The Aspirin and Heme-binding Sites of Ovine and Murine Prostaglandin Endoperoxide Synthases*

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Acetylation of Ser-530 of sheep prostaglandin endoperoxide (PGG/H) synthase by aspirin causes irreversible inactivation of the cyclooxygenase activity of the enzyme. To determine the catalytic function of the hydroxyl group of Ser-530, we used site-directed mutagenesis to replace Ser-530 with an alanine. Cos-1 cells transfected with expression vectors containing the native (Ser-530) or mutant ( Ala-530) cDNAs for sheep PGG/H synthase expressed comparable cyclooxygenase and hydroperoxidase activities. $K_m$ values for arachidonate (8 mM) and $I_D_{50}$ values for reversible inhibition by the cyclooxygenase inhibitors, flurbiprofen (5 mM), flufenamate (20 mM), and aspirin (20 mM), were also the same for both native and mutant PGG/H synthases; however, only the native enzyme was irreversibly inactivated by aspirin. Thus, the “active site” Ser-530 of PGG/H synthase is not essential for catalysis or substrate binding. Apparently, acetylation of native PGG/H synthase by aspirin introduces a bulky sidechain at position 530 which interferes with arachidonate binding. In related studies, a cDNA for mouse PGG/H synthase was cloned and sequenced. A sequence of 35 residues with Ser-530 at the midpoint was identical in the two proteins. Thus, Ser-530 does lie in a highly conserved region, probably involved in cyclooxygenase catalysis.

Sequence comparisons of mouse and sheep PGG/H synthase also provided information about the heme-binding site of the enzyme. The sheep HYPR sequence (residues 274–277), which had been proposed to form a portion of the distal heme-binding site, is not conserved in the mouse PGG/H synthase, suggesting that this region is not the distal heme-binding site. One sequence, TIWLREHNRV (residues 303–312 of the sheep enzyme), is very closely related to the sequence TLW(L)REHNRV, common to thyroid peroxidase and myeloperoxidase. The histidine in this latter sequence is the putative axial heme ligand of these peroxidases. We suggest that the histidine (His-309) of sheep PGG/H synthase sequence is the axial heme ligand of this enzyme.

Prostaglandin endoperoxide (PGG/H)$^1$ synthase (E.C. 1.14.99.1) catalyzes the conversion of arachidonate to prostaglandin H$_2$ (PGH$_2$), the immediate precursor of PGE$_2$, PGF$_2$, PGD$_2$, prostacyclin, and thromboxane A$_2$. PGG/H synthase has two different enzymatic activities (1–3): (a) a bis oxygenase (cyclooxygenase) which mediates the formation of PGG$_2$ from two molecules of oxygen and a molecule of arachidonate, and (b) a hydroperoxidase which catalyzes a net two electron reduction of the 15-hydroperoxyl group of PGG$_2$ yielding PGH$_2$. PGG/H synthase is a membrane-associated protein and can only be removed from membrane preparations with detergents (1, 4, 5). The subunit molecular weight of the sheep PGG/H synthase is about 65,000 as calculated from its primary amino acid sequence (6–8); in addition, the molecule contains an average of three asparagine-linked, mannose-containing oligosaccharides (1). The enzyme from sheep vesicular gland is the one which has been studied most extensively. It exists as a homodimer in detergent solution (1, 9, 10). Immunochemical analyses indicate that PGG/H synthase from various species are closely related (11). Finally, PGG/H synthase is a hemoprotein having a protoporphorin IX prosthetic group which is required for both cyclooxygenase and hydroperoxidase activities (12–16). Heme binding appears to involve bis-imidazole coordination (17).

PGG/H synthase is the pharmacological target site of non-steroidal antiinflammatory drugs (18, 19). Most of these agents are instantaneous, reversible, competitive inhibitors of arachidonate binding (19). Aspirin is a non-steroidal antiinflammatory drug that exhibits an additional, time-dependent secondary effect, which is to irreversibly inactivate PGG/H synthase (18–21). Aspirin causes acetylation of the side chain of Ser-530, the “active site” serine (6–8, 20), and there is a close correspondence between the rate of acetylation and the rate of cyclooxygenase inactivation (21). Interestingly, inhibition of cyclooxygenase activity by competitive inhibitors of arachidonate binding has no appreciable effect on hydroperoxidase activity (22, 23), indicating that the cyclooxygenase and hydroperoxidase active sites are distinct.

