Microsomal Prostaglandin E Synthase Type 2 (mPGES2) Is a Glutathione-dependent Heme Protein, and Dithiothreitol Dissociates the Bound Heme to Produce Active Prostaglandin E\textsubscript{2} Synthase \textit{in Vitro}\textsuperscript{5}

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An x-ray study indicated that microsomal prostaglandin E synthase type 2 (mPGES2) is a heme-bound protein and catalyzes prostaglandin (PG) H\textsubscript{2} degradation, but not PGE\textsubscript{2} formation (Yamada, T., and Takusagawa, F. (2007) Biochemistry 46, 8414 – 8424). In response to the x-ray study, Watanabe et al. claimed that mPGES2 is a heme-free protein and that both the heme-free and heme-bound proteins have PGE\textsubscript{2} synthesis activity in the presence of dithiothreitol (Watanabe, K., Ito, S., and Yamamoto, S. (2008) Biochem. Biophys. Res. Commun. 367, 782 – 786). To resolve the contradictory results, the heme-binding scheme of mPGES2 was further characterized \textit{in vivo} and \textit{in vitro} by absorption and fluorescence spectroscopies. A substantial amount of heme-bound mPGES2 was detected in cell extracts. The heme content in mPGES2 was increased along with an increase in Fe\textsuperscript{3+} in the culture medium. Heme-free mPGES2 was converted to the heme-bound form by mixing it with pig liver extract, indicating that mPGES2 is capable of forming a complex with heme in mammalian cells. Heme binds to mPGES2 only in the presence of glutathione. The newly determined heme dissociation constant (2.9 ns) supports strongly that mPGES2 is a heme-bound protein in \textit{in vivo}. The bound heme was not dissociated by oxidation by H\textsubscript{2}O\textsubscript{2} or reduction by glutathione or 2-mercaptoethanol. However, reduction by dithiothreitol (an artificial reducing compound) induced the bound heme to dissociate from mPGES2 and released heme-free mPGES2, which exhibited PGE\textsubscript{2} synthesis activity \textit{in vitro}. Imidazole bound to mPGES2 by stacking on the bound heme and inhibited heme oxidation by H\textsubscript{2}O\textsubscript{2} and reduction by dithiothreitol.

Prostaglandin E\textsubscript{2} (PGE\textsubscript{2})\textsuperscript{2} is a paracrine hormone and is ubiquitously distributed in virtually all mammalian tissues and organs. Prostaglandin E synthase (PGES) catalyzes isomerization of prostaglandin H\textsubscript{2} (PGH\textsubscript{2}) to PGE\textsubscript{2}. To date, three PGES enzymes, microsomal PGES1 (mPGES1) (1) and mPGES2 (2) and cytosolic PGES (3), have been identified. However, as described below, studies of mPGES2 (EC 5.3.99.3) have produced contradictory results.

mPGES2 was initially found in and purified from the microsomal fraction of bovine heart (2), and mRNAs encoding human and monkey homologs were subsequently identified (4, 5). Unlike mPGES1, mPGES2 was reported to be a GSH-independent enzyme (2, 4, 5). mPGES2 is synthesized as a Golgi membrane-associated protein, and the proteolytic removal of the N-terminal hydrophobic domain leads to the formation of a mature cytosolic enzyme (5). N-terminally truncated recombinant mPGES2 purified from \textit{Escherichia coli} was reported to have the same enzymatic properties as the enzyme purified from bovine heart microsomes (5).

However, an x-ray study of mPGES2 (6) produced results that differ from those described above. N-terminally truncated recombinant mPGES2 purified from \textit{E. coli} has a dark brown color, and the visible and EPR spectra of the purified protein suggest that mPGES2 is a heme-bound protein. Although the previous study indicated that mPGES2 is a GSH-independent enzyme (2, 4, 5), a characteristic sequence (VPxL.... DSxxI) seen in the GST family (7) was found in the mPGES2 amino acid sequence. The recombinant protein was crystallized, and its structure was determined at 2.8 Å resolution. A GSH molecule binds at the predicted amino acid residues (H\textsuperscript{148}PxL.... H\textsuperscript{166}SxxI), and a heme is attached to the bound GSH by an iron–sulfur bond. mPGES2 containing a GSH-heme complex catalyzes PGH\textsubscript{2} degradation to (12S)-hydroxy-(5Z,8E,10E)-heptadecaatrienoic acid (HHT) and malondialdehyde (MDA) with a catalytic efficiency of 4.8 $\times$ 10\textsuperscript{5} M\textsuperscript{-1} s\textsuperscript{-1}, but not PGE\textsubscript{2} formation. On the basis of the x-ray study and the previous mPGES2 studies, I reported that mPGES2 is the first example of a dual-function enzyme (heme-free mPGES2 and heme-bound mPGES2 catalyze isomerization of PGH\textsubscript{2} to PGE\textsubscript{2} and conversion of PGH\textsubscript{2} to HHT and MDA, respectively). Recently, an mPGES2-deficient

\textsuperscript{5} This article contains supplemental Figs. S1–S4.

