Essential Histidines of Prostaglandin Endoperoxide Synthase

HIS-309 IS INVOLVED IN HEME BINDING*

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Prostaglandin endoperoxide (PGH) synthase has a single iron protoporphyrin IX which is required for both the cyclooxygenase and peroxidase activities of the enzyme. At room temperature, the heme iron is coordinated at the axial position by an imidazole, and about 20% of the heme iron is coordinated at the distal position by an imidazole. We have used site-directed mutagenesis to investigate which histidine residues are involved in PGH synthase catalysis and heme binding. Individual mutant cDNAs for ovine PGH synthases were prepared with amino acid substitutions at each of 13 conserved histidines. cos-1 cells were transfected with each of these cDNAs, and the cyclooxygenase and peroxidase activities of the resulting microsomal PGH synthases were measured. Mutant PGH synthases in which His-207, His-309, or His-388 was replaced with either glutamine or alanine lacked both activities. Gln-386 and Ala-386 PGH synthase mutants exhibited cyclooxygenase but not peroxidase activities. Other mutants exhibited both activities at varying levels.

Because binding of heme renders native PGH synthase resistant to cleavage by trypsin, we examined the effects of heme on the relative sensitivities of native, Ala-204, Ala-207, Ala-309, Ala-386, and Ala-388 mutant PGH synthases to trypsin as a measure of the heme-protein interaction. The Ala-309 PGH synthase mutant was notably hypersensitive to tryptic cleavage, even in the presence of exogenous heme; in contrast, the native enzyme and the other alanine mutants exhibited similar, lower sensitivities toward trypsin and, except for the Ala-386 mutant, were partially protected from trypsin cleavage by heme. Preincubation of the native and each of the alanine mutant PGH synthases, including the Ala-309 mutant, with indomethacin protected the proteins from trypsin cleavage. Thus, all the mutant proteins retain sufficient three-dimensional structure to bind cyclooxygenase inhibitors.

Our results suggest that His-309 is one of the heme ligands, probably the axial ligand, of PGH synthase. Two other histidines, His-207 and His-388, are essential for both PGH synthase activities suggesting that either His-207 or His-388 can serve as the distal heme ligand; however, the trypsin cleavage measurements imply that neither His-207 nor His-388 is required for heme binding. This is consistent with the fact that only 20% of the distal coordination position of the heme iron of PGH synthase is occupied by an imidazole side chain.

Prostaglandin endoperoxide (PGH) synthase1,2 (EC 1.14.99.1) catalyzes the committed step in the formation of prostaglandins and thromboxane from arachidonic acid. This enzyme has two activities, including a cyclooxygenase involved in converting arachidonate to PGG2 and a peroxidase which mediates the reduction of PGG2 to PGH2 (1-3). PGH synthase was first purified from solubilized microsomal membranes from bovine (4) and ovine (1, 5) vesicular glands. It exists in detergent solution as a homodimer with a subunit molecular mass of 72 kDa. Complementary DNAs for sheep (6-8), mouse (9), and human (10) PGH synthases have recently been cloned, and the primary sequences of the proteins have been deduced. These sequences are closely related, with 88% amino acid sequence identity between the sheep and mouse enzymes and 91% sequence identity between the sheep and human enzymes (11).

The availability of cDNAs coding for the complete amino acid sequence of PGH synthase has facilitated studies of structure-function interrelationships in this protein. For example, recent results have shown that acetylation of Ser-530 of PGH synthase by aspirin places a bulky group in the enzyme which protrudes into the cyclooxygenase active site (9, 12). In addition, Tyr-385 has now been identified as being essential for the cyclooxygenase activity, but not the peroxidase activity, of PGH synthase (12, 13).

In this report, we describe studies designed to determine the relative importance of the various histidine residues of PGH synthase. The impetus for this work is that both the cyclooxygenase and peroxidase activities of PGH synthase require heme for activity (14-17) and that heme appears to be bound to the protein via imidazole side chains of histidines at both the 5th and 6th coordination positions of the heme iron (17-19). Thus, our ultimate goal is to identify which histidine residues are involved in the binding of heme to PGH synthase. As reported here, we have used site-directed mutagenesis to replace each of the 13 histidine residues common to known PGH synthases (11) and have compared the properties of the mutant enzymes with those of native PGH synthase.