To study the involvement of Ser-530 in cyclooxygenase catalysis, we have used site-directed mutagenesis to replace this serine with an alanine. As a supplement to these studies, we isolated and sequenced a mouse cDNA for PGG/H synthase, in part so that we could compare the sequence of the mouse and sheep enzymes around the active site serine. Importantly, these sequence comparisons have also permitted us

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1 The abbreviations used are: PGG/H synthase, prostaglandin endoperoxide synthase; PG, prostaglandin; DMEM, Dulbecco’s modified Eagle medium; TMPD, N,N,N',N'–tetramethyl-p-phenylenediamine; kb, kilobase.
to identify a putative proximal heme-binding site in another region of the PGG/H synthase molecule.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco's modified Eagle medium (DMEM) and fetal calf serum were obtained from Gibco. Chloroquine, bovine hemoglobin, Tween 20, N,N,N',N'-tetramethylethylene diamine, acetyletrylic acid, hematin, and arachidonic acid were from Sigma. [α-32P]dCTP (800 Ci/mmol) and [α-32P]dATP (500 Ci/mmol) were purchased from Du Pont-New England Nuclear. [32P]labeled Protein A (66 μCi/μg) was from ICN: BA85 (0.45 μm) nitrocellulose was from Schleicher & Schuell; Moloney murine leukemia virus-reverse transcriptase was from Bethesda Research Laboratories; restriction endonucleases, exonuclease III, methvlases, T4 DNA ligase, T4 DNA polymerase, and Klenow exnuclease were from Boehringer Mannheim or New England Biolabs. The Muta-Gen kit was from Bio-Rad. Nucleotides, the Okayama and Berg expression vectors pL1 and pCD1, DEAE-dextran, and oligo(dt) Sepharose were purchased from Pharmacia LKB Biotechnology Inc. CsCl was from Var Lac Oil Chemical Company. Flufenamic acid was generously provided by the Upjohn Company. Fluorescin acid was from Aldrich Chemical Com-pany. Nucleotides used in the New England Biolabs' kits were purchased from the Lopata et al. (33). Each 100-mm dish (≈3 x 10^6 cells) was washed three times with DMEM and incubated for 1 h at 37°C under a water-saturated 7% CO2 atmosphere with 750 μg/ml DEAB-dextran and 30 μg of either PCD-PGHS or PCD-PGHS in 3 ml of DMEM. Chloroquine (0.2 μg/ml) in 7 ml of DMEM containing 10% fetal calf serum was added, and the samples were incubated for 5 h at 37°C. DNAse digestion was performed for 1 h at 37°C in a water-saturated 7% CO2 atmosphere. The cells were removed from the culture dishes with a rubber policeman and centrifuged at 300 × g for 10 min. Cells (~3 × 10^6 from 10 dishes) were resuspended in 4.5 ml of 0.1 M Tris-Cl, pH 7.4, 250 μg of hematin, and 250 μg of microsomal protein. Reactions were initiated by adding enzyme preparations. One unit of cyclooxygenase activity is defined as that which will catalyze the oxygenation of 1 nmol of arachidonate to 50 μM 12-HETE (34). The extent of conversion was determined at 37°C using a Perkin-Elmer model 552A Double Beam UV/VIS spectrophotometer, measuring the oxidation of tetramethylphenylenediamine (TMPD; (35)) at 605 nm. Each hydroperoxidase assay mixture contained 290 μM O2, 1 mM hematin, 1 μM TMPD, and 25 μg of microsomal protein in a final volume of 0.25 ml of 0.1 M Tris-Cl, pH 8.0. 100 μM arachidonate, and 250 μg of microsomal protein. Reactions were initiated by adding enzyme preparations. One unit of cyclooxygenase activity is defined as that which will catalyze the oxygenation of 1 nmol of arachidonate to 50 μM 12-HETE (34). The extent of conversion was determined at 37°C using a Perkin-Elmer model 552A Double Beam UV/VIS spectrophotometer, measuring the oxidation of tetramethylphenylenediamine (TMPD; (35)) at 605 nm. Each hydroperoxidase assay mixture contained 290 μM O2, 1 mM hematin, 1 μM TMPD, and 25 μg of microsomal protein in a final volume of 0.25 ml of 0.1 M Tris-Cl, pH 8.0. Reactions were initiated by adding 50 μl of 1 mM H2O2 to the sample and reference cuvettes. At least two concentrations of protein from each membrane sample were used to measure rates, thereby assuring linearity of the rates with added protein. An extinction coefficient of 15.5 liter mm^-1 cm^-1 at 611 nm was used to calculate rates of TMPD oxidation (35).
Inactivation of PGG/H Synthase with Aspirin—To determine the rate of inactivation of PGG/H synthase by aspirin, enzyme samples (~5 mg of microsomal protein/ml) were incubated plus and minus 0.1 mM aspirin in 0.1 M Tris-Cl, pH 8.0, at 37 °C, and aliquots were removed at 0, 10, 20, 30, 40, 60, and 90 min to measure cyclooxygenase activity. The values were determined from plots of the logarithm of activity versus time. Less than 10% of the cyclooxygenase activity was lost in the absence of aspirin during a 90-min incubation at 37 °C.