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\textsuperscript{2} The abbreviations used are: PGE\textsubscript{2}, prostaglandin E\textsubscript{2}; PGES, prostaglandin E synthase; PGH\textsubscript{2}, prostaglandin H\textsubscript{2}; mPGES, microsomal PGES; HHT, (12S)-hydroxy-(5Z,8E,10E)-heptadecaatrienoic acid; MDA, malondialdehyde.
mouse experiment showed no alteration of PGE₂ levels in several tissues (including liver, kidney, heart, and brain) or in LPS-stimulated macrophages (8), suggesting that mPGES2 is not essential for PGE₂ synthesis in vivo.

In response to the x-ray study of mPGES2, Watanabe et al. (9) claimed that heme-bound mPGES2 was obtained only from the cloned E. coli cells cultured in LB medium containing additional Fe(NO₃)₃ and that mPGES2 purified from the cloned E. coli cells cultured in LB medium was heme-free, suggesting that the heme-bound mPGES2 found in the x-ray study was an artifact. They also showed that both the heme-free and heme-bound proteins had significant PGE₂ synthesis activity in the presence of DTT and that heme-bound mPGES2 catalyzed degradation of PGH₂ to HHT and MDA only in the absence of DTT, and they concluded that mPGES2 is a heme-free protein and is involved in PGE₂ synthesis.

Although Watanabe et al. (9) confirmed that heme-bound mPGES2 has PGH₂ degradation activity, they denied the presence of heme-bound mPGES2. Before the x-ray structure was revealed, Watanabe et al. (10) found that Cys-110 is an essential amino acid residue for PGE₂ synthesis. In the crystal structure, Cys-110 is heavily involved in binding the GSH-heme complex in mPGES2, so Cys-110 cannot participate in the PGE₂ synthesis reaction. Thus, heme-bound mPGES2 is not able to be involved in PGE₂ synthesis. In this regard, it is difficult to accept that both heme-free mPGES2 and heme-bound mPGES2 have PGE₂ synthesis activity (9). The other problem is that the PGE₂ synthesis activity was observed only in the presence of DTT in the reaction mixture. Because DTT is not present in vivo, the PGE₂ synthesis activity of mPGES2 is limited to in vitro. To resolve the contradictory results, it is essential to determine whether mPGES2 is a heme-free or heme-bound protein and whether mPGES2 in solution has a structure similar to that found in the crystal. We further characterize how a heme binds to mPGES2 in E. coli and in pig liver extract using absorption and fluorescence spectroscopies.

**EXPERIMENTAL PROCEDURES**

**Materials**—The chemicals used were reagent-grade or higher. LB medium was prepared from LB broth (USB Corp., Cleveland, OH) dissolved in distilled water (20 g/liter), was autoclaved, and supplemented with 100 mg of ampicillin before use. A minimal medium composed of (NH₄)₂SO₄ (1.0 g), KH₂PO₄ (4.5 g), K₂HPO₄ (10.5 g), sodium citrate (0.5 g), 20 L-amino acids (40 mg each), adenine (125 mg), guanosine (125 mg), thymine (125 mg), uracil (125 mg), glucose (5 g), MgSO₄ (0.12 g), d-ribotin (4 mg), thiamine (4 mg), and ampicillin (100 mg) in 1 liter was prepared as follows. 100 ml of solution containing glucose, MgSO₄, d-ribotin, thiamine, and ampicillin was prepared and sterilized by filtration, and 500 ml of solution containing the other components was autoclaved. The two sterilized solutions were mixed, and water was added to 1 liter.

