-PGE2.

Scheme 1. Metabolism of arachidonic acid by prostaglandin H synthase. Arachidonic acid is oxidized by the cyclooxygenase activity of prostaglandin H synthase to the unstable endoperoxide prostaglandin G2. PGG2 is reduced to prostaglandin H2 by the peroxidase activity of prostaglandin H synthase, and PGH2 then decomposes intramolecularly to prostaglandin E2. When the peroxidase activity is limited, the PGG2, which is not reduced to prostaglandin H synthase, is decomposed to the hydroperoxide 15-hydroperoxy-PGE2.
previous study on arachidonic acid oxidation to PGG, and PGH2 by ram seminal vesicle microsomes (14), which demonstrated that PGE2 and 15-HP-PGE2 are the major decomposition products of these compounds as determined by GC-MS. As shown in Table I, the addition of the reducing cosubstrate aminopyrine increased the oxidation of arachidonic acid by prostaglandin H synthase. For example, approximately 44 µM PGG2 was formed in the presence of 50 µM aminopyrine compared with 24 µM formed in the absence of a reducing cosubstrate. The formation of the AP* appeared to be in agreement with the ratio of 15-HP-PGE2 to PGE2 as determined by HPLC analysis. However, at 500 µM aminopyrine the formation of the AP+ underestimated the reduction of PGG2. At this aminopyrine concentration, higher concentrations of the AP+ are achieved, which decays at a faster rate than observed at lower concentrations (13). The rapid speed of the decay results in an underestimation of the AP+ concentration.

Additional experiments were conducted with purified prostaglandin H synthase using varying concentrations of reducing cosubstrate. These results differed significantly from those obtained with microsomal prostaglandin H synthase. Regardless of the amount of phenol (0–500 µM) only PGE2 was observed in these incubations as measured by HPLC analysis (data not shown). Maximum enzymatic activity was observed at 500 µM phenol, with approximately 10 µM PGG2 being formed at this cosubstrate concentration (Table I). Cyclooxygenase activity was reduced significantly at lower phenol concentrations (data not shown). This is in agreement with previously published reports demonstrating the high sensitivity of purified prostaglandin H synthase to peroxide initiated inactivation in the absence of a reducing cosubstrate (20).

Effect of Exogenous PGG2 on Prostaglandin H Synthase—The effect of exogenous PGG2 on the cyclooxygenase and peroxidase activities of prostaglandin H synthase was examined using RSV microsomes as the source of the enzyme. Previous data (20) indicated that exogenous PGG2 and other peroxides could inactivate prostaglandin H synthase. This inhibition could interfere in competition experiments between endogenous and exogenous PGG2. The addition of PGG2 60 s before the addition of arachidonic acid caused a significant inhibition of prostaglandin H synthase activity as measured by oxygen incorporation with RSV microsomes as seen in Fig. 2. A 5 µM concentration of PGG2 produced a 50% inhibition, which is in agreement with published data (20). However, the simultaneous addition of PGG2 and arachidonic acid from a single solution did not inhibit the cyclooxygenase activity even at PGG2 concentrations as high as 100 µM (Fig. 2). Thus, under these conditions the simultaneous addition of exogenous PGG2 does not contribute to the inhibition of cyclooxyg

### Table I

| Incubation conditions | PGG2 | PGE2/PGG2 |
|-----------------------|------|-----------|
|                       | µM   |           |
| RSV + AA              | 24 ± 3 | 0.5       |
| RSV + AA + AP (50 µM) | 44 ± 3 | 0.3–0.6   |
| RSV + AA + AP (500 µM)| 78 ± 3 | >0.9      |
| RSV + AA + phenol (50 µM)| 19 ± 1 | 0.5       |
| PHS + AA + phenol (500 µM)| 8.3 ± 2.2 | >0.95   |

* Calculated from the total oxygen incorporated in 100 µM arachidonic acid.
* Based upon HPLC analysis of the metabolites obtained from incubations with 1H-arachidonic acid. AA, arachidonic acid.
Coupling of Cyclooxygenase with Peroxidase

Fig. 2. The effect of exogenous PGG₂ on cyclooxygenase activity in RSV microsomes. The cyclooxygenase activity was measured by determining the incorporation of molecular oxygen into arachidonic acid (100 μM) with a Clark electrode with 0.15 mg/ml RSV in the presence of 50 μM aminopyrine. Panel A, microsomes were preincubated with PGG₂ (0–50 μM) for 1 min before the addition of arachidonic acid. Panel B, arachidonic acid and PGG₂ (0–50 μM) added from a single stock solution.

genase which occurs during arachidonic acid oxidation by prostaglandin H synthase.

