Molecular Characterization of a Novel Type of Prostamide/Prostaglandin F Synthase, Belonging to the Thioredoxin-like Superfamily

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Prostaglandin F (PGF) ethanolamide (prostamide F) synthase, which catalyzed the reduction of prostamide H₂ to prostamide F₂α, was found in mouse and swine brain. The enzyme was purified from swine brain, and its amino acid sequence was defined. The mouse enzyme consisted of a 603-bp open reading frame coding for a 201-amino acid polypeptide with a molecular weight of 21,669. The amino acid sequence placed the enzyme in the thioredoxin-like superfamily with Cys44 being the active site. The enzyme expressed in Escherichia coli as well as the native enzyme catalyzed not only the reduction of prostamide H₂ to prostamide F₂α but also that of PGH₂ to PGF₂α. The Vₘₐₓ and Kₘ values for prostamide H₂ were about 0.25 μmol/min/mg of protein and 7.6 μM, respectively, and those for PGH₂ were about 0.69 μmol/min/mg of protein and 6.9 μM, respectively. Neither PGF₂ nor PGD₂ served as a substrate for this synthase. Based on these data, we named the enzyme prostamide/PGF synthase. Although the enzyme showed a broad specificity for reductants, reduced thioredoxin preferentially served as a reducing equivalent donor for this enzyme. Moreover, Northern and Western blot analyses in addition to the prostamide F synthase activity showed that the enzyme was mainly distributed in the brain and spinal cord. The immunohistochemical study in the spinal cord showed that the enzyme was found mainly in the cytosol. These results suggest that prostamide/PGF synthase may play an important functional role in the central nervous system.

Arachidonoyl 1-ethanolamide (anandamide) is one of the natural ligands for the cannabinoid receptors and is an electrophysiologically neutral derivative of arachidonic acid (1–5). It is biologically and pharmacologically quite distinct from arachidonic acid and is a substrate for cyclooxygenase-2 (6–8). The resultant products of anandamide are prostaglandin (PG) ethanolamides (prostamides), which are a pharmacologically novel class of substances in which the COOH group of a PG is replaced by an ethanolamide group. Prostamides potently stimulated cat iris contraction with a potency closely approaching that of the corresponding PGs; however, prostamide F₂α exhibited no meaningful interaction with the cat recombinant FP receptor (9). It has been suggested based on studies on prostamide F₂α as an agonist that a prostamide F₂α receptor may exist and that it is different from the prostanoid FP receptor (9). The validity of this suggestion has recently been confirmed at the pharmacological level by the discovery of prostamide antagonists (AGN 204396 and AGN 211334) that selectively block the effects of prostamide F₂α, without affecting PGF₂α-mediated responses (10, 11). Although the physiological and pharmacological roles of prostamide F₂α appear to be distinct from those of PGF₂α (9–14), the nature of the enzyme responsible for the synthesis of prostamide F₂α from prostamide H₂ has not yet been clarified. Kozak et al. (15) indicated prostamide formation indirectly by using a coupling system of cyclooxygenase-2 and individual PGE, PGD, PGI, and thromboxane synthases to catalyze the isomerization of prostamide H₂. Although they reported that prostamide F₂α was detected in HCA-7 cells treated with anandamide, the enzyme responsible for prostamide F₂α synthesis was not determined. PGF synthase (EC 1.1.1.188) is a dual-function enzyme that catalyzes the reduction of not only PGD₂ but also that of PGH₂ in the presence of NADPH (16–22) and belongs to the aldo-keto reductase family (18, 21). It forms 9α,11β-PGF₂α, a stereoisomer of PGF₂α, from PGD₂ (PGD₂ 11-ketoreductase activity) and PGF₂α from PGH₂ (PGH₂ 9,11-endoperoxide reductase activity) on the same molecule (16, 19, 20, 22). We also demonstrated that PGF synthase reduced prostamide D₂ to 9α,11β-prostamide F₂ and that the prostamide F₂ analog and anti-glaucoma agent Bimatoprost potently inhibited PGD₂ 11-ketoreductase, PGH₂ 9,11-endoperoxide reductase, and prostamide D₂ 11-ketoreductase activities (23). However, we did not examine the synthesis of prostamide F₂α directly from prostamide H₂. Because prostama-
mide H₂ is not commercially available and would nonenzymatically degrade to prostamides D₂, E₂, and to a lesser extent to prostamide F₂₀. Due to the instability of its endoperoxide group, it has been difficult to examine the enzyme catalyzing the synthesis of prostamide F₂₀ from prostamide H₂ directly. In the present study we synthesized [1⁴C]prostamide H₂ from [¹⁴C]anadamide by using cyclooxygenase-2. With [¹⁴C]prostamide H₂ as a substrate, we measured the prostamide F₂₀ synthase activity of PGF synthase (EC 1.1.1.188) directly. Although PGF synthase also has prostamide H₂ 9,11-endoperoxide reductase activity with Kₘ and Vₘₐₓ values for prostamide H₂ of about 10 μM and 60 nmol/min·mg of protein, respectively, this synthase is mainly localized in the lungs and liver (24). On the other hand, prostamide F synthase activity was mainly localized in the spinal cord and brain. Thus, we found a novel enzyme that directly synthesizes prostamide F₂₀ from prostamide H₂. This enzyme catalyzed only the reduction of the endoperoxide group of PGH₀ or prostamide H₂, but did not catalyze the reduction of the keto-group of PGD₂ or PGE₂. We demonstrated that the enzyme belongs to the thioredoxin-like superfamily, the members of which basically have the CXXC motif as their active site and that thioredoxin served as a reducing equivalent donor for this enzyme.