**Overexpression and Purification**—The mRNA sequence from *Macaca fascicularis* was obtained from the NCBI database (GenBank™ accession number AB046026.1, GI:9280107, *M. fascicularis* mRNA for membrane-associated prostaglandin E synthase-2, complete coding sequence, clone Qc CE16688). The cDNA of the soluble section of monkey mPGES2 (residues 88–378) was constructed from the mRNA sequence, and two restriction sites (BamHI (GGATCC) and EcoRI (AGAATT C)) were appended to the 5’- and 3’-ends, respectively, of the cDNA. The DNA (886 bp) synthesized by a commercial vendor was cloned into the pTrc-HisA vector (Invitrogen) and transformed in HB101 competent *E. coli* cells (Takara Bio Inc.) and BL21 competent *E. coli* cells (New England Biolabs). The DNA sequence in the plasmid was confirmed by DNA sequencing. Because the cDNA was cloned in the pTrc-HisA vector, the recombinant protein was expected to have a His tag at the N terminus.

For the cell extract spectrum experiment, the cloned *E. coli* cells were grown at 30 °C in 50 ml of either LB medium alone or containing 0.25 mM Fe(NO₃)₃ and 1.5 mM ð-aminoethylvulinate. When the cell turbidity measured at 600 nm reached an absorbance of 0.6, isopropyl ß-D-thiogalactopyranoside was added to a final concentration of 1 mM, and the culture was continued for an additional 15 h. Cells were harvested by centrifugation, washed three times with 5 ml of lysis buffer (50 mM Tris-HCl (pH 7.5) and 300 mM NaCl), and stored at −80 °C. *E. coli* cells were suspended into 5 ml of lysis buffer, and lysis was carried out by freezing and thawing. The mixture was subjected to sonication for 180 s at 30% power output using a Vibra-Cell processor (Sonic & Materials, Inc.) in an ice bath. Cell debris was removed by centrifugation. The supernatant was used directly for the absorption spectrum measurement. For SDS-PAGE, 20 µl of the cell extract was dissolved in 80 µl of SDS-PAGE sample buffer, and 5-µl aliquots were loaded.

For purification of the protein, cell growth was scaled up 20-fold. LB medium alone and containing 0.25 mM Fe(NO₃)₃ and 1.5 mM ð-aminoethylvulinate were used for the *E. coli* cell cultures. Because heme-free protein from *E. coli* cells cultured in iron-free minimal medium aggregated during purification, minimal medium containing 5% LB medium was used for heme-free protein purification. Cells were harvested by centrifugation and suspended in 60 ml of buffer containing 50 mM Tris-HCl (pH 7.5) and 0.5 mM EDTA. Cell lysis was carried out by treatment with egg white lysozyme (1 mg/ml of suspension at 0 °C for 1 h), followed by freezing and thawing. The mixture was subjected to brief sonication. The centrifuged supernatant was treated with 300 g/liter ammonium sulfate, and the precipitated protein was recovered by centrifugation. Ammonium sulfate, EDTA, and Tris-HCl were removed by dialysis in buffer A (30 mM potassium phosphate (pH 7.2) and 0.2% Tween 20). The protein was loaded onto a DE52 column (2.4 × 10 cm) equilibrated with buffer A. The protein was eluted by a linear gradient between 100 ml each of 30 and 200 mM potassium phosphate (pH 7.2) containing 0.2% Tween 20. Fractions having a red color were pooled, and imidazole was added to a final concentration of 10 mM. The solution was mixed with 8 ml of nickel-chelating resin (Ni-CAM™ HC resin, Sigma) equilibrated with 30 mM potassium phosphate (pH 7.2) containing 10 mM imidazole. After 1 h of stirring at 4 °C, the resin was collected by centrifugation and packed into a column (1.0 × 10.0 cm). The column was washed with 30 mM potassium phosphate (pH 7.2) containing 10 mM imidazole and 0.01% n-dodecyl-ß-D-maltopyranoside until the absorbance at 280 nm of the elute reached that of the washing buffer. The protein was eluted by a...
linear gradient between 50 ml each of 10 and 200 mM imidazole in 30 mM potassium phosphate (pH 7.2) containing 0.01% n-dodecyl-β-D-maltopyranoside. The red fractions were pooled and dialyzed in 30 mM potassium phosphate (pH 7.2) containing 0.01% n-dodecyl-β-D-maltopyranoside. The protein with a His tag was concentrated to 33 mg/ml (1 mM) using an Amicon concentrator with a 30-kDa cutoff membrane and stored in a −85 °C freezer. Protein purity was checked by SDS-PAGE.