Immunoprecipitation of PGG/H Synthase—Suspensions (10% w/v) of IgG(day-1)-Staphylococcus aureus (control) and IgG(cyo-3)-S. aureus (anti-PGG/H synthase) complexes in 0.1 M Tris-Cl, pH 7.4, containing 0.1% Tween 20, were prepared as described previously (11). Microsomes prepared from transfected cos-1 cells were solubilized by adding one volume of 0.1 M Tris-Cl, pH 8.0, containing 2% Tween 20 to the microsomal suspension. Samples of solubilized microsomes (50–100 μl) were incubated for 2 min at 24 °C with 5–50 μl aliquots of 10% suspensions of IgG(day-1)- or IgG(cyo-3)-S. aureus complexes and then centrifuged for 2 min at 5000 × g. Aliquots of the resulting supernatants were assayed for cyclooxygenase and hydroperoxidase activities as described above.

Western Transfer Blotting—Solubilized microsomal membranes from sham-transfected cos-1 cells and cos-1 cells transfected with pCD-PGHSov or pCD-PGHSov-A530 were resolved by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose filters (0.45 μM) essentially as described by Towbin et al. (36). Filters were blocked in Tris-buffered saline (TBS, 0.1 M Tris-Cl, pH 7.4, containing 0.15 M NaCl) containing 3% bovine serum albumin and then incubated overnight with a 1:20 dilution of monoclonal rabbit anti-PGG/H synthase serum (37) or nonimmune rabbit serum. The filters were washed six times with 0.1% Tween 20 in TBS and then incubated with 10–30 μCi of 125I-Protein A in 3% bovine serum albumin in TBS for 2 h. Finally, the filters were washed four to six times with 0.1% Tween 20 in TBS and twice in TBS, air-dried, and subjected to autoradiography using Kodak XRP-5 x-ray film.

RESULTS AND DISCUSSION

Oligonucleotide-directed Mutagenesis of Ser-530—Acetylation of the hydroxyl group of Ser-530 of sheep PGH synthase by aspirin causes irreversible inactivation of the cyclooxygenase activity of the enzyme (6, 8, 21, 29). To determine what role the hydroxyl group of Ser-530 might play in cyclooxygenase catalysis, we used the following approach to replace Ser-530 with Ala-530 and to compare the native and mutant enzymes: (a) starting with an M13mp19 construct containing the coding region of sheep PGG/H synthase (6), the codon for Ser-530 (nucleotides 1687–1689 (6)) was altered to the codon for alanine using the method of Kunkel et al. (29); (b) the coding regions of the native (Ser-530) and mutant (Ala-530) enzymes were removed from the two M13mp19 constructs and used to prepare Okayama/Berg pCD expression vectors (31, 32); (c) the pCD vectors (designated pCD-PGHSov and pCD-PGHSov-A530) were used to transfect host cos-1 cells; and finally, (d) the cyclooxygenase and hydroperoxidase activities of the native and mutant PGG/H synthases expressed by the transfected cell populations were characterized.