Thioredoxin (Trx), a small protein of ~100 amino acid residues with the CXXC active site, is ubiquitously present and has been evolutionarily conserved from prokaryotes to higher eukaryotes such as plants and vertebrates (25). In Escherichia coli, thioredoxin was first identified as an electron donor for ribonucleotide reductase. In eukaryotic cells, thioredoxin has been involved in a wide variety of biochemical and biological functions. It can function as a reducing equivalent donor to its target proteins, can facilitate refolding of disulfide-containing proteins, and can modulate the activity of some transcription factors such as NF-κB and AP-1. Moreover, thioredoxin is an efficient antioxidant that can reduce hydrogen peroxide, scavenge free radicals, and protect cells against oxidative stress. Human cytosolic thioredoxin, which is a key component of cellular redox biochemistry and regulation, acts as a co-cytokine upon leaderless secretion (26).

Prostamide/PGF synthase is, thus, a novel enzyme different from PGF synthase previously reported, based on its amino acid sequence, substrate specificity, reductant requirement, and distribution. In this present study we clarified that prostamide/PGF synthase synthesized prostamide/PGF₂₀ from prostamide/PGH₂ by using thioredoxin as a reducing equivalent donor. Consequently, we suggest that this system should be added as a new physiological role of thioredoxin.

**EXPERIMENTAL PROCEDURES**

**Materials**—[1⁴C]Anadamide (arachidonyl-¹⁴C, 2 GBq/mmol) was obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO). [5,6,8,9,12,14,15-³H]PGD₂ (6.2 TBq/mmol) and [5,6,8,11,12,14,15-³H]PGE₂ (5.9 TBq/mmol) were purchased from PerkinElmer Life Sciences. [1⁴C]PGH₂ was prepared by Daichi Pure Chemicals Co. Ltd. (Tokyo, Japan) from [¹⁴C]arachidonic acid as described previously (16) with acetone powder of sheep vesicular gland microsomes (Ran Biochemicals, Tel Aviv) used as a source of PG endoperoxide synthase. Human cyclooxygenase-2 (prostaglandin H synthase) was obtained from Cayman Chemical (Ann Arbor, MI), pColdII bacterial expression vector was from TAKARA BIO INC. (Otsu, Shiga, Japan), and His₅ monoclonal antibody was from BD Biosciences. pET-32a, which is a pET Trx Fusion System 32, was kindly donated by Dr. Takashi Tamura of Okayama University. Other materials and commercial sources were as follows; NADP, NADPH, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase from bakers’ yeast (type IX) were from Sigma; Q Sepharose (Hiload 26/10), HiTrap Blue, HiTrap Q, HiTrap heparin, and phenyl-Superose PC 1.6/5 were from GE Healthcare; precoated silica gel glass plates (F254) were from Merck KGaA. Other chemicals were at least of reagent grade.

**Preparation and Identification of Prostamide H₂**—Preparation of prostamide H₂ is described in the supplemental material. Prostamide H₂ was identified by the liquid chromatographic-electrospray ionization-mass spectrometry (JEOL, Tokyo, Japan) technique under the condition of a mobile phase consisting of acetonitrile/H₂O/acetic acid (115/185/0.5; v/v/v). The liquid chromatographic-electrospray ionization-mass spectrometry procedures were carried out as described previously (23). Prostamide H₂ exhibited distinct chromatographic and mass spectral characteristics. Supplemental Fig. 1A shows its negative-ion mass chromatograms. Prostamide H₂ had m/z 376.1 ([M-H-H₂O]⁻) as the most abundant ion (supplemental Fig. 1B) followed by m/z 430.1 ([M+Cl⁻]), 358.0 ([M-H-2H₂O]⁻), 393.9 ([M-CH₃]⁻), and 454.1 ([M+CH₃COO⁻]⁻) in this order. These ions identified the compound synthesized from anadamide by cyclooxygenase-2 as prostamide H₂. [¹⁴C]Prostamide H₂ was used as a substrate for prostamide F synthase.