To investigate the effect of exogenous PGG₂ on the peroxidase we measured the formation of the AP⁺⁺ by RSV microsomes. Reactions were initiated by the addition of either arachidonic acid, PGG₂ or a mixture of arachidonic acid and PGG₂ under conditions described above except that 500 μM aminopyrine was included. As shown in Fig. 3, the addition of PGG₂ to solubilized RSV microsomes produced a very rapid appearance of the AP⁺⁺ which then decayed as described previously (13). The peak absorbance value in the curve was used to estimate the total formation of AP⁺⁺ and thus the reduction of PGG₂ to PGG₁ based on a stoichiometry of 1 mol of peroxide/2 mol of AP⁺⁺ (13). At a PGG₂ concentration of 50 μM approximately 65 μM AP⁺⁺ was formed whereas at 100 μM arachidonic acid approximately 96 μM AP⁺⁺ was observed. The data presented demonstrated that the simultaneous addition of arachidonic acid and PGG₂ did not alter the AP⁺⁺ compared with arachidonic acid alone, consistent with the data in Fig. 2. Table II shows the quantitative comparison between the formation of the AP⁺⁺ at the various incubation conditions. The simultaneous addition of 100 μM arachidonic acid and PGG₂ at concentrations as high as 60 μM PGG₂ did not alter the level of AP⁺⁺ formation. This result may be explained by the fact that the enzyme appeared to be operating at near capacity for reduction of PGG₂ as observed in additional experiments (data not shown). However, the addition of PGG₂ did not alter the reduction of the PGG₂ derived from labeled arachidonic acid. As seen in Fig. 3 insets, HPLC analysis indicated essentially the same quantity of PGG₁.

These results suggest that the AP⁺⁺ formation catalyzed by the peroxidase was supported by PGG₂ derived from arachidonic acid rather than exogenous PGG₂.

Analysis of Reduction Products of d₆- and d₇-PGG₂—After isolation by HPLC, d₆- and d₇-PGE₂ formed from the decomposition of d₆- and d₇-PGH₂ were analyzed via negative chemical ionization GC-MS as described above and used as an index for estimating PGG₂ reduction to PGH₂. Two peaks at m/z 524 corresponding to the methoxime derivative stereoisomers of d₆-PGE₂ were detected at about 19 min 4 s and 19 min 54 s (Fig. 4, top). Methoxime isomers of d₇-PGE₂ were detected at m/z 531 as two peaks eluting slightly earlier than those detected at m/z 524 (Fig. 4, middle). The internal standard, d₆-PGF₃, was detected as a single peak eluting at 18 min 40 s at m/z 573 (Fig. 4, bottom).

Reduction of Endogenous and Exogenous PGG₂—The isotopic composition of PGE₂ was used to estimate the reduction exogenous and endogenous PGG₂. We initially used purified
The formation of the aminopyrine cation free radical (AP⁺) was measured spectrophotometrically at 565 nm as described previously (12, 13). The quantity of AP⁺ formed was calculated from the extinction coefficient and the peak in the absorbance curve as seen in Fig. 3. Reaction mixtures contained 0.15 mg/ml RSV microsomes and 500 μM aminopyrine in 0.1 M phosphate buffer, pH 7.8. Reactions were initiated by the addition of either arachidonic acid, PGG₂, or ethanolic solution of arachidonic acid and PGG₂ at the concentrations indicated.

| Arachidonic acid | PGG₂ | AP⁺ |
|------------------|------|-----|
| μM               | μM   | μM  |
| 0                | 50   | 65.0* |
| 100              | 0    | 95.6 ± 5.3 |
| 100              | 10   | 100.3 ± 2.1 |
| 100              | 20   | 98.4 ± 1.9 |
| 100              | 30   | 101.3 ± 1.3 |
| 100              | 45   | 102.6 ± 1.9 |
| 100              | 60   | 103.7 ± 2.8 |

* Mean of two determinations. Other values are mean ± S.D.; n = 3 separate incubations.