Shown in Fig. 2 is a autoradiogram of a sequencing gel comparing the nucleotide sequences of the native and mutant PGG/H synthase in the region coding for amino acid residue number 550 (nucleotides 1687–1689). These sequences are 5'-TCC and 5'-GCC for the native (Ser-530) and mutant (Ala-530) cDNAs, respectively.

Cultures of cos-1 cells were transfected with each of the expression vectors (pCD-PGHSov and pCD-PGHSov-A530), and 48 h after transfection, microsomal membranes were prepared from each group of transfected cells. As shown by Western transfer blotting (Fig. 3), both groups of cells contained proteins of the same size (Mr, 70,000) which interacted with a rabbit anti-PGG/H synthase antisera. This serum was shown previously to be monospecific for PGG/H synthase (37). As judged by the labeling intensity, similar concentrations of native and mutant PGG/H synthases were present in microsomal membranes prepared from the transfected cells, while little or no immunoreactivity was apparent in membranes from sham-transfected cells (Fig. 3). No radiolabeling of any proteins was seen in a control blot treated with nonimmune rabbit serum in place of anti-PGG/H synthase serum.

Table I compares the cyclooxygenase and hydroperoxidase activities of membranes from sham-transfected cos-1 cells and cos-1 cells transfected with expression vectors for either the native (pCD-PGHSov) or mutant (pCD-PGHSov-A530) PGG/H synthases. Consistent with results obtained previously, we found essentially no PGG/H synthase activity in sham-transfected cells, but comparatively high levels of both cyclooxygenase and hydroperoxidase activities in cells transfected with pCD-PGHSov which contains the cDNA coding for the native enzyme (32). The striking new result was that the mutant enzyme exhibited a cyclooxygenase specific activity quite similar to that found for the native enzyme. Further comparisons of the cyclooxygenase activities of the native and mutant enzymes indicated that these two activities have quite similar kinetic constants. Both enzymes had essentially identical K_m values for arachidonate; furthermore, the ID_50 values for n-
a bulky, uncharged amino acid such as asparagine or glutamine at position 530 would also prevent the cyclooxygenase reaction from occurring; preliminary results suggest that this is the case (38).

Although the Ala-550 mutant is insensitive to inactivation by aspirin, both the Ala-530 mutant and the native enzyme underwent time-dependent, irreversible inactivation when incubated with flurbiprofen (data not shown). Thus, irreversible inactivation of PGG/H synthase by flurbiprofen does not require the hydroxyl group of Ser-530.

Finally, as shown in Table I, all of the cyclooxygenase activity and approximately 95% of the hydroperoxidase activity solubilized from membranes from transfected cos-1 cells was precipitated by a monoclonal antibody (IgG (γ 0-3); (11, 34)) to PGG/H synthase. This result verifies that both the cyclooxygenase and hydroperoxidase activities being measured in these experiments can be attributed to PGG/H synthase.

**Structure of a Mouse 3T3 Cell PGG/H Synthase**—Although Ser-530 appears to play no obvious role in the cyclooxygenase reaction, we found, after sequencing a mouse PGG/H synthase, that the region surrounding Ser-530 is very highly conserved. Fig. 4 shows the nucleotide sequence and deduced amino acid sequence of a cDNA coding for a mouse PGG/H synthase. The mouse cDNA shown in Fig. 4 was cloned from a λgt 10 cDNA library which was prepared from poly(A)+ RNA isolated from Swiss mouse 3T3 cells; the library was screened with a 1.6-kb EcoRI restriction fragment prepared from the sheep PGG/H synthase cDNA (6). The mouse cDNA for PGG/H synthase contains 2757 bases (Fig. 4). An open reading frame of 1806 bases begins at nucleotide 37 and extends to the stop codon TGA, at nucleotides 1843–1845. There is 81% nucleotide sequence identity between the mouse cDNA (Fig. 4) and the sheep cDNA for PGG/H synthase (6–8) in the sequences containing the protein coding regions (nucleotides 1–2000); there is 50% identity between the sequences in the region (nucleotides 2000–2757) encompassing the 3′-noncoding region.