**Preparation of Various Mouse Tissues**—Mice (C57BL/6J, 8 weeks) weighing about 30 g (male) or 23 g (female) were sacrificed by perfusion with ice-cold saline under anesthesia with sodium pentobarbital (50 mg/kg of body weight), and various tissues were removed quickly. All procedures were carried out at 0–4 °C. The tissues were weighed and then cut into small pieces and mixed with 3 volumes of 30 mm potassium phosphate buffer (KPB [pH 7.0]). Each mixture was homogenized with a Polytron homogenizer, and after centrifugation at 10,000 × g for 10 min, the supernatant was centrifuged at 100,000 × g for 1 h. The supernatant was used as the enzyme source.

**Purification of Prostamide F Synthase from Swine Brain and Its Partial Amino Acid Sequence**—Purification of prostamide F synthase from swine brain is described in the supplemental material. The partial amino acid sequence of the purified enzyme was identified by Peptide Mass Fingerprint Analysis (Shimadzu Biotech, Tsukuba, Japan). The purified enzyme was digested with trypsin, and the molecular weights of the resulting peptides were determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The amino acid sequences of these peptides were analyzed by using Mascot.

**Construction of Expression Vector**—A Mus musculus brain cDNA clone (Riken Fantom clone ID: 1500010G21, accession number AK005188) was used as a template. The full amino acid sequences of these peptides were analyzed by using Mass Spectrometry.
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sequence deduced from the cDNA clone was named PM/PGFS.

The PM/PGFS fragment was amplified by the PCR using primers PM/PGFS NdeI forward (5'-GGACGGAGCATATGATGAGAATGTGGTGACC-3') and PM/PGFS EcoRI reverse (5'-CAGGGTGAATTTCTACCTCCCACACACC-3'). The conditions for the PCR were as follow: 1 cycle at 98 °C for 1 min and 30 cycles of 10 s at 98 °C, 30 s at 54 °C, and 1 min at 72 °C. The amplified PM/PGFS fragment was digested with NdeI/EcoRI and inserted into the Ndel/EcoRI site of pColdII as pColdII-PM/PGFS.

Purification of the Expressed Prostamide/PGF Synthase—BL21 cells were cultured at 37 °C for 6 h in 500 ml of LB medium (1% Tryptone, 0.5% yeast extract, and 1% NaCl) containing 50 µg/ml ampicillin. Cold shock at 15 °C was applied for 30 min to cultures having 0.4–0.5 absorbance at 600 nm. This was followed by the addition of 1 ml isopropyl 1-thio-β-D-galactopyranoside and culturing at 15 °C overnight. The cells were then harvested by centrifugation at 3000 × g for 20 min and stored at −80 °C until the purification was begun. All procedures were carried out at 0–4 °C. The cells were thawed and suspended in 30 ml KPB (pH 7.0) containing 150 mM NaCl, 0.1 mM β-mercaptoethanol, and 3% glycerol (buffer A). They were then sonicated and centrifuged at 10,000 × g for 10 min. The resulting supernatant was loaded on to a Ni2⁺-agarose column (ProBond, Invitrogen) previously equilibrated with buffer A, and the expressed protein was eluted with a gradient from 0 to 0.5 M imidazole in buffer A. The fractions with prostamide F synthase activity were concentrated by Centriprep-10, and buffer A was exchanged for 10 ml Tris-HCl (pH 7.8) containing 0.1 mM β-mercaptoethanol and 3% glycerol (buffer B). The concentrated enzyme was loaded onto a HiTrap Q column that had been previously equilibrated with buffer B. The expressed protein was eluted with a gradient from 0 to 0.6 M KCl in buffer B. The purified enzyme in 10 ml KPB (pH 7.0) containing 0.1 mM β-mercaptoethanol and 3% glycerol was used as the expressed prostamide/PGF synthase.