Catalytic Activities of mPGES2 Containing Various Amounts of Heme—The following stock solutions were prepared: buffer solution (50 mM potassium phosphate (pH 7.2) containing 1 mM GSH), mPGES2 solution (14 μM in the buffer solution), and PGH2 solution (0.1 mg/ml acetone solution from Cayman Chemical). 10 μl of mPGES2 and 5 μl of PGH2 were added to a tube containing 85 μl of the buffer solution. The enzyme reaction was carried out for 100 s at 4 °C. The reaction was stopped by adding 100 μl of 0.35% TFA. The reaction mixture was poured onto a pre-equilibrated PrepSep C18 100 mg column (Fisher), and the column was washed with 2 ml of 0.01% TFA and eluted with 300 μl of acetonitrile. The collected solution was diluted with 300 μl of 0.01% TFA, of which 150 μl was injected into an HPLC system (Shimadzu LC-6A) with a NovaPak C18 column (3.9 × 150 mm, Waters) and eluted with 50% acetonitrile containing 0.01% TFA as the mobile phase. The enzyme reaction was repeated 11 times, and 12 fluorescence emission spectra were obtained. Correction for light scattering was not done because the corresponding spectra of heme did not show an emission spectrum. Protein-heme binding was analyzed from the quenching of the intrinsic Trp fluorescence intensity of mPGES2 used in a previous study (6).

Heme Binding Measurements—The intrinsic fluorescence of the Trp residues of mPGES2 was measured after the addition of 5 μl of 5 μM hematin (heme) in dimethyl sulfoxide to 3.0 ml of 50 mM heme-free mPGES2 in 100 mM sodium phosphate (pH 7.2) containing 100 μM GSH. The titration was repeated 10 times, and 12 fluorescence emission spectra were obtained. Correction for light scattering was not done because the corresponding spectra of heme did not show an emission spectrum. Protein-heme binding was analyzed from the quenching of the intrinsic Trp fluorescence intensity of mPGES2 used in a previous study (6).

Oxidation of mPGES2 by H2O2—1 ml of 10 μM heme-bound mPGES2 solution containing 100 mM sodium phosphate (pH 7.2) was prepared in a cuvette at room temperature. An absorption spectrum was recorded, and 10 μl of 100 mM H2O2 was then added to the cuvette. After every 10 min, an absorption spectrum was recorded. Measurement of the absorption spectrum was stopped after 120 min because the spectrum change became negligible. Using the same procedures except for the solution volume (3 ml) and heme-bound mPGES2 concentration (1 μM), the oxidation process of dark brown mPGES2 purified from the cloned E. coli cells cultured in LB medium (referred to as mPGES2-hH) was monitored by the fluorescence spectrum for 120 min.

Reduction of mPGES2 by GSH, 2-Mercaptoethanol, and DTT—Heme-bound mPGES2 was reduced by GSH, 2-mercaptoethanol, and DTT following the same procedures used for heme-bound mPGES2 oxidation by H2O2.

Absorption Spectrum Measurements—All absorption spectra were measured at 23 °C using a JASCO 560V spectrophotometer. The spectrophotometer was calibrated with the appropriate buffer before each measurement. Spectra were recorded at 1-nm intervals at a scan speed of 40–100 nm/min.

Fluorescence Spectrum Measurements—Trp fluorescence spectra were measured at 23 °C using a Cary Eclipse fluorescence spectrophotometer (Varian). Emission spectra were recorded at a interval of 1 nm between 300 and 450 nm with an excitation wavelength of 280 nm at a slit width of 5 nm.

RESULTS

Heme Content in Recombinant mPGES2 Increases along with an Increase in the Iron Concentration in the Culture Medium—The cloned E. coli cells were cultured in three different media containing different amounts of iron. (Minimal medium containing 5% LB medium had a nearly zero iron content, LB medium had an average iron content, and LB medium containing 0.25 mM Fe(NO3)3 had a high iron content.) SDS-PAGE of the cell extracts indicated that different iron contents did not alter the mPGES2
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expression level (supplemental Fig. S1). A substantial amount of heme-bound mPGES2 was detected in extracts of E. coli cells cultured in media containing iron (supplemental Fig. S2). The purified recombinant proteins had three different colors and are designated as mPGES2-zH (light yellow), mPGES2-lH (brown), and mPGES2-hH (dark brown). The absorption spectra of mPGES2-zH, mPGES2-lH, and mPGES2-hH are shown in Fig. 1. Except for the typical protein absorption peak at 280 nm, a relatively broad heme peak (corresponding to the $\gamma$-peak of cytochrome) is observed at 390 nm. The relative peak heights (peak height ratios of $A_{390}/A_{280}$) are 0.042, 0.412, and 0.527, respectively.

