Mutation of a Critical Arginine in Microsomal Prostaglandin E Synthase-1 Shifts the Isomerase Activity to a Reductase Activity That Converts Prostaglandin H$_2$ into Prostaglandin F$_2\alpha$

Received for publication, November 3, 2008 Published, JBC Papers in Press, November 3, 2009, DOI 10.1074/jbc.M808365200

Tove Hammarberg$^{1,2}$, Mats Hamberg$^{3,2}$, Anders Wetterholm$, Henrik Hansson$, Bengt Samuelsson$, and Jesper Z. Haeggström$^{1,2}$

From the $^{1}$Department of Medical Biochemistry and Biophysics, Division of Chemistry II, Karolinska Institutet, S-171 77 Stockholm, Sweden and the $^{2}$Department of Molecular Biology, Swedish University of Agricultural Sciences, 751 24 Uppsala, Sweden

Microsomal prostaglandin E synthase type 1 (mPGES-1) converts prostaglandin endoperoxides, generated from arachidonic acid by cyclooxygenases, into prostaglandin E$_2$. This enzyme belongs to the membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) family of integral membrane proteins, and because of its link to inflammatory conditions and preferential coupling to cyclooxygenase 2, it has received considerable attention as a drug target. Based on the high resolution crystal structure of human leukotriene C$_4$ synthase, a model of mPGES-1 has been constructed in which the tripeptide co-substrate glutathione is bound in a horseshoe-shaped conformation with its thiol group positioned in close proximity to Arg-126. Mutation of Arg-126 into an Ala or Gln strongly reduces the catalytic activity of mPGES-1 with its thiol group positioned in close proximity to Arg-126. Mutation of a neighboring Arg-122 does not have any significant effect. Interestingly, R126A and R126Q mPGES-1 exhibit a novel, glutathione-dependent, reductase activity, which allows conversion of prostaglandin H$_2$ into prostaglandin F$_2\alpha$. Our data show that Arg-126 is a catalytic residue in mPGES-1 and suggest that MAPEG enzymes share significant structural components of their active sites.

Prostaglandin E$_2$ (PGE$_2$) is an abundant lipid mediator that signals via four receptors (EP1 to -4), resulting in an array of important biological actions in physiology as well as pathophysiology (1, 2). Biosynthesis of PGE$_2$ proceeds from arachidonic acid, which is converted to the unstable endoperoxide, prostaglandin H$_2$, by cyclooxygenase type 1 and 2. PGH$_2$ is further isomerized into PGE$_2$ by three distinct enzymes, cytosolic PGE synthase, microsomal PGE synthase type 1 (mPGES-1), and microsomal PGE synthase type 2 (3–5). Although cytosolic PGE synthase and microsomal PGE synthase type 2 seem to provide a basal synthesis of PGE$_2$, mPGES-1 appears to account for PGE$_2$ synthesis under proinflammatory conditions. Thus, mPGES-1 is up-regulated by mitogens and cytokines and is functionally coupled to cyclooxygenase type 2 (4, 6). Due to its key role in PGE$_2$ synthesis, mPGES-1 has attracted attention as a potential drug target in the areas of inflammation, pain, fever, and cancer (7).

mPGES-1 is a member of the MAPEG superfamily of enzymes (8), which also includes two key proteins in the leukotriene cascade, viz. 5-lipoxygenase-activating protein and leukotriene C$_4$ synthase (LTC4S). Since all MAPEG members are integral membrane proteins, structural information on this family has been scarce. Recently, however, significant progress has been made in this area, and several high and low resolution structures have been solved by three-dimensional as well as two-dimensional crystallography (9–12). The crystal structures of human LTC4S clearly provided the most detailed structural information, inter alia a unique, horse-shoe shaped binding conformation of GSH, a hydrophobic crevice presumably binding the lipid substrate leukotriene A$_4$, and an Arg residue, possibly involved in the activation of the GSH thiol. Here we used a homology model, based on the LTC4S structure, and site-directed mutagenesis to identify Arg-126 as a key catalytic residue in mPGES-1. Our data are consistent with the notion that MAPEG members share a structurally similar binding site to accommodate a horse-shoe shaped GSH involved in different catalytic reactions.