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1 The abbreviations used are: PGH synthase, prostaglandin endoperoxide synthase; PG, prostaglandin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PHP, 5-phenyl-4-pentenyl-1-hydroperoxide.
2 The numbering of amino acids in PGH synthase begins with methionine at the deduced translational start signal. Thus, there are 600 amino acids. The mature PGH synthase from which the signal peptide has been removed consists of 576 amino acids.
EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium and fetal calf serum were obtained from GibCO. Calf serum was from HyClone Laboratories. Cholesterol, bovine hemoglobin, Tween 20, hematin, guanidine, trypsin (Type IX), chicken egg white trypsin inhibitor, and arachidonic acid were from Sigma. 5-Phenyl-4-pentenyl-1-hydroperoxide (PPHP) was from ICN Biomedicals. BA85 (0.45 μl) nitrocellulose was from Schleicher & Schuell. Restriction enzymes (T4 DNA ligase, T4 DNA polymerase, and Klenow fragment), and CsCl were from Boehringer Mannheim or New England Biolabs. Prestained SDS-PAGE molecular mass markers and the Muta-Gene kit were from Bio-Rad. Sequenase was from U. S. Biochemical Corp. DEAE-dextran was purchased from Pharmacia LKB Biotechnology Inc. The oligonucleotides were used as primers for preparing the mutants of PGH synthase and for DNA sequencing were prepared by the Michigan State University Macromolecular Structure and Sequencing Facility. All other reagents were from standard sources.

Preparation of PGH Synthase Mutants—The coding region of a PGH synthase with unique Sau restriction sites in both the 5' and 3' untranslated sequences was as described previously (9). Mutants were prepared starting with M13mpl9-PGHS0, according to the method of Saiki et al. (21) using a Bio-Rad kit essentially as described previously (9). Mutations were as described in Table I. The replica plate of M13mpl9-PGHS0 containing each of the desired mutations was isolated (23) and digested with SalI, and the 2.3-kilobase fragment was purified (9, 20), which had been cleaved with Sau3A and pretreated with calf intestinal alkaline phosphatase. Aliquots of the ligation mixture were used to transform competent Escherichia coli DH5a. Minipreparations of the resulting clones were screened by restriction analyses of Sau and PstI digests. The presence of two Sau fragments corresponding to the PGH synthase insert (2.5 kilobase) and psV77 (3.6 kilobase) indicate successful insertion into psV77. The correct orientation of the insert yields PstI fragments of 89, 220, and 830 base pairs (9). The bacteria obtained containing plasmid (pSV77-PGHs0) were grown in 2YT medium in the presence of ampicillin (75 μg/ml), and the plasmid was isolated and purified by CsCl gradient centrifugation. Reconfirmation of the plasmid was then incubated with or without 10 μM indomethacin (2.5 mM stock solution in dimethyl sulfoxide) for 10 min. Reconfirmation of the plasmid was then incubated with or without 10 μM indomethacin (2.5 mM stock solution in dimethyl sulfoxide) for 10 min. The bacteria obtained containing plasmid (psV77-PGHs0) were grown in 2YT medium in the presence of ampicillin (75 μg/ml), and the plasmid was isolated and purified by CsCl gradient centrifugation. Reconfirmation of the plasmid was then incubated with or without 10 μM indomethacin (2.5 mM stock solution in dimethyl sulfoxide) for 10 min. The bacteria obtained containing plasmid (psV77-PGHs0) were grown in 2YT medium in the presence of ampicillin (75 μg/ml), and the plasmid was isolated and purified by CsCl gradient centrifugation. Reconfirmation of the plasmid was then incubated with or without 10 μM indomethacin (2.5 mM stock solution in dimethyl sulfoxide) for 10 min. The bacteria obtained containing plasmid (psV77-PGHs0) were grown in 2YT medium in the presence of ampicillin (75 μg/ml), and the plasmid was isolated and purified by CsCl gradient centrifugation. Reconfirmation of the plasmid was then incubated with or without 10 μM indomethacin (2.5 mM stock solution in dimethyl sulfoxide) for 10 min.