**Structures of mPGES2-zH and mPGES2-hH**—The initial crystal structure of mPGES2 was determined by the multiwavelength anomalous dispersion method using SeMet-mPGES2 purified from the cloned E. coli cells cultured in minimal medium containing SeMet instead of Met. The crystal structure of mPGES2 reveals that mPGES2 forms a dimer and that each subunit is composed of two domains, the N-terminal domain (residues 100–175) and the C-terminal domain (residues 220–370), which are connected to form a V-shaped hydrophobic cavity (12). There is no significant electron density in the V-shaped cavity of SeMet-mPGES2. Because mPGES2-zH was obtained from the same minimal medium containing Met instead of SeMet, it is reasonable to assume that the structure of mPGES2-zH is similar to that of SeMet-mPGES2.

In the heme-free structure, the sulfhydryl group of the essential amino acid residue Cys-110 for PGE$_2$ synthesis is exposed to an aqueous environment (Fig. 2A). In contrast, in the mPGES2-hH structure (Protein Data Bank code 2PB), a GSH molecule binds tightly to amino acid residues in a GSH-binding motif ($^{148}$VPxL...$^{164}$DSxxI) at the N-terminal domain via seven H-bonds (including an SH--S H-bond with Cys-110) and hydrophobic interactions. A heme is attached to the bound GSH with an iron–sulfur bond (Fig. 2B).

Heme Cannot Bind to mPGES2 in the Absence of GSH—Using heme-free mPGES2-zH, binding of heme to mPGES2 in the presence of GSH was examined by following the increase in absorbance at 390 nm. The absorption spectrum was similar to those of purified mPGES2-lH and mPGES2-hH (Figs. 1 and 3A). When a binding experiment was carried out in the absence of GSH, the peak profiles were significantly different from those of mPGES2-lH and mPGES2-hH (Fig. 3B). The peak profiles were similar to those of the sum of the spectra of mPGES2-zH and heme itself (Fig. 3C), indicating that heme does not have a specific interaction with mPGES2 in the absence of GSH. Other sulphydryl compounds (DTT and 2-mercaptoethanol) were unable to promote heme binding to mPGES2. These results confirm that GSH is essential for heme binding to mPGES2. This finding is consistent with the presence of the GSH-binding motif ($^{148}$VPxL...$^{164}$DSxxI) in the amino sequence.

Interestingly, purified mPGES2-zH did not contain sufficient GSH, suggesting that GSH is readily dissociated from mPGES2, although mPGES2 has a GSH-binding motif. For GSH to bind tightly to mPGES2, GSH must form a complex with heme in the V-shaped cavity. In this regard, it would be interesting to know mPGES2 is the only protein structure in the Protein Data Bank that contains a GSH–heme complex.

Heme-free mPGES2 Becomes Heme-bound in a Mammalian Cell—It was important to determine whether the characteristics of mPGES2 in mammalian cells are similar to those characterized in the E. coli model described above. Because the transcript for mPGES2 has been found abundantly in various organs, including liver (5), pig liver was used to make a pseudo-mammalian cell environment for characterization of mPGES2 by absorption spectroscopy.

The absorption spectrum of mPGES2-zH in the liver extract was different from the calculated spectrum obtained by summing those of mPGES2-zH and the liver extract (supplemental Fig. S3), suggesting that mPGES2-zH converts to heme-bound mPGES2 in liver extract solution. To confirm this conclusion, mPGES2-zH was mixed with the liver extract, and mPGES2-zH was re-purified by nickel-chelating resin chromatography. An absorption spectrum of the fraction with the highest $A_{280}$ showed a significant peak at 415 nm (Fig. 4), indicating that mPGES2 forms a complex with heme in pig liver extract. As described below, the heme peak was shifted from 390 to 415 nm in the presence of a high concentration of imidazole (200 mM).

The Heme Dissociation Constant Is Determined to Be 2.9 mM—It has been shown that mPGES2 has strong heme affinity in E. coli and in pig liver extract. To analyze the heme affinity quantitatively, the heme dissociation constant was determined to be 2.9 mM using a fluorescence quenching method (6). The details of dissociation constant determination are given in supplemental Fig. S4. The cellular levels of free heme have been

FIGURE 1. Absorption spectra of purified mPGES2 from E. coli cells grown in medium containing various concentrations of Fe$^{3+}$. Solid line, mPGES2-hH from LB medium containing 0.25 mM Fe(NO$_3$)$_3$ (protein concentration of 18 $\mu$M); dashed line, mPGES2-lH from LB medium alone (protein concentration of 13 $\mu$M); dotted line, mPGES2-zH from minimal medium containing 5% LB medium (protein concentration of 10 $\mu$M). The $A_{390}/A_{280}$ peak height ratios of mPGES2-HH, mPGES2-lH, and mPGES2-zH are 0.527, 0.412, and 0.042, respectively.
reported to be submicromolar (13–17). With this heme dissociation constant, >97% of mPGES2 is predicted to be in the heme-bound form if the concentrations of free heme and mPGES2 in the cells are 100 and 1 nM, respectively.

