The Heme-binding Properties of Prostaglandin Synthetase from Sheep Vesicular Gland*

Gerald J. Roth‡, Edward T. Machuga, and Philipp Strittmatter

From the Division of Hematology, Department of Medicine and the Department of Biochemistry, University of Connecticut School of Medicine, Farmington, Connecticut 06032

Purified, apoprostaglandin synthetase was prepared from sheep vesicular gland and studied in terms of its heme-binding properties. The enzyme binds a single heme group per enzyme monomer, \( M_r = 70,000 \). When reconstituted with heme, the enzyme has an absorption maximum at 412 nm and an absorption coefficient, \( \varepsilon_{412} = 120 \text{ M}^{-1} \text{cm}^{-1} \). The binding of heme to the apoenzyme was accompanied by a proportional increase in enzyme activity up to the point of heme-binding saturation. This reconstituted holoenzyme forms prostaglandin \( H_2 \) from arachidonate. We conclude that prostaglandin synthetase possesses the heme-binding properties of a "typical" heme protein and that a single heme group mediates both the oxygenase and the peroxidase activities of the enzyme.

Prostaglandin synthetase (cyclooxygenase, prostaglandin endoperoxide synthetase) is the first enzyme of prostaglandin synthesis, catalyzing the oxygenation of arachidonic acid to \( \text{PGG}_2 \) and the reduction of \( \text{PGG}_2 \) to \( \text{PGH}_2 \). Both the former oxygenase activity and the latter peroxidase activity are contained in a single polypeptide chain (5, 6). Aspirin (acetylsalicylic acid) inhibits oxygenase activity by acetylatyng a single internal serine residue (5-7). The enzyme has also been characterized as a membrane-bound protein which requires heme for activity and undergoes self-inactivation during catalysis (8-11). In 1970, Yoshimoto and co-workers (8) initially demonstrated that the enzyme requires heme for activity. Additional work has confirmed and extended this observation (9-11). However, the enzyme has been reported to possess unusual heme-binding properties; namely, a stoichiometry of 2 hemes/mol of protein, a relatively low absorption maximum in the \( \gamma \) region (\( \varepsilon_{412} = 61 \text{ M}^{-1} \text{cm}^{-1} \)), and a dissociable heme group (11). In addition, the heme requirement of the apoenzyme can be supplied by a second heme protein such as hemoglobin (8, 10).

Heme dissociation was observed during earlier purifications of the enzyme (3, 4, 9), possibly due to a concomitant denaturation of the apoenzyme in procedures using a variety of detergents. Therefore, the heme-binding characteristics noted above could reflect a mixture of apoprotein species with variable heme-binding properties. The mixture may include both active and inactive heme proteins in which heme association is either tight or readily reversible. To avoid this problem, we included a reversible inhibitor, flufenamic acid, to stabilize enzyme activity and avoid heme loss during purification (9). Heme was then removed from the purified holoprostaglandin synthetase by incubation with the hydrophilic, heme-binding peptide segment of cytochrome \( b_5 \) (12) under conditions that yield apo-PG synthetase which will bind heme to produce an active enzyme with both spectral and enzymatic properties of the native enzyme. This purified apoenzyme binds heme with a 1:1 stoichiometry and shows a typical absorption maximum in the \( \gamma \) region (absorption coefficient, \( \varepsilon_{412} = 120 \text{ M}^{-1} \text{cm}^{-1} \)). The single enzyme-bound heme group provides for both oxygenase and peroxidase activity in the holoenzyme.

MATERIALS AND METHODS

The initial purification procedure for prostaglandin synthetase (7) was modified by the addition of flufenamic acid to stabilize the enzyme. All steps were performed at 4 °C. Frozen seminal vesicles (Wellington Abattoir, Wellington, New Zealand) were homogenized in 0.1 m potassium phosphate, pH 8.0, 0.5 m KCl, 1 mM FLF, 1 mM NADH, 10 mM EDTA, filtered through gauze, and centrifuged (12,000 \( \times g \), 10 min). The supernatant was applied to a DEAE-cellulose column equilibrated and eluted with the same buffer used for dialysis except for the Tween concentration for the Tween concentration of 0.1%. The eluate was dialyzed for 14 h against 0.02 m potassium phosphate, pH 7.4, 1 mM FLF, 0.1 mM NADH, 0.1 mM EDTA, and 2% Tween 20, incubated for 30 min, and centrifuged (100,000 \( \times g \), 40 min). The supernatant was applied to a DEAE-cellulose column equilibrated with the same buffer used for dialysis. After sample application, the column was washed with 2 column volumes of the equilibration buffer. Enzyme was eluted in an exclusion volume with 0.5 m Tris-acetate, pH 8.1, containing the same additional steps noted above. The first enzyme fractions totaling 2 ml in volume were pooled and incubated with a 4-fold molar excess of apocytochrome \( b_5 \), hydrophilic peptide for 2 h at 30 °C. The enzyme lacked all oxygenase activity in the absence of heme following this incubation. Deoxycholate (3% final concentration) and Triton X-100 (2% final concentration) were added and 2 ml of the mixture was applied to a gel filtration column (Sephadex G-100), equilibrated, and eluted with 0.02 m Tris-acetate, pH 8.1, 0.2% deoxycholate, 80 mM FLF, 100 mM NADH, and 10 mM EDTA. The first enzyme-containing fractions totaling 2 ml in volume were pooled and applied to a second gel filtration column (Sephadex G-25), equilibrated, and eluted with 0.02 m Tris-acetate, pH 8.1, 0.2% deoxycholate. The pooled enzyme fractions appearing in the void volume of this column constituted the final preparation of purified active apo-PG synthetase.