The numbering of amino acids in PGH synthase begins with methionine at the deduced translational start signal. Superscripts indicate the nucleotide number of the cDNA for sheep PGH synthase. Mutant codons are marked with underline.

Cyclooxygenase Assays—Cyclooxygenase assays were performed at 37° C by monitoring the initial rate of O2 uptake using an O2 electrode as described previously (9). The assay mixture contains 3 ml of 0.1 M Tris-HCl, pH 7.2, 3.6 mM guaiacol, 85 μg of hemoglobin (as a source of heme), and 100 μM arachidonic acid. Reactions were initiated by adding 5-100 μg of microsomal protein.

 Peroxidase Assays—Peroxidase activity of microsomal preparations was measured spectrophotometrically essentially as described by Marnett et al. (20). The reaction mixture contains 100 mM Tris-HCl, pH 7.2, 5.6 mM guaiacol, 85 μg of hemoglobin (as a source of heme), and 100 μM arachidonic acid. Reactions were initiated by adding H2O2 or PPHP to a final concentration of 400 or 133 μM, respectively, and the progress of the reaction was monitored at 436 nm. The rate of increase in absorption at 436 nm due to the formation of 3,3'-dime thoxyphenyl-4,4'-quinone (ε = 6 4 mM^-1 cm^-1) (26) was calculated from the slope of a tangential line drawn at the initial phase of the time course.

Western Transfer Blotting—Solubilized microsomal membranes from sham-transfected cos-1 cells and cos-1 cells transfected with psV77-PGHs0, native or psV77-PGHs0, mutant vectors were resolved by one-dimensional SDS-PAGE and transferred electrophoretically to nitrocellulose filters (0.45 μM) essentially as described previously (9). Filters were incubated overnight with a 1:100 dilution of monospecific rabbit anti-PGH synthase serum (27). The filters were then washed and incubated with 125I-Protein A. After drying in a x-ray film.

Tryptic Cleavage of PGH Synthase—Microsomes prepared from cos-1 cells transfected with psV77 plasmsid containing the coding regions for the native and variant PGH synthase were incubated with or without 20 μM hematin (1.6 mM stock solution in dimethyl sulfoxide) for 10 min. In some experiments, the microsomes were then incubated with or without 10 μM indomethacin (2.5 mM stock solution in methanol) for 1 h at room temperature. Tryptic cleavage of microsomal PGH synthases from transfected cos-1 cells was performed essentially as described by Kuimac and Wu (28). Aliquots of microsomes (1 mg of protein/ml) from transfected cos-1 cells were incubated with trypsin (6 μg of trypsin/mg of microsomal protein) reconfirmed after subcloning into psV77 by double-stranded sequencing using Sequenase (version 2.0) and the protocol described by the manufacturer.

Transfection of cos-1 Cells with psV77 Vectors—cos-1 cells (ATCC CRL-1650) were grown in Dulbecco's modified Eagle's medium containing 5% fetal calf serum. The cells were transfected with plasmids containing mutated coding regions of the sheep PGH synthase (psV77-PGHs0), or with plasmids containing mutated coding regions of the sheep PGH synthase (psV77-PGHs0, mutant). Microsomal membranes were prepared from transfected (9).
for 10, 30, and 60 min at room temperature. The reactions were quenched with a 33-fold molar excess of trypsin inhibitor and chilled on ice. Intact enzyme (72 kDa) and tryptic fragments (33 and 38 kDa) were separated by SDS-PAGE, subjected to Western transfer blotting with monospecific rabbit anti-PGH synthase antibody and visualized on x-ray film at -80°C for 6 h with an intensifying screen.

Protein Determinations—Determination of protein concentrations was done using the method of Bradford (29).