EXPERIMENTAL PROCEDURES

Materials—The I.M.A.G.E. clones were obtained from the Medical Research Council Geneservice (Cambridge, UK). Enzymes, Escherichia coli TOP10 cells, and pPICZA were from Invitrogen. His$_6$-pSP19T7LT was a kind gift from Dr. Sipra Saha. Anti-mPGES-1 antiserum was purchased from Cayman Chemical (Ann Arbor, MI). Platinum Pfx polymerase was from Invitrogen, and Pfu polymerase was from Promega (Madison, WI). PGH$_2$ (purity in excess of 95%) was purchased from Laro-
dane Fine Chemicals (Malmö, Sweden).

Cloning and Plasmid Construction—The human mPGES-1 gene was subcloned into pPICZA from the His$_6$-pSP19T7LT

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$^{1}$ The work was supported by Swedish Research Council Grants 10350, 20854, Linnéus Grant CERIC, and 2001-2553, the CIDaT consortium (Vinnova), the Söderberg Foundation, and European Commission FP6 Grant LSHM-CT-2004-005033. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

$^{2}$ Both of these authors contributed equally to this work.

$^{3}$ To whom correspondence should be addressed: Dept. of Medical Biochemistry and Biophysics, Division of Chemistry II, Karolinska Institutet, S-171 77 Stockholm, Sweden. Tel.: 46-8-5248-7612; Fax: 46-8-736-0439; E-mail: jesper.haeggstrom@ki.se.

$^{4}$ *The work was supported by Swedish Research Council Grants 10350, 20854, Linnéus Grant CERIC, and 2001-2553, the CIDaT consortium (Vinnova), the Söderberg Foundation, and European Commission FP6 Grant LSHM-CT-2004-005033. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The abbreviations used are: PGE$_2$, prostaglandin E$_2$; PGH$_2$, prostaglandin H$_2$; LTC4S, leukotriene C$_4$ synthase; MAPEG, membrane-associated proteins in eicosanoid and glutathione metabolism.
vector. The coding part of the gene, supplemented with an N-terminal in frame sequence encoding a His<sub>6</sub> tag, was PCR-amplified using the primer pair 5'<CGAACCCTTGAAGACTTCAACAAATGTCACATCAAGCCATCCTGCTGCAC-AAGCCCTGG and 5'<CAAGGCCGTCCTTTGACCAGCAGCCAT-GTGCCCGGCGCAGGTAGAAGTTGTG. The final pellet was homogenized with glass beads (0.5 mm), and the slurry was filtered through nylon filters (180 μm). Also, the linearized vector was PCR-amplified. For this Pfu polymerase, the primers 5'<CAAGACCGGTCTTCTCAGATGAGG and 5'<GTCGACCCCTGTCCTTGAGC were used. The PCR products were co-transformed into CaCl<sub>2</sub>-competent E. coli TOP10 cells, utilizing the endogenous recombinase activity of E. coli to recombine the fragments (13).

The protein coding part of the resulting expression vector, pPICZ-hisMPGES, was sequenced for verification.

**Mutagenesis**—Selected amino acids in pPICZ-hisMPGES were mutated using the QuickChange site-directed mutagenesis kit from Stratagene (La Jolla, CA).