Immunological Procedure—A polyclonal antibody against prostamide/PGF synthase was raised in a rabbit by the same method as described previously (16), with peptide GDKVLL-HFVQKSPGDY, amino acids 155–170, of the enzyme used as the immunogen. For Western blot analysis, the enzyme was subjected to SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane (PROTRAN BA85, Whatman GmbH, Dassel, Germany). Protein bands were immunostained with the anti-prostamide/PGF synthase antibody (IgG) and visualized by using an enhanced chemiluminescence kit, Lumi-Light PLUS and Lumi Image (Roche Diagnostics).

Site-directed Mutagenesis—Site-directed mutagenesis was performed by using a QuikChange site-directed mutagenesis kit II (Stratagene, La Jolla, CA). The primers for the C44S, C47S, and C44S,C47S mutants are shown in supplemental Table 1. The plasmid containing PM/PGFS cDNA was amplified with Pfu Ultra polymerase and 2 complementary primers containing the desired mutation by using a thermal cycler (1 cycle at 95 °C for 30 s and 18 cycles of 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 1.5 min). After amplification, mutated plasmids containing staggered nicks were generated. The products were digested with DpnI and used to transform E. coli BL21 cells. The nucleotide sequences of the amplified products were verified. The expressed proteins were purified by the same method as described above under “Purification of the Expressed Prostamide/PGF Synthase.”

Construction of Expression Vector of E. coli Trx Reductase (TrxR) and Purification of the Expressed Trx and E. coli TrxR—Construction of expression vector of E. coli TrxR and Purification of the expressed Trx and E. coli TrxR are described in the supplemental materials.

Enzyme Assays—The standard reaction mixture for prostamide F synthase activity contained 40 µM [1-14C]prostamide H₂ (4.1 kBq), 0.1 mM KPB (pH 7.0), Trx generating system (0.5 mM NADPH, 2 mM Trx, 3.3 mM TrxR) (27), 1 mM ammonium sulfate, and enzyme in a total volume of 50 µl. Incubation was carried out at 24 °C for 1 min. The reaction was started by the addition of prostamide H₂ and terminated by that of 5 µl of 2 M HCl, and termination was followed by immediate extraction with 250 µl of chloroform:methanol (20:1). Prostamide F₂o (about 10 µg) was added to the solution as an authentic marker. The organic phase was subjected to TLC, and the chromatogram was developed with the solvent system of benzene/dioxane/acetic acid (30/50/1). The positions of resultant products (prostamides) on the chromatogram were visualized with iodine vapor, and the radioactivity on the TLC plate was monitored with a bioimaging analyzer FLA-2000. The silica gel was scraped off in those sections corresponding to prostamide F₂o and others, and radioactivity was measured with a Packard liquid scintillation analyzer (TRI-CARB 2100TR, PerkinElmer Life Sciences) using Emulsifier-Scintillator Plus (PerkinElmer Life Sciences). The PGH₂ 9,11-endoperoxide reductase, PGD₂ 11-ketoreductase, and PGE₂ 9-ketoreductase activities were measured as described previously (16, 17, 20–22) except for the addition of 1 mM ammonium sulfate and thioredoxin system instead of NADPH. The hydrogen peroxide reductase activities were measured spectrophotometrically at 24 °C by following the decrease in the absorbance at 340 nm of NADPH in the assay mixture consisting of 0.1 mM KPB (pH 7.0), Trx generating system, 1 mM ammonium sulfate, various concentrations of substrate (hydrogen peroxide, t-butyl hydroperoxide, or cumene hydroperoxide) and enzyme in a total volume of 0.5 ml. Protein concentrations were determined by using a DC protein assay kit (Bio-Rad) with bovine serum albumin as standard.

Northern Blot Analysis—Total RNA was extracted from various mouse tissues, and 1 µg/lane was electrophoresed in 1.2% (w/v) agarose gels containing 0.66 M formaldehyde and transferred to Nylon positively charged membranes (Roche Diagnostics). As a probe for hybridization, mouse cDNA (606 bp) for the coding region of prostamide/PGF synthase was amplified by PCR using primers mouse PM/PGFS F (5'-GGACGGAGCATATGATGAGAATGTGGTGACC-3') and mouse PM/PGFS R (5'-TAATACGACTCACTATAGGGTGACTCCTCCACACACCTCTTC-3'), and the sequence was confirmed. The PCR conditions were 1 cycle at 98 °C for 1 min and 30 cycles for 10 s at 98 °C, 30 s at 55 °C, and 1 min at
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RESULTS