Cytochrome \( b_5 \) was purified from steer liver and assayed as described (12). The hydrophilic peptide was obtained by trypsic digestion of the intact protein (13). Heme was removed from the heme peptide by acid/acetone treatment followed by gel filtration on Seph-
RESULTS AND DISCUSSION

The enzyme was purified about 20-fold from microsomes by the procedure outlined under "Materials and Methods." The isoelectric focusing step used in earlier purifications (3–5) was omitted in order to shorten the time required for purification. Enzyme was incubated at 30 °C in air-equilibrated 0.1 M potassium phosphate, pH 8.0, containing 0.67 mM phenol. Arachidonate (100 μM) was added and the rate of O2 uptake was determined, expressed as the initial linear rate of substrate consumed/min, assuming that the enzyme preparation contained 4.3 pmol of O2. A gel of a final preparation of apo-PG synthetase is shown to the right. The middle gel was run with a sample of the freshly prepared enzyme assayed by oxygen electrode. Enzyme was incubated with 100 μM NADH, 80 μM PLF, in 0.02 M Tris/acetate, pH 8.1, followed by the addition of 100 μM arachidonate. The oxidation of NADH was measured by an absorbance change at 340 nm and expressed as the initial linear rate of NADH oxidized/min at 30 °C.

Enzyme activity was assayed by several means: PGH2 formation, arachidonic acid consumption by O2 electrode, and NADH oxidation. PGH2 formation was assayed by a modification of the method of Ogino and co-workers (14). Enzyme, with or without added heme, in 0.1 ml of 0.1 M potassium phosphate, pH 8.0, containing 0.67 mM phenol, was incubated with 100 μM [14C]arachidonate, 5 Ci/mol, for 1 min at 24 °C. Cold diethyl ether/methanol/0.4 M citric acid (30:4:1, v/v) (0.3 ml) was added, followed by 0.5 g of Na2SO4. An aliquot of the dried organic phase was spotted on a Silica Gel G thin layer chromatography plate and products were separated by ascending TLC in hexane/ethyl acetate/acetic acid (100:100:0.5, v/v) at room temperature. PGH2 was measured as radioactivity with the appropriate migration, Rf = 0.51. In a separate experiment, 0.1 ml of purified apoenzyme was reconstituted with a saturating amount of heme and incubated for 1 min at 30 °C with 2 μmol of [14C]arachidonate.

Products were extracted and isolated as described by Gorman and co-workers (15). PGH2 was identified as described (6) by TLC analysis, Rf = 0.51 as above, reduction to PGF2α, by triphenylphosphine, isomerization to PGF2β by sheep vesicular gland microsomes in the presence of 1 mM glutathione, isomerization to PGF2α and PGD2 in buffer, and ability to aggregate aspirin-treated platelets. For assays utilizing oxygen consumption, enzyme was incubated at 30 °C in air-equilibrated 0.1 M potassium phosphate, pH 8.0, containing 0.67 mM phenol. Arachidonate (100 μM) was added and the rate of O2 uptake was determined, expressed as the initial linear rate of substrate consumed/min, assuming 2 mol of O2 are consumed per mol of substrate (1, 4).

We also found that substrate addition to PG synthetase in the presence of NADH and FLF led to NADH oxidation at a rate comparable to substrate consumption as measured by O2 electrode. Enzyme was incubated with 100 μM NADH, 80 μM FLF, in 0.02 M Tris/acetate, pH 8.1, followed by the addition of 100 μM arachidonate. The oxidation of NADH was measured by an absorbance change at 340 nm and expressed as the initial linear rate of NADH oxidized/min at 30 °C.