The Bound Heme Is Oxidized by H$_2$O$_2$, but It Does Not Dissociate from mPGES2—The absorption spectrum of heme itself showed that the heme peak at 390 nm gradually decreased and disappeared upon the addition of H$_2$O$_2$ (Fig. 5A). Because hydroperoxides produced in living cells might release the bound heme from mPGES2 by oxidation, it would be important to examine mPGES2 in an oxidation state. mPGES2-hH was oxidized in 1 mM H$_2$O$_2$, and its process was monitored by absorption spectroscopy. The heme peak of mPGES2-hH grad-
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Imidazole Stacks on the Bound Heme and Inhibits Heme Oxidation by H2O2. Heme Dissociation by DTT, and Catalytic Activity—The γ-peaks in the absorption spectra of cytochromes are above 400 nm (415–440 nm) (18), whereas the corresponding heme peak of heme-bound mPGES2 is below 400 nm (390 nm) (Fig. 1), indicating that the bound heme is in a relatively hydrophilic environment. A modeling study suggested that a flat aromatic molecule such as imidazole can bind between the bound heme and the protein. Indeed, the addition of imidazole to the mPGES2-hH solution shifted the heme peak in the absorption spectrum from 390 to 415 nm (Fig. 7A). A red shift in the heme peak indicates that a flat imidazole molecule inserts between the bound heme, the protein expels water molecules in the space, and consequently, the environment of the bound heme becomes hydrophobic. The heme peak was further shifted from 415 to 423 nm by increasing the GSH concentration (Fig. 7B). A similar heme peak shift was observed with other sulfhydryl compounds (DTT and 2-mercaptoethanol).

In the presence of 200 mM imidazole, mPGES2-hH was not oxidized by H2O2 and dissociation of the bound heme by DTT was also not observed. Furthermore, the catalytic activity (conversion of PGH2 to HHT and MDA) of mPGES2-hH was inhibited by 200 mM imidazole. These observations indicate that imidazole, PGH2, H2O2, and DTT bind competitively to the space between the bound heme and the protein (Fig. 2).

Heme-free mPGES2 Catalyzes Isomerization of PGH2 to PGE2 whereas Heme-bound mPGES2 Catalyzes Conversion of PGH2 to HHT and MDA—The catalytic activities of mPGES2 containing different amounts of heme were examined by HPLC, and the results confirmed that mPGES2 has dual functions as described previously (6). The catalytic reactions were carried out in the presence of 1 mM GSH instead of DTT because DTT is not present in vivo. The results are given in Table 1, and the HPLC chromatograms are shown in Fig. 8.
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![Absorption spectra showing downfield shift of the heme peak upon titration with imidazole and GSH.](image)

**TABLE 1**

| mPGES2       | GSH-heme content | Major product from PGH₂ |
|--------------|------------------|-------------------------|
| mPGES2-zH    | ~8%              | PGE₂                    |
| mPGES2-IH    | ~78%             | HHT + MDA               |
| mPGES2-hH    | ~100%            | HHT + MDA               |
| mPGES2-oh⁴   | ~100% oxidized heme | None                   |

⁴ mPGES2-oh oxidized in 1 mM H₂O₂ for 2 h.

activity, whereas mPGES2-IH and mPGES2-hH (that contains a large amount of heme) converted PGH₂ to HHT and MDA. mPGES2-hH oxidized by H₂O₂ did not catalyze PGE₂ synthesis or HHT and MDA formation. The peak height of PGE₂ by mPGES2-zH is relatively low (Fig. 8A), suggesting that the PGE₂ synthesis activity of mPGES2-zH is relatively weak. The ratio of the specific activity for PGE₂ synthesis and HHT and MDA formation for the heme-free and heme-bound enzymes, respectively, is 1:7.6 on the basis of the peak area of the HPLC chromatograms (Fig. 8, A and B).