Tissue Distribution of Prostamide F Synthase—We previously showed that NADPH or glutathione (GSH) are required for PGF synthase activity (28). Therefore, the prostamide F synthase activity of the cytosolic fraction of various mouse tissues was measured in the presence of NADPH or GSH. As shown in Fig. 1A, the prostamide F synthase activity in the presence of NADPH was highest in the ovary, with decreasing activity in the uterus, testis, and vesicular glands in this order, and was almost the same as that in the presence of GSH. In organs besides the genital ones, prostamide F synthase activity in the presence of NADPH was high in thymus, brain, and heart (Fig. 1B). The activity in the presence of GSH was the highest in the heart and was almost the same in other organs, including the eyes. In the presence of GSH, the enzyme activity was detected in most tissues as that in the presence of NADPH. Because the distribution of prostamide F synthase activity was different from that of PGF synthase (PGD 11-ketoreductase) activity (24), an enzyme specific for the synthesis of prostamide F₂α was, thus, expected. For the purification of the enzyme, we tested swine tissues as enzyme sources: brain, heart, lung, and eye. The enzyme activity of the cytosolic fraction of each tissue was measured in the presence of NADPH. These specific activities of swine tissues were almost the same as those of mouse tissues, and the activity in swine brain was the highest (data not shown). Therefore, swine brain was used for the purification of prostamide F synthase as an enzyme source.

Purification of Prostamide F Synthase from Swine Brain—During purification, in addition to prostamide F synthase activity, PGF synthase activity was also followed. About 70% of the prostamide F synthase activity was localized in the cytosolic fraction of swine brain. Although about 85% of the activity was found in the 30–80% ammonium sulfate fraction, the enzyme activity decreased to 30–50% during overnight dialysis. The enzyme activity was completely retained on Q Sepharose and was eluted at 0.4–0.6 M KCl (supplemental Fig. 2A). The enzyme activity was separated from other proteins on HiTrap Blue (supplemental Fig. 2B), HiTrapQ (supplemental Fig. 2C), HiTrap heparin (supplemental Fig. 2D), and phenyl Superose (supplemental Fig. 2E) columns. The enzyme activity was eluted from the HiTrap heparin and phenyl Superose columns together with a protein band of molecular mass of about 20 kDa on SDS-PAGE (supplemental Fig. 2, D and E). Because PGF synthase (PGH₂ 9,11-endoperoxide reductase) activity was purified together with prostamide F synthase (prostamide H₂ 9,11-endoperoxide reductase) activity, we called this enzyme prostamide/PGF synthase. A representative protocol of the enzyme purification is summarized in supplemental Table II. About a 102- and 152-fold purification of the prostamide F synthase and PGF synthase activities, respectively, was achieved from the swine brain (90 g) with a yield of 1.4 and 2%, respectively.

Amino Acid Sequence and Expression of Prostamide/PGF Synthase—The band with a molecular mass of 20 kDa from the HiTrap heparin column (supplemental Fig. 2D) was used for the peptide mass fingerprint analysis of prostamide/PGF synthase. Based on the Mascot analysis, the molecular weights of six peptides of the purified enzyme digested with trypsin were identical to those of the corresponding portions of the amino acid sequence deduced from the mouse cDNA (Fig. 2), which encodes a hypothetical protein with unknown function (accession number AK005188). The mouse amino acid sequence showed about 90% identity with those sequences of human.
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(AF425266), ox (BC114900), and pig (AB329665), indicating that prostamide/PGF synthase is widely distributed among various species. These sequences had high homology to those of proteins belonging to the thioredoxin-like superfamily and had CXXC, which is basically the active site of the enzymes belonging to the thioredoxin-like superfamily. Fig. 3 shows SDS-PAGE (Fig. 3A) and Western blot (Fig. 3B) results for each purification step for prostamide/PGF synthase from swine brain. A protein with a molecular mass of about 20 kDa was detected with the anti-prostamide/PGF synthase antibody raised against the peptide as described under “Experimental Procedures.” To further confirm this result, we constructed an expression plasmid (pCold II-PM/PGFS) using polyhistidine-tagged peptide-fused vector and mouse cDNA encoding a 201-amino acid polypeptide with Mr of 21,669 and expressed the protein in E. coli (BL21). The Mr of the expressed protein was 23,234, a little larger than that of the enzyme purified from swine brain due to the 12 amino acid residues of the His tag. We purified the expressed protein to apparent homogeneity by Ni²⁺/H⁺-agarose and Q Sepharose column chromatography (Fig. 3C). Prostamide F synthase and PGF synthase activities of the purified enzyme showed a specific activity of 0.25 and 0.69 H/mg of protein, respectively (Table 1). Zinc staining (Fig. 3C) of the gel indicated that an ~23-kDa protein was produced in the cells harboring pCold II-PM/PGFS, and Western blot analysis also revealed that this protein was recognized by antibody against the peptide sequence of prostamide/PGF synthase (Fig. 3D) or by His₆ monoclonal antibody (data not shown). No protein from the control E. coli bearing pCold II vector without the inserted DNA interacted with either antibody (data not