The formation of the aminopyrine cation free radical (AP⁺) was increased by approximately 50% as can be seen on comparing the middle panel, selected ion monitoring at m/z 524, with the top panel, selected ion monitoring at m/z 521. Characteristic fragment ion of deuterated PGE₂ observed as two peaks eluting about 19:00 and 19:50 min. The two peaks observed for the non- and heptadeuterated PGE₂ represent syn- and antiisomers of the methoxime derivatives. Bottom panel, selected ion monitoring at m/z 573. Characteristic fragment ion of tetradeuterated PGE₂, eluted as a single peak at 18 min 40 s.

Prostaglandin H synthase to determine if endogenous and exogenous PGG₂ competed for reduction by the peroxidase. A cosubstrate such as phenol was required for the purified enzyme to oxidize arachidonic acid to the endoperoxides (20). Under these conditions, the percentage of PGE₂ as d⁵-PGE₂ decreased proportionately to the concentration of d⁵-PGG₂ added as shown in Fig. 5, panel A. At a d⁵-PGG₂ concentration of 10 μM the percentage was decreased by approximately 50%. Under these conditions approximately 10 μM d⁵-PGG₂ was formed from the d⁵- arachidonic acid as estimated from the oxygen incorporation (Table I). Further inspection of the data indicated that the experimental curve paralleled the theoretical dilution curve calculated from the amount of PGG₂ formed from arachidonic acid and the amount of exogenous PGG₂ added to the incubation. This theoretical dilution curve would be expected if both the exogenous and endogenous PGG₂ have equal access for the peroxidase for reduction, a result that was obtained in the experiment shown in panel A. With purified prostaglandin H synthase and 500 μM phenol, all the endogenous and exogenous PGG₂ was reduced to PGH₂. Hence, simple dilution of the isotope occurred in this experiment. Since all the peroxide was reduced, these experimental conditions do not permit us to address accurately whether or not endogenous PGG₂ is the preferred substrate for the peroxidase, but they do provide evidence for the validity of the techniques and experimental approach.

With the purified prostaglandin H synthase we attempted to establish conditions in which competition between the endogenously generated PGG₂ and exogenous PGG₂ could be measured. These attempts were unsuccessful with the purified enzyme but were achieved with ram seminal vesicle microsomes that were washed to reduce any contaminating endogenous cosubstrate for the peroxidase, as described above. Fig. 5, panels B, C, and D, are the results obtained from two experiments with different batches of microsomes and different cosubstrates, phenol and aminopyrine. The results obtained from incubations of RSV microsomes with phenol (50 μM) in the presence of varying concentrations of PGG₂ are shown in panel B. In this experiment approximately 18 μM endogenous PGG₂ was formed and approximately 50% of the peroxide was reduced to PGH₂ as determined by HPLC analysis using radiolabeled arachidonic acid as a substrate. The percentage of d⁵-PGE₂ of the total PGE₂ decreased to only 93% as increasing concentrations of exogenous PGG₂ were added. For comparison, the theoretical curve based upon equal competition between the endogenous and exogenous PGG₂ is also shown in this panel. Clearly there is a significant difference between the experimental and theoretical curves. The data from this experiment indicate that little or no competition occurred between endogenous and exogenous PGG₂. Fig. 5, panels C and D, shows the results of additional experiments with a different batch of microsomes with 50 or 500 μM aminopyrine as the cosubstrate. Under these conditions the total oxidation of arachidonic acid and the ratio of endogenous PGE₂ to endogenous 15-HP-PGE₂, as measured by HPLC analysis of the [3H]arachidonic acid incubations, was not altered by the simultaneous addition of PGG₂. Furthermore, the formation of AP⁺, which serves as a measure of the peroxidase activity of prostaglandin H synthase, was not altered by the addition of exogenous PGG₂ as compared with incubations with arachidonic acid alone (Table II). For both 50 and 500 μM aminopyrine the peroxidase was active. At 50 μM aminopyrine, approximately 44 μM d⁵-PGG₂ was generated in the incubation of 100 μM d⁵-arachidonic acid with RSV microsomes. With no exogenous d⁵-PGG₂ added, all of the PGE₂ formed was heptadeuterated as expected. As the concentrations of exogenous d⁵-PGG₂ were increased to 60 μM, a slight dilution of the d⁵-PGE₂ (to about 70%) was observed (Fig. 5, panel C). Also shown is the theoretical curve predicted for equal competition between the endogenous and exogenous PGG₂. These data demonstrate clearly that less of the exogenous PGG₂ was reduced than expected under competitive conditions. At 500 μM aminopyrine, a concentration that supported the formation of 80 μM d⁵-PGG₂ from 100 μM d⁵-arachidonic acid, the percentage of d⁵-PGE₂ decreased to only 85% as the concentration of exogenously added d⁵-PGG₂ increased to 60 μM (Fig. 5D). A dilution of about 52% was expected for 80 μM endogenous and 60 μM exogenous PGG₂ (Fig. 5D, open curves), again indicating less reduction of exogenous PGG₂ than would be expected if simple dilution occurred. It must be noted that as the data are presented the magnitude of the difference between the experimental and theoretical values was reduced as a larger quantity of endogenous PGG₂ was formed. Thus, when larger amounts of endogenous PGG₂ are formed the slope of theoretical dilution curves decreases as can be seen on comparing the panel A with D in Fig. 5.
Prostaglandin H synthase has two enzymatic activities present within a single homodimer: the cyclooxygenase and a peroxidase. The cyclooxygenase catalyzes the oxidation of arachidonic acid to a peroxide, PGG₂, and the peroxidase catalyzes the two-electron reduction of the peroxide PGG₂ to the corresponding alcohol PGHz (Scheme 1). The peroxidase activity of prostaglandin H synthase has the ability to reduce a wide variety of exogenous peroxides at the expense of reducing cosubstrates (1-8). In this paper, we attempted to answer the following question: Is the product of the first enzymatic activity the preferred substrate for the second enzymatic activity?