Heme Binding to Sheep Prostaglandin Synthetase

RESULTS AND DISCUSSION

The enzyme was purified about 20-fold from microsomes by the procedure outlined under "Materials and Methods." The isoelectric focusing step used in earlier purifications (3–5) was omitted in order to shorten the time required for purification. Using the oxygen electrode assay for activity and the Lowry protein determination (16), the final preparations had a specific activity of about 35 μmol of substrate consumed per min per mg of protein, corresponding to a turnover number of approximately 2500, as described below.

Sodium dodecyl sulfate polyacrylamide gels of typical enzyme preparations are shown in Fig. 1. The gel to the left (gel A) was run with a sample of enzyme purified through ion exchange chromatography on DEAE-cellulose. The major band of M, = 70,000 is PG synthetase. The middle gel was run on a sample of the same enzyme preparation used in gel A following addition of cytochrome b5, hydrophilic peptide, M, = 10,000. A gel of a final preparation of apo-PG synthetase is shown to the right (gel C). The apoenzyme used in the heme-binding studies was at least 90% pure by the criterion of gel electrophoretic analysis.

Heme binding of the apo-PG synthetase was initially followed spectrophotometrically (Fig. 2A). The concentration of the enzyme in these titrations was 5.5 μM as determined by absorbance at 280 nm (ε280 nm of approximately 130 mm−1 cm−1) and by Lowry protein determination. The initial spectrum obtained prior to the addition of heme showed broad, indistinct absorption at
Heme Binding to Sheep Prostaglandin Synthetase

**Fig. 2. Absorption spectra of PG synthetase.** A, absorption spectra of apo-PG synthetase reconstituted with heme. Aliquots of heme (0.062 nmol in 1 μl) were added successively to purified apo-PG synthetase (5.5 μM) at 5 °C in 0.5 ml of buffer (0.02 M Tris/acetate, pH 8.1, 0.2% deoxycholate). Absorption spectra were obtained with a Bausch and Lamb split-beam recording spectrometer, using protein-free buffer as reference. The first spectrum was obtained without added heme, and 7 heme aliquots were added thereafter. B, absorbance at 412 nm with addition of heme to apo-PG synthetase. The data are taken from A, plotting absorbance versus heme concentration. The enzyme concentration is 5.5 μM. The inflection point at 4.3 μM heme corresponds at an absorbance of 0.51, giving an absorption coefficient ε412 of 120 molar cm⁻¹ bound heme. Assuming the inflection point reflects saturation of heme binding, oxygen electrode assay of freshly prepared enzyme gave an activity of 39 μmol of substrate consumed/mg of heme-binding enzyme/min, corresponding to a turnover of 2800 mol of substrate consumed/mol of enzyme-bound heme/min.

**Fig. 3. Activity of PG synthetase with heme addition.** Heme (0 to 7 mol/mol of enzyme) was added to apoenzyme and incubated with 100 μM [³H]arachidonate for 1 min at 24 °C. Products were extracted and separated by TLC and PGG₂ was measured as radioactivity migrating with authentic PGG₂ as noted under "Materials and Methods." PGG₂ formed (mol/mol of enzyme-bound heme/min) is plotted versus heme added, expressed as a ratio of heme added/heme bound. "Heme bound" is the amount of heme required to saturate the binding site of the enzyme. It was determined by spectral analysis in Fig. 2, A and B (4.3 μM heme/5.5 μM enzyme).

expressed as a ratio with the denominator of "heme bound" referring to the amount of heme bound at saturation according to the spectral analysis (Fig. 2, A and B). The rate of PGG₂ formation reached a nearly maximal level at a heme added/heme bound ratio of 1. The slightly greater activity seen with additional heme may represent a kinetic problem in reaching complete saturation or the intrinsic heme-binding constant of the apoenzyme. These results indicate that the inflection point seen in Fig. 2B does correspond to virtual saturation of the heme-binding site of the enzyme. The main product formed by the enzyme proved to be PGG₂, identified as outlined under "Materials and Methods." PGG₁ was not formed in a significant amount. Therefore, the single heme group appears to mediate peroxidase activity, in addition to oxygenase activity, since PGG₂ is the product of the peroxidase function of the enzyme.