To examine the heme catalytic activity, PGH₂ was incubated in the reaction solution containing 50 mM phosphate buffer, 1 mM GSH, and various concentrations of heme for 100 s at 4 °C and then analyzed by HPLC (Fig. 8C). Conversion of PGH₂ to HHT was not detected with 1.4 μM heme (the same concentration of mPGES2-hH used for the above catalytic experiment), whereas a significant amount of HHT was detected upon incubation with 140 μM heme. The results indicate that heme can catalyze conversion of PGH₂ to HHT and MDA, but the catalytic efficiency is ~1/100 of that of mPGES2-hH.

Endoperoxide PGH₂ is a very unstable molecule, and its half-life at 37 °C is known to be 5 min (19). It has been reported that PGH₂ converts non-enzymatically to various prostaglandins, including HHT and MDA (20, 21). As shown in Fig. 8B, a control experiment carried out at 4 °C for 100 s exhibited a detectable HHT peak at a retention time of 10.5 min. Two additional control experiments were carried out at 37 °C for 50 and 100 s. Neither experiment altered the peak height of HHT, indicating that non-enzymatic conversion of PGH₂ to HHT and MDA was negligible and that the PGH₂ used in this study was contaminated with a small amount of HHT. Similar results were reported by Gryglewski (22).

**DISCUSSION**

In this study, we have shown the following. (a) Recombinant mPGES2 converts to the heme-bound form if free heme is available. The supporting evidences are 1) the heme content in recombinant mPGES2 was increased along with an increase in the iron concentration in the culture medium; 2) a substantial amount of heme-bound mPGES2 was detected in cell extracts; 3) heme-free mPGES2 was changed to the heme-bound form in a mammalian tissue extract; and 4) mPGES2 has a strong heme affinity (Kₐ = 2.9 nm). (b) mPGES2 in solution has a similar heme binding cavity as observed in the crystal structure. The supporting evidences are 1) bound heme is in a relatively hydrophilic environment; 2) heme cannot bind to mPGES2 in the absence of GSH; 3) the hydrophobicity of the bound heme environment is increased by imidazole; and 4) imidazole inhibits heme oxidation by H₂O₂ and reduction by DTT. (c) The catalytic activities of heme-bound mPGES2 (conversion of PGH₂ to HHT and MDA) and heme-free mPGES2 (conversion of PGH₂ to PGE₂) were confirmed.

These results overwhelmingly confirm that recombinant mPGES2 is a heme-bound protein as seen in the crystal structure. In mammalian cells, mPGES2 is constitutively expressed, so the concentration of mPGES2 in cells is expected to be very low in comparison with that of overexpressed mPGES2 in E. coli cells. Therefore, mPGES2 in mammalian tissue is expected to be a heme-bound form.

The cavity of heme-free mPGES2 is abnormally large for PGH₂ binding because PGH₂ can fit into the space between the bound heme and the protein (Fig. 2B). Furthermore, mPGES2 catalyzes conversion of PGH₂ to PGE₂ without GSH, although mPGES2 has a GSH-binding motif in the cavity. These abnor-
malities in structures support the idea that mPGES2 is not a heme-free protein.

In the crystal structure, the sulfhydryl group of Cys-110 forms an iron–sulfur bond with the heme iron (6). Because Cys-110 is an essential amino acid residue for PGE₂ synthesis (10), heme-bound mPGES2 is not able to participate in PGE₂ synthesis. However, if DTT is utilized during the protein purification and the enzyme assay, DTT induces the bound heme to dissociate from mPGES2 and releases heme-free mPGES2 that exhibits PGE₂ synthesis activity. Therefore, mPGES2 exhibited false PGE₂ synthesis activity in an in vitro experiment. Because DTT is an artificial compound and not present in vivo, heme dissociation would not occur in vivo. In this regard, this conclusion is consistent with an mPGES2-deficient mouse experiment that showed no alteration of PGE₂ levels in several tissues (8).

Why do the other sulfhydryl compounds (GSH and 2-mercaptoethanol) not dissociate heme from mPGES2? One possibility is that GSH and 2-mercaptoethanol do not have a polarized sulfhydryl group that could attack the heme iron to form an iron–sulfur bond. In heme-bound mPGES2, the sulfhydryl group of the bound GSH is polarized by forming an SH/H₁₈₅₂₈/H₁₈₅₂₈/H₁₈₅₂₈SH-bonding network among Cys-113/H₁₈₅₂₈/H₁₈₅₂₈/H₁₈₅₂₈Cys-110/H₁₈₅₂₈/H₁₈₅₂₈/H₁₈₅₂₈GSH and forms an iron–sulfur bond with the bound heme. To break the iron–sulfur bond, a strong nucleophile that attacks the heme iron from the opposite side is needed (Fig. 9). DTT has two sulfhydryl groups and is able to polarize one of the sulfhydryl groups by forming an SH/H₁₈₅₂₈/H₁₈₅₂₈/H₁₈₅₂₈H-bond. The resulting -S⁻ can attack the heme iron and break the opposite side of the iron–sulfur bond. In vivo, the major reducing compound GSH cannot dissociate the bound heme because it has only one sulfhydryl group. If a sufficient amount of natural dithiol