The data presented here indicate for the microsomal enzyme that endogenously formed PGG₂ is preferentially reduced by the peroxidase compared with exogenously added PGG₂, but some competition occurred between the endogenous and exogenous PGG₂. This conclusion is supported by several observations. First, incubations of prostaglandin H synthase with low micromolar concentrations of peroxides such as PGG₂ inactivated the enzyme (20). However, the simultaneous addition of PGG₂ at concentrations up to 100 μM with arachidonic acid did not enhance the inhibition of the cyclooxygenase activity which occurs during arachidonic acid oxidation. Since the peroxidase turnover has been linked to inactivation of the cyclooxygenase, this finding indicates that the exogenous PGG₂ was not accessible to or reduced by the peroxidase in the presence of arachidonic acid.

Second, the addition of PGG₂ did not alter the formation or reduction of endogenous PGG₂ as measured by O₂ consumption (Fig. 2) or by HPLC analysis of ³H-metabolites formed from labeled arachidonic acid. Moreover, measurement of the amount of peroxide reduced by the peroxidase, as estimated by the quantitative formation of the AP⁻⁺, was similar for reactions initiated by arachidonic acid alone or by arachidonic acid and PGG₂ simultaneously (Table II). These data indicate that the endogenous PGG₂ produced by the cyclooxygenase from arachidonic acid was supporting the oxidation of the aminopyrine by the peroxidase.

Finally, direct measurement of the reduction of PGG₂ was accessed by measuring the isotopic composition of the PGH₂ decomposition product, PGE₂, by gas chromatography-mass spectrometry. These results indicate that endogenous PGG₂ was the preferred substrate for the peroxidase, but some competition between the endogenous and exogenous PGG₂ occurred. This conclusion was based on the results of two experiments with different preparations of ram seminal vesicle microsomes and using either phenol or aminopyrine as the cosubstrate for the peroxidase. Some variability in the extent to which the exogenous PGG₂ was able to compete with endogenous PGG₂ was observed in these experiments. In one experiment (Fig. 5, panel B), the exogenous PGG₂ was essentially not reduced by the peroxidase in the presence of endogenous PGG₂. In the second experiment (Fig. 5, panels C and D) with different amounts of the peroxidase cosubstrate present in the incubation some reduction of exogenous PGG₂ occurred. Clearly in both experiments the experimental results were significantly different from the calculated theoretical values based on the assumption that if the endogenous PGG₂ dissociated from the enzyme it would freely mix with the exogenous PGG₂ and then be reduced by the peroxidase to PGH₂. The preference of the peroxidase for endogenous