Two other enzyme assays were performed to test the catalytic activity of the reconstituted enzyme. In Fig. 4, both NADH oxidation and substrate consumption activity are plotted against the amount of heme added to the enzyme. Other investigators have found that pyridine nucleotides do not enhance oxygenase activity and are generally not oxidized during *in vitro* PG synthesis (1, 8). We confirmed this observation but found that NADH was oxidized in the presence of
80 μM FLF. This concentration of FLF was found to be optimal in these assays, whereas higher concentrations result in inhibition of synthetase activity as observed previously (18). As noted under "Materials and Methods," the rate of NADH oxidation under appropriate conditions is approximately equal to the rate of substrate consumption measured with an oxygen electrode. NADH oxidation appeared to relate to the peroxidase function of the enzyme since it occurred in the presence of hydroperoxide such as 15-OOH arachidonate. In Fig. 4, the amount of heme added is again expressed as a ratio related to heme bound at saturation according to the spectral studies. The lines in Fig. 4 are drawn as best visual fit to the data points. The result indicates that nearly maximal activity is reached with both assays in the presence of a saturating amount of heme. The result reinforces the conclusion that the inflection point in the spectral analysis (Fig. 2, A and B) does represent saturation of the heme-binding site of the enzyme. The maximal turnover of 2000 noted in Fig. 4 was observed using enzyme which had been stored at 0 or −70 °C, and the activity is about 70% of that seen with the freshly prepared enzyme. In the absence of FLF, the apoenzyme lost activity with time, even at 0 °C.

The absorption spectrum of the reconstituted enzyme was also determined in the presence of FLF. The conditions of the study were identical with those used previously (Fig. 2, A and B) except for the presence of 80 μM FLF. The spectra shown in Fig. 5A were developed by adding successive aliquots of heme to apoenzyme. The enzyme concentration was 4.7 μM. The low absorption peak at 350 nm is probably due to enzyme-bound FLF. The spectra are again marked by a prominent absorption peak at 412 nm. In Fig. 5B, the increase in absorbance at 412 nm (data taken from Fig. 5A) is plotted against the concentration of added heme. The inflection point occurred at 3.9 μM heme, consistent with 1:1 stoichiometry of heme binding to the enzyme. The absorbance at 412 nm with 3.9 μM heme was 0.59 corresponding to an absorption coefficient, ε_{412 nm}, of 145 mm⁻¹ cm⁻¹. Enzyme activity, as assayed by NADH oxidation and substrate consumption, reached maximal levels when a "saturating" amount of heme was added (3.9 μM heme/4.7 μM enzyme, the inflection point of Fig. 5B). The turnover of the enzyme preparation used in Fig. 5, A and B was about 3400 mol/mol of enzyme-bound heme/min, indicating that this enzyme preparation was composed almost entirely of active PG synthetase. The spectrum of reconstituted enzyme in FLF containing 1 mol of heme is not only virtually identical with purified enzyme in FLF prior to addition of the apocytochrome β, but also shows a similar turnover number. Thus, the binding of 1 mol of heme constitutes both the spectral and the catalytic characteristics of the holoenzyme. The increased absorption of the heme protein in FLF (ε_{412 nm} of 145 versus 120 mm⁻¹ cm⁻¹) is consistent with the stabilization of heme binding to the enzyme by FLF which we observed in a qualitative fashion in developing the purification procedure.

The observed absorption maximum at 412 nm differs from that found in an earlier study, 408 nm (11), in which the bound heme group could be dissociated by octaethylene glycol dodecyl ether and in which binding was termed hydrophobic (19). On the other hand, our results are consistent with those of O'Brien and Rahimtula (20), who describe an absorption trough at 412 nm in the spectrum of the microsomal enzyme, following substrate addition. The finding appears to relate to destruction of heme during arachidonate oxygenation (20).

Aspirin-mediated acetylation and inactivation of PG synthetase could interfere with heme binding to the enzyme and a series of heme binding experiments were performed with the aspirin-acetylated enzyme. The absorption spectrum showed a major absorption peak at 412 nm as in Fig. 2A and 5A, but the absorption coefficient was somewhat lower with the acetylated enzyme (ε_{412 nm} = 95 mm⁻¹ cm⁻¹). Although the result suggests that aspirin modification may alter heme binding, the initial homogenization of the starting material for the acetylated preparation was necessarily carried out in the absence of FLF. Therefore, the lower absorption coefficient may reflect some denaturation of the enzyme during purification rather than the acetylation itself.