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**FIGURE 8.** HPLC chromatograms showing conversion of PGH₂ (28 μM) to PGE₂ and HHT catalyzed by various forms of mPGES2 and heme at 4 °C in 100 s reaction. A, conversion of PGH₂ to PGE₂ by various forms of mPGES2 (1.4 μM). From bottom to top: mPGES2-zH, mPGES2-lH, mPGES2-hH, mPGES2-oH (mPGES2 oxidized in 1 mM H₂O₂ for 2 h), control (no enzyme), and authentic marker (14 μM PGE₂). By comparing the PGE₂ peak area to that of the PGE₂ marker, it was determined that 3.8% of the PGH₂ was converted to PGE₂ by mPGES2-zH. B, conversion of PGH₂ to HHT and MDA by various stages of mPGES2. From bottom to top: mPGES2-zH, mPGES2-lH, mPGES2-hH, mPGES2-oH, control (no enzyme), and authentic marker (28 μM HHT). By comparing the HHT peak area to that of the HHT marker, it was determined that 28.8% of the PGH₂ was converted to HHT and MDA by mPGES2-hH. C, conversion of PGH₂ to HHT and MDA by mPGES2-hH and various concentrations of heme. From bottom to top: 1.4 μM mPGES2-hH, 1.4 μM heme, 140 μM heme, control (no heme), and authentic marker (140 μM heme).
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FIGURE 9. Putative mechanism of heme dissociation by DTT. A, model structure showing DTT (cyan) in a space between the bound heme and the protein. B, one of the sulfhydryl groups of DTT is polarized by forming an SH–S H bond with another sulfhydryl group, attacks the heme iron, and forms an iron–sulfur bond. The bond formation weakens the iron–sulfur bond in the GSH-heme complex, and subsequently, the DTT-heme complex is released from mPGES2.

components such as lipoic acid (which has vicinal dithiols) is available, it might induce the bound heme to dissociate from mPGES2 in vivo.

mPGES2 is constitutively produced in mammalian cells (23). Heme-bound mPGES2 catalyzes PGH2 degradation to HHT and MDA, so it participates in the decrease in PGH2 (6). A considerable increase in mPGES2 expression is observed in human colorectal cancer and bone marrow stromal cells (23–26) and in the pyramidal neurons of brains from familial Alzheimer disease patients (27). Expression of mPGES2 might be due to uncontrolled PGH2 biosynthesis in these cancer cells and neurons.

It is known that HHT is produced from PGH2 by thromboxane A2 synthase and cyclooxygenase-1 and -2. Thromboxane A2 synthase converts PGH2 to thromboxane A2, HHT, and MDA in a 1:1:1 ratio (28, 29). Cyclooxygenase-1 and -2 convert 88 and 78% of arachidonic acid, respectively, to HHT and MDA through PGH2 in the presence of GSH (1 mM) (11). These findings suggest that the cells are capable of producing HHT. Thus, is it necessary to have an additional enzyme to degrade PGH2 to HHT and MDA? To answer this question, it will be important to elucidate the real function of mPGES2.

In response to our X-ray study of mPGES2 (6), Watanabe et al. (9) claimed that mPGES2 is a heme-free enzyme and that the heme-bound enzyme is produced only from cloned E. coli cells cultured in LB medium containing excess iron. They reported also that both heme-free mPGES2 and heme-bound mPGES2 have significant PGE2 synthesis activity (9). Obviously, their findings do not agree with the conclusion of this study. Perhaps Watanabe et al. reached these conclusions because they obtained heme-free mPGES2 by utilizing DTT during the protein purification and the enzyme assay. In the absence of DTT, they indeed obtained heme-bound mPGES2 that degraded PGH2 to HHT and MDA. However, the absorption spectrum of heme-bound mPGES2 exhibited a heme peak at 415 nm, which differs from the peak at 390 nm obtained in this study. If the absorption spectrum had been measured using a fraction from a nickel column without removing imidazole, the heme peak position at 415 nm would agree with this study.

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