**Protein Expression and Microsome Preparation**—Human recombinant mPGES-1 was overexpressed in Pichia pastoris. For most experiments, the strain KM71H was used, but strain X-33 also worked. The expression vector was transformed into competent P. pastoris cells using the Pichia EasyComp Transformation kit (Invitrogen). Recombinant cells were cultivated in baffled flasks in 2.5 liters of minimal yeast medium with glycercol (Invitrogen) at 27 °C. When A<sub>600</sub> reached 10–12, the cells were resuspended in 0.5 liters of minimal yeast medium with 0.5% methanol. After another 72 h, cells were harvested by centrifugation (2,500 × g, 7 min) and resuspended in 15 mM Tris-HCl, pH 7.8, 0.25 mM sucrose, and 1 mM GSH. Cells were homogenized with glass beads (0.5 mm), and the slurry was filtered through nylon filters (180 μm; Millipore) and centrifuged (5,000 × g, 10 min). The supernatant was ultracentrifuged (100,000 × g, 65 min), and microsomes were prepared from the pellet by homogenization in 20 mM Tris-HCl, pH 7.8, in a glass homogenizer. The microsome suspension was kept in aliquots at −20 °C. Prior to incubations with GSH analogues, the microsomes were washed from residual GSH: 100 μl of microsomes were diluted 10-fold, and 0.1 mM Tris-HCl, pH 7.8, kept on ice for 15 min, and ultracentrifuged (100,000 × g, 30 min); this procedure was repeated 3-fold, and the final pellet was homogenized in 100 μl of buffer.

Expression of both nonmutated and mutated mPGES-1 was confirmed by Western blot analysis (Fig. 1) using a commercial antiserum (Cayman 160140) specific for mPGES. We could not observe any cross-reactivity with other recombinant MAPEG proteins produced in P. pastoris.

**Western Blot**—The proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes using a Pharmacia Phast system. Antiserum against mPGES-1 (catalog number 160140; Cayman) and horseradish peroxidase-linked anti-rabbit IgG (catalog number NA934; Amersham Biosciences) were used together with an ECL Plus detection kit (Amersham Biosciences) to visualize the proteins.

**Enzyme Activity Assay**—Microsomal suspensions (1.5 μl) were incubated on ice for 5 min with 10 μM PGH<sub>2</sub> and 2.5 mM GSH in 300 μl of 0.1 M potassium phosphate buffer, pH 7.4. The reaction was terminated by the addition of 1.2 ml of 20 mM FeCl<sub>2</sub> and 50 mM citric acid, pH 2–3. A solution of [3,3,4,4-<sup>2</sup>H<sub>4</sub>]<PGF<sub>2α</sub> (1 μg) and [3,3,4,4-<sup>2</sup>H<sub>4</sub>]<PGF<sub>2α</sub> (1 μg) in 200 μl of ethanol was added, and the mixture was applied to a 1-ml Chromobond C18 column for solid phase extraction. Material eluted with 80% methanol was esterified by treatment with diazomethane and trimethylsilylated by treatment with trimethylchlorosilane/hexamethyldisilazane/pyridine (2:1:2, v/v/v). Aliquots of the derivatized material were subjected to gas chromatography-mass spectrometry using a Hewlett-Packard model 5970B mass selective detector connected to a Hewlett-Packard model 5890 gas chromatograph equipped with a 5% phenylmethylsilicone capillary column (12 m, 0.33-μm film thickness). Helium was used as the carrier gas, and the column temperature was increased from 120 to 300 °C at a rate of 10 °C/min. The mass spectrometer was operated in the selected ion monitoring mode using the ions m/z 439 and 443 (unlabeled and deuterated PGF<sub>2α</sub>, respectively) and m/z 423 and 427 (unlabeled and deuterated PGF<sub>2α</sub>, respectively). The amounts of PGF<sub>2α</sub> and PGF<sub>2α</sub> were calculated in the usual manner using ion intensity ratios and the appropriate standard curves. Unreacted PGH<sub>2</sub> was decomposed into 12-hydroxy-heptadecatrienoic acid and malondialdehyde by FeCl<sub>3</sub> in the stop solution.

**Homology Modeling**—To obtain the mPGES-1 model structure, kindly provided by Professor Pär Nordlund, structure-based sequence alignment of MAPEG members and homology modeling were carried out as described (12, 14). PGG<sub>2</sub> was manually fitted in the active site of this model using the computer program O.