RESULTS

There are 13 histidines which are conserved among sheep, mouse, and human PGH synthases (12). In order to determine which histidine residues are essential for cyclooxygenase and peroxidase catalysis, we first replaced each histidine individually with a glutamine residue using site-directed mutagenesis. Next, we prepared pSVT7 constructs of each mutant, transfected cos-1 cells with each construct, and then assayed microsomal membranes from the transfected cells for cyclooxygenase and peroxidase activities. The results of these experiments are summarized in Table II. Three mutant PGH synthases, those with glutamine residues at positions 207, 309, or 388, lacked both cyclooxygenase and peroxidase activities. Gln-204 and Gln-386 mutants exhibited cyclooxygenase activity but had low or undetectable peroxidase activity. Other mutants exhibited both catalytic functions in the range of 44-176% of native cyclooxygenase activities and 22-114% of native peroxidase activities.

To determine if the results obtained with mutants lacking either or both activities were unique to the Gln mutations, we constructed and assayed PGH synthase mutants having alanine residues at positions 204, 207, 309, 386, and 388 (Table III). The results were qualitatively the same as those obtained with the glutamine mutants. Mutants with alterations at positions 207, 309, or 388 (Ala-207, Ala-309, or Ala-388) completely lacked both activities. The Ala-204 mutant retained a trace amount of peroxidase activity when tested using H2O2 as the oxidant substrate, but the Ala-386 mutant completely lacked both activities when tested with either H2O2 or PHPP. The results of Western transfer blotting with monospecific rabbit anti-PGH synthase antibody indicated that the native enzyme (M, = 72,000) as well as all of the glutamine- and alanine-containing mutant enzymes were expressed to similar extents by cos-1 cells (data not shown).

Proteases such as trypsin cleave purified and microsomal PGH synthases at Arg-277, yielding 33- and 38-kDa fragments corresponding to N- and C-terminal peptides (28, 30-32). When microsomes from cos-1 cells expressing native or alanine mutant PGH synthases were treated with trypsin, there was in each case a rapid decrease in the amount of immunoreactive PGH synthase migrating with an M, of 72,000 (Fig.1A); there was also a decrease in the intensity of a less prominent band migrating with an M, of 68,000.3 Associated with these decreases was concomitant increases in the appearance of two major immunoreactive bands migrating with approximate M, = 38,000 and 33,000. The one striking exception was the Ala-309 mutant; trypsin treatment caused an almost complete loss of the bands at M, = 72,000 and 68,000 within 10 min, but bands at M, = 38,000 and 33,000 did not appear; presumably, this lack of appearance of the smaller peptides was due to their further degradation by trypsin.

Heme is reported to protect PGH synthase from cleavage by trypsin (25, 28, 31, 32). Pretreatment of microsomal enzymes with heme altered the pattern of trypsin cleavage for all but the Ala-309 and Ala-386 PGH synthase mutants (Fig. 1B). The 72- and/or 68-kDa subunits of the Ala-204, Ala-207, and Ala-388 mutants, and the native enzyme were partially protected to varying degrees by the presence of heme. Effects of heme on the proteolytic cleavage of different PGH synthase mutants were also determined using pSVT7 constructs containing cDNAs coding for PGH synthases. Normally, this band represents about 5% of the protein present in the band at M, = 72,000. We do not yet know if the band at M, = 68,000 is a splice variant or results from a posttranslational modification.

| Mutant     | Cyclooxygenase | Peroxidase | C/P   |
|------------|----------------|------------|-------|
|            | nmol/min/mg    | nmol/min/mg|       |
| Native     | 171            | 100        | 1.0   |
| His-90 → Gln | 168            | 98         | 0.9   |
| His-95 → Gln | 95             | 56         | 0.5   |
| His-134 → Gln | 101            | 59         | 2.7   |
| His-204 → Gln | 107            | 63         | 1.6   |
| His-207 → Gln | 0             | 0          | 0     |
| His-226 → Gln | 75             | 44         | 1.7   |
| His-232 → Gln | 301            | 176        | 1.8   |
| His-300 → Gln | 0             | 0          | 0     |
| His-320 → Gln | 234            | 137        | 1.7   |
| His-386 → Gln | 16             | 9          | 1.1   |
| His-388 → Gln | 0             | 0          | 1.1   |
| His-443 → Gln | 93             | 54         | 1.1   |
| His-446 → Gln | 212            | 124        | 1.1   |