FIGURE 2. Deduced amino acid sequences of pig (AB329665), ox (BC114900), mouse (AK005188), and human (AF425266) prostamide/PGF synthases. The amino acid sequences of bovine, mouse, and human prostamide/PGF synthase are compared with that sequence of the enzyme purified from swine brain and determined by peptide mass fingerprint analysis and Mascot analysis as described under “Experimental Procedures.” Bars denote sequences corresponding to the peptide fragments obtained by tryptic digestion of the swine brain enzyme. The box shows the CXXC active site motif, and the asterisk indicates the mutated Cys. We determined the amino acid sequence of swine prostamide/PGF synthase (AB329665) by reverse transcriptase-PCR based on that of mouse prostamide/PGF synthase.

FIGURE 3. SDS-PAGE (A and C) and Western blot analysis (B and D) for each step of the purification of native swine prostamide/PGF synthase (A and B) and for the purified murine enzyme expressed in E. coli (C and D). The active fractions at each step (1.7 µg of protein) and the purified expressed enzyme (0.5 µg of protein) were subjected to 15% PAGE in the presence of 0.1% SDS followed by staining with Zn stain. The Western blot analysis was performed with the anti-prostamide/PGF synthase antibody. Lane 1, 100,000 g supernatant of swine brain homogenate; lane 2, 30–80% ammonium sulfate fraction; lane 3, Q Sepharose; lane 4, HiTrap Blue; lane 5, HiTrap heparin; lane 6, phenyl Superose. The positions of the molecular mass standards for A and B (SeeBlue Plus2 Pre-Stained Standard, Invitrogen) are shown at the left of A: bovine serum albumin (98 kDa), glutamic dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa), carbonic anhydrase (36 kDa), myoglobin red (22 kDa), lysozyme (16 kDa), aprotinin (6 kDa). The positions of the molecular mass standards for C and D (Precision Plus Protein Standards, Bio-Rad) are shown at the left of C.
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TABLE 1
Substrate specificity for prostamide/PGF synthase
The reductase activities for prostamide H2 and PGH2, PGD2, and PGE2, were measured by the radiochemical methods described under “Experimental Procedures” and those for H2O2, butyl hydroperoxide, and cumene hydroperoxide by the spectrophotometric methods also described under “Experimental Procedures.” ND, no activities toward PGD2 and PGE2, were detected even at the concentration of 1.5 mM for either substrate.

| Substrate          | Vmax | kcat | km  | kcat/Km |
|--------------------|------|------|-----|---------|
|                    | μmol/min/mg protein | min⁻¹ | mM | min⁻¹/mM |
| Prostamide H2      | 0.25 | 5.4  | 0.0076 | 713 |
| PGH2               | 0.685 | 14.8 | 0.0069 | 2151 |
| PGD2               | ND   | ND   | ND  | ND      |
| PGE2               | ND   | ND   | ND  | ND      |
| H2O2               | 0.281 | 6.1  | 0.16 | 0.17    |
| t-Butyl hydroperoxide | 0.248 | 5.4  | 0.4  | 13.4    |
| Cumene hydroperoxide | 0.281 | 6.1  | 0.026 | 234    |

These results suggest that prostamide/PGF synthase expressed in E. coli was identical to that from swine brain.

Enzymatic Properties of Prostamide/PGF Synthase Purified from Swine Brain and of Murine Enzyme Expressed in E. coli—The specific activities for prostamide F synthase and PGF synthase of the purified enzyme from swine brain were about 53 and 62 nmol/min/mg of protein, respectively, and those for PGD2, and PGE2 were about 510 and 797 nmol/min/mg of protein, respectively. The specific activity for prostamide F synthase purified from swine brain were about 2500 and 713 nmol/min/mg of protein, respectively, and the Km values were 14 and 7 μM, respectively. Almost all enzymatic properties of prostamide/PGF synthase purified from swine brain were the same as those of murine enzyme expressed in E. coli.