**RESULTS AND DISCUSSION**

Microsomal PGES-1 is critical for production of PGF<sub>2α</sub> during inflammatory reactions and has attracted considerable attention as a drug target. Since this enzyme is an integral membrane protein, information on structure-activity relationships has been scarce and essentially limited to a projection map obtained by two-dimensional crystallography, demonstrating a trimeric quaternary structure (15). However, a rapid and significant progress in this area was recently achieved when the crystal structures of MGST-1, 5-lipoxygenase-activating protein, and LTC4S were reported (9–12). In particular, the LTC4S structures allowed a detailed view of the enzyme’s active center and...
revealed a hydrophobic cleft that accommodates the lipid sub-
strate and a deeper pocket for GSH bound in a horseshoe-
shaped conformation. In addition, an Arg residue seemed to be
positioned for activation of the GSH thiol and formation of a
thiolate anion.

A Model of mPGES-1 Identifies Arg-126 as a Potential Cata-
lytic Residue—Based on these new three-dimensional struc-
tures of MAPEG members, a structure-based sequence align-
ment was performed (12), from which a model of mPGES-1
with bound GSH could be generated (14). When compared
with the structure of LTC4S, the lipid binding crevice appears
wider (Fig. 2), as one would expect from the chemistry of PGH2
as compared with leukotriene A4. The co-substrate GSH is
bound in a horseshoe conformation, and Arg-126 has a position
compatible with a role in catalysis (Fig. 2). In the primary struc-
ture, mPGES-1 contains two additional amino aids in the
stretch Tyr-117 to Arg-126, as compared with LTC4S. This
difference between mPGES-1 and LTC4S has been disregarded
in the model, since the two residues do not appear to have any
impact on the α-helical structure carrying Arg-126.

Mutation of Arg-126 Strongly Reduces the Isomerase Activity
of mPGES-1—Arg-126 in human mPGES-1 was exchanged for
an Ala or Gln residue by site-directed mutagenesis. As a con-
trol, a neighboring Arg-122 was also mutated into an Ala or Gln
residue, respectively. Microsome preparations from yeast cells
expressing nonmutated and mutated enzyme were assayed for
PGE2 synthase activity. Nonmutated mPGES-1 converted
almost all added PGH2 (10 μM) to PGE2 when incubated on ice
for 5 min. However, when R126A or R126Q mPGES-1 were
incubated with PGH2, a pronounced loss (85–95%; n = 6) of
conversion into PGE2 was observed (Fig. 3A). In contrast,
R122A and R122Q mPGES-1 retained the activity measured for
wild type enzyme.

Exchange of GSH for Its Ethylester Is Compatible with Catal-
ysis, whereas Substitution for a Thiolester Does Not Allow any
PGE2 Synthase Activity—To gain further mechanistic informa-
tion, we exchanged GSH for various analogues during the incu-
bation with PGH2 (Fig. 3B). S-methyl-GSH did not result in any
PGE2 formation, indicating that the thiol group of GSH is cru-
cial for the reaction. Incubations with GSH-ethylester (esteri-
fied at the C-9 hydroxyl of Glu), on the other hand, did still result in some
PGE2 production (about 28% of nonmutated), which suggests
that minor changes of the GSH binding conformation, at least
near its Glu carboxylate, still allows a productive position of the
GSH free thiol. These data support the proposed role of the
thiolate anion as the attacking species of GSH during conver-
sion of PGH2 to PGE2.

Mutation of Arg-126 Allows a Reductase Activity That Con-
verts PGH2 into PGF2α—Interestingly, when microsome prepa-
rations containing mutants of Arg-126 were incubated with
PGH2, we detected a reductase activity rather than an isomer-
ase activity, such that PGF2α appeared as the major product in
the gas chromatography-mass spectrometry analysis (Figs. 4
and 5A). Both R126A and R126Q mPGES-1 converted PGH2 to
PGF2α, a prostanoid with a distinct spectrum of bioactions, dif-
ferent from those of PGE2. This reductase activity also required
the presence of GSH and its free thiol, as assessed from incuba-
tions with GSH and S-methyl-GSH (Fig. 5B).