1 The immunoreactive band at M, = 68,000 is present to varying extents in microsomal preparations of cos-1 cells transfected with the indicated pSVT7 constructs containing cDNAs coding for PGH synthases. Normally, this band represents about 5% of the protein present in the band at M, = 72,000. We do not yet know if the band at M, = 68,000 is a splice variant or results from a posttranslational modification.
Fig. 1. Trypsin cleavage of native PGH synthase and Ala mutant of PGH synthases in microsomes from transfected cos-1 cells. Experimental details of the trypsin treatments and analyses by SDS-PAGE are presented under “Experimental Procedures.” Microsomes prepared from cos-1 cells transfected with pSVT7 plasmids containing the indicated mutation were incubated with or without 20 μM hematin for 10 min and with or without 10 μM indomethacin for 1 h at room temperature. Microsomes (A), heme-treated microsomes (B), and indomethacin-treated microsomes (C) were incubated with trypsin (5 μg/mg of total microsomal protein) for the time indicated in the figure. Reactions were quenched with trypsin inhibitor and chilled on ice. Samples (20 μg of protein) were subjected to SDS-PAGE and transferred to nitrocellulose and incubated with anti-PGH synthase serum and 125I-labeled protein A. The filters were all washed with 0.1% Tween 20 in Tris-buffered saline, air-dried, and exposed on x-ray film for 6 h at -80 °C with an intensifying screen.
of heme were also apparent at the level of the 33- and 38-kDa peptides. In several cases, the presence of heme led to an increase in the amount of immunoreactive 33-kDa peptide relative to the 38-kDa peptide (i.e. native, Ala-204, and Ala-388). In contrast, the relative amount of 33-kDa peptide seemed to be decreased in the presence of heme in the case of the Ala-207 mutant; apparently, heme affects secondary cleavage of the 33-kDa peptide by trypsin. In contrast to these results, cleavage of the Ala-309 and Ala-386 mutants by trypsin seemed unaffected by heme, suggesting that heme is not interacting with either of these mutant PGH synthases in a way that alters the structures of these proteins.

The effects of the cyclooxygenase inhibitor, indomethacin, on the sensitivity of PGH synthases to trypsin were also examined (Fig. 1C). Indomethacin, like heme, can render both purified and microsomal ovine PGH synthases resistant to trypsin, although indomethacin binds to a site distinct from the heme binding site (32, 33). With both the native and mutant PGH synthases, the amounts of immunoreactive peptide of M_r = 72,000 were greater in the indomethacin-pretreated microsomes. The relative amounts of immunoreactive fragments of 33 and 38 kDa in most cases differed from those observed with trypsin-treated microsomes which had not been pretreated with indomethacin. Importantly, indomethacin attenuated the digestion of the Ala-309 PGH synthase mutant by trypsin. This suggests that the Ala-309 mutant retains some degree of three-dimensional structure, although, as shown above (Fig. 1B), there is no evidence that the Ala-309 mutant PGH synthase can interact with heme.

DISCUSSION

PGH synthase has an iron protoporphyrin IX which is required for both cyclooxygenase and peroxidase activities (14, 34). There appears to be one catalytically functional heme per subunit (14–17). Spectral evidence indicates that the heme iron is coordinated by histidine residues at both the axial and distal positions (18, 19), although at room temperature only about 20% of the heme is in a form with bis-imidazole coordination (15). One would expect that substitution of histidine residues essential for heme binding would eliminate both the cyclooxygenase and peroxidase activities of PGH synthase. We have shown here that three histidines (His-207, His-309, and His-388), of the 13 histidine residues common to known PGH synthases, qualify as potential heme ligands on the basis of this criterion (Tables II and III).