The activity of the purified recombinant enzyme was stimulated about 4-fold by 1.5 mM ammonium sulfate (supplemental Fig. 3A). The effect of ammonium sulfate was also observed that on the native swine enzyme (data not shown). This result suggests that the enzyme may not be a monomer but, rather, a dimer or oligomer (29). Although the enzyme was most active with 1.5 mM ammonium sulfate (supplemental Fig. 3A), this high concentration was unsuitable for a conventional assay, and 1 mM ammonium sulfate was added to the standard assay system. The prostamide/PGF synthase activity was proportional to the enzyme amount at least up to about 2 μg (data not shown) and proceeded linearly for at least 1 min (data not shown). The optimal pH for the prostamide F synthase activity was about 7.0 (supplemental Fig. 3B). The Km value for prostamide H2 and the specific activity were about 7.6 μM and about 0.25 μmol/min/mg of protein, respectively, and those for PGH2 were 6.9 μM and 0.69 μmol/min/mg of protein, respectively (Table 1, supplemental Fig. 3, C and D). When the enzyme activity was measured in the presence of various reductants, it was most stimulated, about 9-fold, by the thioredoxin-generating system (supplemental Fig. 3E). The recombinant enzyme also required thioredoxin as a reducing equivalent donor more efficiently than NADPH, NADH, or GSH, which have been reported as hydride donor or reducing equivalent donor for the various PG reductases to date (28). The Km value for reduced thioredoxin was about 1 μM (data not shown). The effects of thioredoxin on the enzyme activity were the same as those on that of the native enzyme (data not shown).

The enzyme catalyzed the reduction of hydrogen peroxide, t-butyl hydroperoxide, and cumene hydroperoxide as well as prostamide H2 and PGH2. As shown in Table 1, the Vmax values for hydrogen peroxide, t-butyl hydroperoxide, and cumene hydroperoxide were about 0.2–0.3 μmol/min/mg of protein, and the Km values (30–1600 μM) were higher than those for prostamide H2 and PGH2, indicating that the catalytic efficiencies (kcat/Km) of these substrates were lower than those for prostamide H2 and PGH2 (Table 1). Moreover, we examined the effects of cumene hydroperoxide on prostamide F synthase activity. Cumene hydroperoxide competitively inhibited prostamide F synthase activity (supplemental Fig. 3, F and G), indicating that the active site for these substrates was the same. Unlike previously reported PGF synthase (16–22) or PGE 9-keotoreductase (30), PGD2 and PGE2 were not reduced to 9α,11β-PGF2 and PGF2α, respectively. These results indicate that prostamide H2 and PGH2 were the best substrates among the substrates tested. The previously reported PGF synthase reacted with prostamide H2 exhibited the Km values of about 10 μM and 60 nmol/min/mg of protein, respectively. These results indicate that prostamide/PGF synthase is a novel enzyme and that the enzyme directly synthesized prostamide/PGF2α from prostamide/PGH2 better than the previously reported PGF synthase (EC 1.1.1.188).

Site-directed Mutation of 44Cys and 47Cys—The amino acid sequence (Fig. 2) of prostamide/PGF synthase indicated that the enzyme belonged to the thioredoxin-like superfamily. The 44CXXC47 motif is known to be the active site of the enzymes belonging to this superfamily. To determine the roles of Cys44 and Cys47 in prostamide/PGF synthase, we constructed a site-directed mutagenesis study using cDNA of the murine enzyme. We constructed three mutants, C44S, C47S, and the double mutant C44S,C47S. These enzymes were expressed in E. coli and purified to homogeneity. The expressed proteins of the wild type and 3 mutants moved to the same position of 23 kDa on SDS-PAGE and were recognized by anti-prostamide/PGF synthase antibody (data not shown). The comparison of the relative activities is given in Fig. 4. C47S retained 63% of the enzyme activity of the wild type, whereas C44S and C44S,C47S showed values below 1% of that activity. These results indicate that Cys44 was essential for the catalytic activity of prostamide/PGF synthase and that Cys47 did not subserve directly the conversion of prostamide/PGH2 to prostamide/PGF2α.

Prostamide/PGF Synthase Activity Assayed with Thioredoxin System as a Reducing Equivalent Donor and Northern and Western Blot Analyses in Various Mouse Tissues—Because the thioredoxin system was effective, the prostamide F synthase...
Prostamide/PGF Synthase Belonging to Thioredoxin-like Superfamily

FIGURE 5. Tissue distribution of prostamide/PGF synthase activity in the presence of thioredoxin system as a reducing equivalent donor (A) and Northern (B) and Western (C) blot analyses of various mouse tissues. A, the preparation of tissues and the enzyme assay were the same as those described in Fig. 1, except that the Trx-generating system was used as a reducing equivalent donor. B, the upper panel shows the 18.5 and 28.5 S of ribosomal RNAs of various tissues, and the lower panel shows the probe hybridized to a 1-kilobase mRNA in various tissues. The probe and total RNA were prepared as described under “Experimental Procedures.” C, Western blot analysis was conducted by the same method as described in Fig. 3, B and D. The mouse multiple tissues contained about 45 μg of protein/lane.