Putative Mechanism of mPGES-1—Conversion of PGH2 into
PGE2 by mPGES-1 is GSH-dependent and involves cleavage of
the endoperoxide O–O bond and elimination of the C-9 hydro-
gen as a proton (16). The mechanism of this conversion may
involve glutathione thiolate as a base in the deprotonation step. Alternatively, it has been suggested that glutathione thiolate attacks the C-9 carbon to produce a thiohemiketal intermediate, which spontaneously rearranges to PGE₂ (17). A third possibility would involve attack by glutathione thiolate on the C-9 endoperoxide oxygen, forming a mixed sulfide. Subsequent deprotonation at C-9 and cleavage of the O–S bond would lead to the formation of PGE₂ and regeneration of the glutathione thiolate (Fig. 6). It is noteworthy that the mixed sulfide intermediate of this mechanism would be prone to reduction (e.g. it could be attacked by a second glutathione thiolate to produce PGF₂α and oxidized glutathione) (Fig. 6). In light of our present data, it seems possible that an important function of the active site amino acid structure of mPGES-1 is to prevent such reduction from taking place and that the Arg-126 mutants are defective in this respect and therefore produce PGF₂α rather than PGE₂. This notion is also supported by the structural implications of replacing Arg-126, as illustrated in Fig. 7. Both R126A and R126Q mPGES-1 leave more space around the GSH thiol group, thereby allowing for a second GSH molecule to approach in agreement with the observed PGF₂α formation. In this context, it may be mentioned that enzymatic conversion of PGH₂ into PGF₂α by a glutathione-dependent microsomal endoperoxide reductase has been reported (18), as well as the formation of PGE₂ and PGF₂α by glutathione S-transferase-promoted conversions of PGH₂ (19).

From the crystal structure of LTC₄S in complex with GSH, it was suggested that Arg-104 is critical for the activation of the GSH thiol and stabilization of the thiolate anion (12). Since our data show that mutation of the corresponding Arg-126 is detrimental for the PGE₂ synthase activity, it is tempting to speculate a similar role for this residue. However, the mutants R126A and R126Q mPGES-1 actually retained a very small, albeit detectable, isomerase activity and also exhibited a significant, GSH-dependent reductase activity, which is likely also to involve formation of a thiolate anion (Figs. 3 and 4). Hence, our data suggest that the primary role of Arg-126 is not activation of the GSH thiol. Further structure-function studies with crystallography and mutagenesis will hopefully clarify the precise role.
of Arg-126 and other residues involved in the molecular mechanism of mPGES-1 catalysis.

During the course of these investigations, Jegerschöld et al. (20) reported a structure of mPGES-1 at 3.5 Å resolution, obtained by electron crystallography. GSH was found to bind in a U-shaped conformation, and, interestingly, the authors could not find an access path for PGH₂ to the bound GSH molecule, indicating that the protein was in a closed conformation and that dynamic changes, involving helices 1 and 4, occur at the active site during binding and turnover of the lipid substrate. A putative structure of an open conformation of the active site, with a critical Arg residue (Arg-126), was obtained through modeling against the crystal structure of human LTC₄S. Hence, the mPGES-1 structure presented by Jegerschöld et al. (20) agrees very well with the results of our own investigation and suggests that further mechanistic studies must take into account the consequences of protein dynamics.

Acknowledgment—We thank Gunvor Hamberg for technical assistance.

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FIGURE 7. Model of structural consequences caused by mutations of Arg-126 in mPGES-1. In the homology model, Arg-126 (green, left) has been replaced by Ala (orange, middle) or Gln (violet, right). In addition to GSH (white), the lipid substrate PGH₂ (blue) has been manually fitted into the putative active site.