Additional studies on the trypsin sensitivities of mutant PGH synthases suggest that His-309 is one of the heme ligands of PGH synthase. The Ala-309 PGH synthase mutant is unable to bind heme as judged from the observation that in contrast to native PGH synthase, the Ala-209 mutant is not protected from trypsin degradation by exogenous heme. The Ala-309 mutant, however, appears to retain some degree of three-dimensional structure because indomethacin partially protects the mutant protein from cleavage by trypsin (Fig. 1). Thus, the lack of heme binding by the Ala-309 PGH synthase cannot be simply attributed to a complete unfolding of this protein. Comparison of the amino acid sequences of PGH synthases from different species with sequences of two other mammalian peroxidases, thyroid peroxidase and myeloperoxidase, has established that His-309 is present in a consensus decameric peptide common to all these proteins (9, 12). In short, His-309 appears to be a heme ligand of PGH synthase, and we speculate that it is the proximal heme ligand.

Our activity assays establish that His-207 and His-388 are essential for both the cyclooxygenase and peroxidase functions of PGH synthase, but the results of the trypsin cleavage experiments imply that mutant PGH synthases in which His-207 or His-388 is replaced by alanine residues bind heme effectively. The "distal" histidine residue of PGH synthase actually occupies the 6th coordination position of the heme only to a limited extent, about 20%, at room temperature (18). Thus, substitution of this distal histidine may not greatly affect the binding of heme to PGH synthase, although it may have a substantial effect on catalysis. Hopefully, it will be possible to gain further insight into the roles of His-207 and His-388 in heme binding by performing spectral studies on the interactions of peroxide activators of the enzyme (33–35) with mutant PGH synthases containing substitutions at positions 207 and 388. At this point, our guess is that His-388 will prove to be the distal histidine of PGH synthase. This speculation is based on the present findings that PGH synthase mutants containing alterations at position 388 are catalytically inactive and very recent evidence that a closely neighboring residue, Tyr-385, is at the cyclooxygenase active site. Tyr-385 is essential for cyclooxygenase but not peroxidase activity, and competitive cyclooxygenase inhibitors shield Tyr-385 from nitration by tetranitromethane (12, 13, 33).

One surprising result of our study is that the peroxidase activities of Ala-386 and Gln-386 PGH synthase mutants are undetectable but that these mutant proteins retain cyclooxygenase activities. Currently, the most plausible model for the interrelationship between the peroxidase and cyclooxygenase activities is that the peroxidase catalyzes a requisite first step in peroxide activation of the cyclooxygenase (34). It is known that peroxidase-deficient PGH synthases such as the Mn^2+-protoporphyrin IX-reconstituted enzyme, which has only about 1–5% of the native peroxidase activity, express cyclooxygenase activity (36). While the Ala-386 and Gln-386 PGH synthases lacked detectable peroxidase activities, one peroxidase oxidation per subunit, which would be undetectable in our assays, may be all that is needed to activate the cyclooxygenase. It will be of interest to determine if the Ala-386- or Gln-386-containing mutants undergo peroxide-induced changes in their heme spectra characteristic of the native enzyme.

Depicted in Fig. 2 is a model for the cyclooxygenase and peroxidase active sites of PGH synthase. This model has evolved from evidence: (1) that there are distinct substrate binding sites for cyclooxygenase and peroxidase substrates.
and inhibitors (13, 35, 37, 38); (b) that there is a single heme group per PGH synthase subunit and that heme is coordinated at both proximal and distal positions by histidine residues (14–19); (c) that there is an essential tyrosine residue (Tyr-358) which can then react with a second molecule of O2 producing PGG2 and regenerating a Tyr-385 radical which can, in turn, initiate another cyclooxygenase catalytic cycle. The PGG2 is reduced to PGH2 by the peroxidase activity. In this model, we speculate that His-309 has a crucial role as the proximal heme ligand and that either His-207 or His-388 serves as the distal heme ligand.

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