The mouse cDNA was found to hybridize with a single species (3 kb) of cytoplasmic RNA from mouse 3T3 cells (data not shown). This RNA species is somewhat larger than the mouse cDNA and larger than the cytoplasmic RNA (2.8 kb) for sheep vesicular gland PGG/H synthase (6). We suspect the difference in the size of our cDNA and the observed mouse message is due to an incomplete 5′-noncoding region in our cDNA. Our reasoning is as follows. Although the 3′-noncoding region of the mouse cDNA lacks the poly(A) tail, it is 87% identical over the last 33 nucleotides with the sheep sequence, which are not identical, only 11 (~2%) result from nonconservative codon changes; three of these differences result from insertions and changes in the putative signal peptide and five others are found in the 12 amino acids at the C terminus. Notable similarities between the sheep and mouse PGG/H synthase sequences include (a) four potential sites of N-glycosylation (asparagines 68 (70), 104 (106), 144 (146), and 410 (412) of the sheep (mouse) enzyme; (b) the presence of a

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**Fig. 3. Western transfer blot of microsomal membranes from cos-1 cells transfected with pCD-PGHS<sub>α</sub> and pCD-PGHS<sub>α</sub>-A530.** Membranes were prepared from transfected and sham-transfected cos-1 cells and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Samples were transferred to nitrocellulose and incubated sequentially with rabbit anti-PGG/H synthase serum and 125<sup>I</sup>-labeled Protein A. The samples were dried and subjected to autoradiography as detailed in the text. Lane A, sheep PGG/H synthase (0.25 μg) purified by immunoaffinity chromatography (6); lane B, microsomes from cos-1 cells transfected with pCD-PGHS<sub>α</sub> (30 μg of protein); lane C, microsomes from cos-1 cells transfected with pCD-PGHS<sub>α</sub>-A530 (30 μg of protein); and lane D, microsomes from sham-transfected cos-1 cells (30 μg of protein).
Comparison of the cyclooxygenase and hydroperoxidase activities of membranes from cos-1 cells transfected with pCD-PGHS<sub>n</sub> and pCD-PGHS<sub>n</sub>-A530.

Measurements of cyclooxygenase and hydroperoxidase activities, \( K_m \) values for arachidonate, ID<sub>so</sub> values for cyclooxygenase inhibitor, and immunoprecipitations with control (IgG<sub>day-1</sub>) and anti-PGG/H synthase (IgG<sub>cyclo-3</sub>) S. aureus complexes were performed as described in the text. The half-life for cyclooxygenase activity in the presence of aspirin was determined at 37 °C with 0.1 mM aspirin. Abbreviations: 20:4, arachidonate; TMPD, tetramethylphenylenediamine; N.D., not determined.

| cos-1 cells transfected with: | Cyclooxygenase activity | Peroxidase activity | \( t_{1/2} \) for irreversible inactivation by aspirin | \( K_m \) for arachidonate | ID<sub>so</sub> values for reversible inhibition of cyclooxygenase activity | % Cyclooxygenase activity precipitated by | % Peroxidase activity precipitated by |
|-----------------------------|-------------------------|---------------------|-----------------------------|-----------------------------|--------------------------------|-------------------------------------|-------------------------------------|
| nml 20:4/min | nml TMPD/min | min | \( \mu M \) | \( \mu M \) | Inhibitor | Flurbiprofen | Flufenamate | Aspirin |
| Sham transfected | 0 | 8.1 | N.D. | 8 | 22 | 20,000 | 0 | 100 | 0 | 93 |
| pCD-PGHS<sub>n</sub> (native) | 278 | 240 | 28 | 20,000 | 0 | 100 | 0 | 93 |
| pCD-PGHS<sub>n</sub>-A530 | 178 | 179 | Stable | 20,000 | 0 | 100 | 0 | 93 |