The retention of both enzyme activity and heme binding during purification shows the usefulness of the stabilizing property of the FLF interaction with PG synthetase. It is not yet clear whether enzyme activity is retained simply by a

![Absorption spectra of PG synthetase in the presence of flufenamate.](image-url)

**Fig. 5.** Absorption spectra of PG synthetase in the presence of flufenamate. A, absorption spectra of apo-PG synthetase reconstituted with heme in the presence of flufenamate. Aliquots of heme (0.53 mM in 1 μl) were added successively to 4.7 μM purified apo-PG synthetase in 0.5 ml of buffer (0.02 M Tris acetate, pH 8.1, containing 0.2% deoxycholate and 80 μM flufenamate acid). Spectra were recorded as noted in Fig. 2 using protein-free buffer with 80 μM flufenamate as reference. The initial spectrum was obtained without added heme and 11 heme additions were made thereafter. B, absorbance at 412 nm with addition of heme to apo-PG synthetase in the presence of flufenamate. The data are taken from A, plotting the increase in absorbance at 412 nm versus heme concentration. The enzyme concentration is 4.7 μM. The inflection point at 3.90 μM heme corresponds to an absorbance of 0.59, giving an extinction coefficient; ε_{412 nm} = 145 mm⁻¹ bound heme cm⁻¹. Assuming the inflection point reflects saturation of heme binding, the NADH and arachidonate oxidation assays gave a turnover of 3400 (moles/mol of enzyme-bound heme/min) and showed nearly maximal activity at a ratio of heme added/heme bound of 1 (data not shown, similar data given in Fig. 4).
direct effect of FLF upon the heme binding constant or by a more subtle stabilization of a larger but fragile structural feature of the enzyme. Reconstitution of apoenzyme prepared under mild conditions clearly identified a single heme group at the active center as sufficient for both the oxygenase and peroxidase functions of the enzyme. This is consistent with a reaction sequence in PGH₂ formation from arachidonate at a single heme site in which PGG₂, the hydroperoxide intermediate, does not dissociate from the enzyme during a normal catalytic cycle but undergoes direct reduction to the hydroxyl-containing end product, PGH₂.

With stable preparations of the holosynthetase in buffers containing dilute deoxycholate, it will now be possible to effect reconstitution of the holoenzyme in phospholipid vesicles free of other microsomal proteins. This represents the methodological prerequisite for studies on the stabilizing effects of the normal phospholipid environment of the enzyme, more direct examination of the reaction sequence, and attempts to define more clearly the normal intracellular electron donors for PGH₂ synthesis.

REFERENCES
1. Sammelsson, B. (1972) Fed. Proc. 31, 1442-1450
2. Nugteren, D. H., and Hazelhof, E. (1973) Biochim. Biophys. Acta 326, 448-461
3. Miyamoto, T., Ogino, N., Yamamoto, S., and Hayaishi, O. (1976) J. Biol. Chem. 251, 2629-2636
4. Van der Ouderna, F. J., Buytenhek, M., Nugteren, D. H., and Van Dorp, D. A. (1977) Biochim. Biophys. Acta 487, 315-331
5. Roth, G. J., Sluk, C. J., and Ozols, J. (1980) J. Biol. Chem. 255, 1391-1394
6. Van der Oudera, F. J., Buytenhek, M., Nugteren, D. H., and Van Dorp, D. A. (1980) Eur. J. Biochem. 109, 1-8
7. Roth, G. J., and Sluk, C. J. (1978) J. Biol. Chem. 253, 3782-3784
8. Yoshimoto, A., It, H., and Tomita, K. (1970) J. Biochem. 68, 487-499
9. Hemler, M., Lands, W. E. M., and Smith, W. L. (1976) J. Biol. Chem. 251, 5575-5579
10. Ogino, N., Ohki, S., Yamamoto, S., and Hayaishi, O. (1978) J. Biol. Chem. 253, 5061-5068
11. Van der Oudera, F. J., Buytenhek, M., Slukkerveer, F. J., and Van Dorp, D. A. (1979) Biochim. Biophys. Acta 572, 29-42
12. Strittmatter, P., Fleming, P., Connors, M., and Corcoran, D. (1978) Methods Enzymol. 52, 97-101
13. Strittmatter, P., and Ozols, J. (1966) J. Biol. Chem. 241, 4787-4792
14. Ogino, N., Miyamoto, T., Yamamoto, S., and Hayaishi, O. (1977) J. Biol. Chem. 252, 890-895
15. Gorman, R. R., Sun, F. F., Miller, O. V., and Johnson, R. A. (1977) Prostaglandins 13, 1043-1053
16. Lowry, O., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
17. Weber, K., and Osborn, M. (1975) in The Proteins (Neurath, H., and Hill, R. L., eds) pp. 180-223, Academic Press, New York
18. Flower, R. J. (1974) Pharmacol. Rev. 26, 33-67
19. Van der Oudera, F. J., Buytenhek, M., and Van Dorp, D. A. (1980) Adv. Prostaglandin Thromboxane Res. 6, 139-144
20. O'Brien, P. J., and Rahimtula, A. D. (1980) Adv. Prostaglandin Thromboxane Res. 6, 145-151