**FIG. 5. Effects of exogenous nondeuterated PGG₂ on production of deuterated PGE₂.** Octadeuterated arachidonic acid (100 μM) was incubated with ram seminal vesicle microsomes or purified prostaglandin H synthase in the presence of aminopyrine or phenol and increasing concentrations of nondeuterated PGG₂ (0-100 μM). The ratio of deuterated to nondeuterated PGE₂ formed was measured via HPLC and GC-MS. Points represent the averages of two determinations or mean ± S.D., n = 3. The dashed lines and the open circles represent the theoretical percentage was calculated using the formula: theoretical % d-PGE₂ = ([d-PGG₂ formed]/([d-PGG₂ added] + [d-PGG₂ formed])) × 100. The solid line and the solid circles are the actual experimental results. Panel A, results obtained from the use of purified prostaglandin H synthase in the presence of 500 μM phenol. Panel B, ram seminal vesicle microsomes and 50 μM aminopyrine. Panel C, solubilized ram seminal vesicle microsomes and 50 μM aminopyrine. Panel D, solubilized ram seminal vesicle microsomes and 500 μM aminopyrine.

**DISCUSSION**

Prostaglandin H synthesis has two enzymatic activities present within a single homodimer: the cyclooxygenase and a peroxidase. The cyclooxygenase catalyzes the oxidation of arachidonic acid to a peroxide, PGG₂, and the peroxidase catalyzes the two-electron reduction of the peroxide PGG₂ to the corresponding alcohol PGHz (Scheme 1). The peroxidase activity of prostaglandin H synthase has the ability to reduce a wide variety of exogenous peroxides at the expense of reducing cosubstrates (1-8). In this paper, we attempted to answer the following question: Is the product of the first enzymatic activity the preferred substrate for the second enzymatic activity?

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Finally, direct measurement of the reduction of PGG₂ was accessed by measuring the isotopic composition of the PGH₂ decomposition product, PGE₂, by gas chromatography-mass spectrometry. These results indicate that endogenous PGG₂ was the preferred substrate for the peroxidase, but some competition between the endogenous and exogenous PGG₂ occurred. This conclusion was based on the results of two experiments with different preparations of ram seminal vesicle microsomes and using either phenol or aminopyrine as the cosubstrate for the peroxidase. Some variability in the extent to which the exogenous PGG₂ was able to compete with endogenous PGG₂ was observed in these experiments. In one experiment (Fig. 5, panel B), the exogenous PGG₂ was essentially not reduced by the peroxidase in the presence of endogenous PGG₂. In the second experiment (Fig. 5, panels C and D) with different amounts of the peroxidase cosubstrate present in the incubation some reduction of exogenous PGG₂ occurred. Clearly in both experiments the experimental results were significantly different from the calculated theoretical values based on the assumption that if the endogenous PGG₂ dissociated from the enzyme it would freely mix with the exogenous PGG₂ and then be reduced by the peroxidase to PGH₂. The preference of the peroxidase for endogenous
PGG₃ is strengthened further if one considers the fact that the peroxidase was exposed essentially instantly to the full concentration of exogenous PGG₂ whereas in contrast the endogenous PGG₂ was formed over a period of time (see Fig. 2) as measured by oxygen consumption.