**Fig. 4.** Nucleotide and deduced amino acid sequence (standard one-letter symbols) of a cDNA coding for a mouse 3T3 cell PGG/H synthase. The numbers above the sequence refer to the amino acid position; the nucleotide position is indicated on the far right. The putative signal peptide (residues 1–26) is underlined; N-glycosylation sites (residues 70, 106, 146, and 412) are marked with filled triangles, and the aspirin-sensitive serine (residue 532) is marked with a closed diamond.
putative signal peptide (amino acids 1-24 (1-26) which is two amino acids longer in the mouse than the sheep, and (c) the active site serine (Ser-530 (532)). In particular, it should be noted that the regions adjoining Ser-530 (532) are those which are most highly conserved between the mouse and sheep protein. Amino acids 514-546 of the sheep (516-548 of the mouse) are identical, and there is only one difference in the sheep and mouse sequences between residues 473 (475) and 546 (548). The high degree of conservation of sequence in this region of PGG/H synthase and the location of the active site serine in this region suggest that this area of the protein may be especially important in cyclooxygenase catalysis.

A notable difference between the sheep and the mouse PGG/H synthase sequences occurs in a region which includes amino acid residues 270-280 (272-282). The sheep enzyme is selectively cleaved by trypsin at Arg-277; however, there is not an homologous arginine in the mouse protein (although it is conceivable that the mouse enzyme could be cleaved at a nonhomologous Arg (276)). The sheep sequence HYPR (amino acids 274-277) postulated to form part of the distal heme-binding site (39) of PGG/H synthase is not present in the mouse enzyme; the sequence of the corresponding region of the mouse is RYP (amino acids 276-279). Since heme binding to PGG/H synthase appears to involve imidazole at both the distal and proximal sites (17), it seems unlikely that the HYPR sequence is directly involved in heme binding. Moreover, preliminary results indicate that a sheep PGG/H synthase mutant having the sequences HFPR has both cyclooxygenase and hydroperoxidase activities (38). A potential axial heme-binding site involves amino acids 303-312 of sheep PGG/H synthase (residues 306-314 of the mouse). This sequence, TIWLRHNRV, is 80 and 70% identical to the related sequences TLWLREHNRL and TLRLREHNRL found in human thyroid peroxidase and human myeloperoxidase (40-43; Table II), respectively. Allowing for conservative substitutions, the thyroid peroxidase and PGG/H synthase sequences are identical, and the myeloperoxidase in 90% homologous to the corresponding thyroid peroxidase and PGG/H synthase sequences. However, the overall amino acid sequence identities between the two PGG/H synthase sequences and the thyroid and myeloperoxidase sequences are less than 20%. The histidine residue present in the TLW(L)LREHNRL sequences of thyroid peroxidase and myeloperoxidase has been proposed as a potential site for axial heme binding (43). By analogy, we suggest that the histidine residue in the TIWLRHNRV sequence of sheep and mouse PGG/H synthases (His-309 of the sheep; His-311 of the mouse) is the axial heme ligand of this enzyme.

The location of the distal histidine is not obvious. In plant and mammalian peroxidases, the distal histidine is present in a sequence of this type. There is, however, one sequence, KALGH (residues 222-226 of the mouse), which is found in sheep and mouse PGG/H synthase sequences (His-309 of the sheep; His-311 of the mouse) is the axial heme ligand of this enzyme.

Fig. 5. Comparison of the amino acid sequences of sheep and mouse PGG/H synthase. Identical residues are marked with a colon (:); residues involving a one-letter change in the codon (i.e. conservative substitutions) are marked with a period (·); nonconservative changes involving two- or three-letter changes in the codon are unmarked. Alignments were performed using the FASTP sequence comparison program (45).

| Enzyme (ref.) | Residue | Similarity* |
|--------------|---------|-------------|
| Sheep PGG/H synthase (6-8) | 303-TIWRHEHNRV-312 | 100 |
| Mouse PGG/H synthase | 305-TIWRHEHNRV-314 | 100 |
| Human thyroid peroxidase (42) | 408-TLRLREHNRL-417 | 100 |
| Human myeloperoxidase (40, 41) | 417-TIWRHNRV-426 | 90 |

*Calculation based on conservative change from isoleucine to leucine and valine to leucine.
served in the sheep and mouse PGG/H synthases. It should be feasible to prepare mutations at all 13 sites to determine which ones are important in heme binding and catalysis (44).

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