Precedent for this behavior of an enzyme with dual catalytic activities exists in the literature. The 5-lipoxygenase that leads to the formation of leukotrienes possesses within a single protein two enzymatic activities (21). The lipoxygenase oxidizes arachidonic acid to a peroxide, 5-HPETE. The leukotriene A₄ synthase present in the same protein then converts the 5-HPETE to leukotriene A₄. Studies by Wiseman et al. (22) and by Puustinen et al. (23) indicate that the endogenously generated 5-HPETE is the preferred substrate for the second enzymatic activity, leukotriene A₄ synthase. In particular Puustinen et al. (23) measured the isotopic composition of the leukotriene A₄ hydrolysis products after incubation of the 5-lipoxygenase with octodeuterated arachidonic acid and exogenous 5-HPETE. They reported that under the conditions of these experiments the 5-lipoxygenase oxidized deuterated arachidonic acid to 20-30% 5-HPETE. In the presence of 80 μM exogenous 5-HPETE only 20% of the leukotriene A₄ was formed from exogenous 5-HPETE. These data are comparable to the results shown in Fig. 5, panel B, in which prostaglandin H synthase oxidized deuterated arachidonic acid to 24 μM PGG₂, but only approximately 10% of the exogenous PGG₂ was reduced. Thus, using methods and techniques similar to those used in our study of prostaglandin H synthase, Puustinen and co-workers (23) obtained results analogous to those reported here; namely, that the endogenously generated peroxide is the preferred substrate for the second enzymatic activity of 5-lipoxygenase.

The relationship between the cyclooxygenase and peroxidase activities of prostaglandin H synthase has been the subject of intensive investigation, and conflicting data exists concerning the association of the enzymatic activities (8). Recently Karthein et al. (24, 25) detected by ESR techniques a tyrosyl radical formed during the oxidation of arachidonic acid by prostaglandin H synthase. The tyrosyl radical was also formed by the addition of peroxides including PGG₂ to the enzyme. They proposed that the tyrosyl radical initiates the oxidation of arachidonic acid by the enzyme and that the tyrosyl radical is formed by an intramolecular radical transfer from the porphyrin cation radical of compound I of the peroxidase. This proposal implies that the cyclooxygenase and peroxidase activities must be closely associated. Our findings that indicate that the PGG₂ formed by the cyclooxygenase is the preferred substrate for the peroxidase also suggest that these two activities are in close proximity on the enzyme. Our results are consistent with but are not evidence for the proposal of Karthein and co-workers.

Marshall and Kulmacz (26) proposed that the binding sites for the peroxidase and cyclooxygenase are different. Their conclusions were based on the observation that docosahexaenoic acid, a competitive inhibitor of arachidonic acid which binds to the cyclooxygenase, does not inhibit peroxide-initiated oxidation of the tetramethyl phenylenediamine, findings that we have recently confirmed.³ The proposal for the existence of two binding sites appears at first glance to be in conflict with our findings presented here. Our results give no indication of the number of binding sites on the enzyme but indicate that the two activities are closely associated since the product of one of the enzymatic activities is the preferred substrate for the second enzymatic activity. Our results suggest that the endogenous PGG₂ does not dissociate from the enzyme and mix freely with any exogenous PGG₂ present in the incubation mixture. However, the observation that endogenous PGG₂ is the preferred peroxidase substrate does not preclude the possibility that two distinct binding sites may exist.

Our results also indicate that significant differences exist between prostaglandin H synthase located in microsomal membranes and isolated purified enzyme used in this study. We attempted to establish conditions with the purified enzyme in which PGG₂ formed by the cyclooxygenase would not be reduced completely by the peroxidase to PGH₂. These attempts were unsuccessful, which prevented us from determining if the endogenous PGG₂ was the preferred substrate for the purified peroxidase. Apparently this preparation of purified ram prostaglandin H synthase is extremely sensitive to inactivation by peroxide, and thus any excess PGG₂ formed inactivates the cyclooxygenase. As a consequence, the cyclooxygenase activity cannot exceed the capacity of the peroxidase. Markey et al. (20) reported that the turnover rate of the peroxidase is 14 times the turnover rate of cyclooxygenase with this preparation of purified enzyme. The peroxidase activity of microsomal prostaglandin H synthase appears to be of the same order as the cyclooxygenase activity. Unlike this preparation of purified prostaglandin H synthase, excess PGG₂ can be generated with the microsomal enzyme by limiting the availability of cosubstrate. Our results suggest that the relationship between the two activities of prostaglandin H synthase could be altered significantly by purification and/or that microsomal membranes may play an unrecognized role in the function of the enzyme.

In conclusion, our results with the microsomal preparation of prostaglandin H synthase indicate that some competition for peroxidase reduction can occur between endogenous and exogenous PGG₂. However, endogenous PGG₂ was the preferred substrate for the peroxidase. This supports the conclusion that these two enzymatic activities within a single protein are closely associated.

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