activity of the cytosolic fraction of various mouse tissues was reexamined in the presence of thioredoxin system. As shown in Fig. 5A, the enzyme activity was the highest in spinal cord and brain followed by thymus, adrenal gland, heart, and genital organs. Northern blot analysis (Fig. 5B) revealed that the probe hybridized to a 1-kilobase mRNA in spinal cord, brain, ovary, or heart. In the thymus and adrenal gland, the probe only slightly hybridized to the 1-kilobase mRNA because the mRNAs of these tissues were slightly degraded. Moreover, Western blot analysis (Fig. 5C) revealed that a protein with a molecular mass of about 20 kDa in extracts of spinal cord, brain, thymus, adrenal gland, or ovary cross-reacted with the anti-prostamide/PGF synthase antibody. It is possible that the enzyme may form a dimer, based on the effect of ammonium sulfate on the enzyme activity. A protein with a molecular mass of about 40 kDa, which was reactive with the antibody in thymus, adrenal gland, heart, and genital organ, may have been a dimer of the enzyme even after SDS-PAGE in β-mercaptoethanol-containing buffer. These results of Northern and Western blot analyses almost coincided with those for the distribution of the prostamide/PGF synthase activity in the presence of the thioredoxin system.

Immunohistochemical Localization of Prostamide/PGF Synthase in Mouse Spinal Cord—Using anti-prostamide/PGF synthase antibody for an immunohistochemical study on mouse spinal cord, we found that the enzyme was localized in the superficial layer of the dorsal horn (Fig. 6A), in motor neurons of the ventral horn (Fig. 6, B and C), and in glia of the white matter of the spinal cord (Fig. 6D), but this immunoreactivity was not found with the antigen-absorbed antibody (insets in Fig. 6, A and B). Immunoreactivity of prostamide/PGF synthase in motor neuron of the ventral horn was diffusely observed in the cytoplasm (Fig. 6C). Fig. 6G shows double labeling created by merging the images obtained for prostamide/PGF synthase (Fig. 6E, red) and protein disulfide isomerase as an endoplasmic reticulum marker (Fig. 6F, green). The immunoreactivity in the motor neurons was found mainly in the cytosol and also appeared to be present on the endoplasmic reticulum. However, studies with electron microscopy are required for more detailed cellular localization.

DISCUSSION

By the use of [14C]prostamide H₂, we found a new enzyme in mouse and swine brain, which catalyzed not only the reduction of prostamide H₂ to prostamide F₂, but also the reduction of PGH₂ to PGF₂α (PGH₂ 9,11-endoperoxide reductase activity). The $K_m$ and $V_{max}$ values for prostamide H₂ were 7.6 μM and 0.25 μmol/min/mg of protein, respectively, and for PGH₂ were 6.9 μM and 0.69 μmol/min/mg of protein, respectively. This enzyme did not catalyze the reduction of the keto group of
PGD$_2$ or PGE$_2$. Although the enzyme showed broad substrate specificity in terms of endoperoxide reduction, prostamide H$_2$ and PGH$_2$ were the best substrates for this enzyme. The enzyme activity was stimulated by ammonium sulfate, suggesting that the enzyme may be a dimer or oligomer instead of a monomer (29). For the native conformation, some components or activators may be required. The lack of some components or coactivators, which have not yet been identified, may be required. The lack of some components or coactivators may explain why the $k_{cat}$ values for prostamide H$_2$ and PGH$_2$ (5.4 and 14.8 min$^{-1}$, respectively) were not so high. This enzyme consisted of a 603-base pair open reading frame coding for a 201-amino acid polypeptide with a $M_r$ of 21,669, and it was placed in the thioredoxin-like superfamily. The enzyme had the $^{44}$CXC motif of this superfamily, and Cys$^{44}$ was proposed to be the active site of this enzyme. Moreover, the enzyme preferentially utilized the thioredoxin/thioredoxin reductase system for the reduction of prostamide/PGH$_2$ to prostamide/PGF$_{2a}$, as a reducing equivalent donor. Based on its enzymatic properties, amino acid sequence, and reductant requirement, we concluded that it was a novel enzyme, and so we named this enzyme prostamide/PGF synthase.

Prostaglandin synthase (EC 1.1.1.188) also catalyzed the reduction of prostamide H$_2$ to prostamide F$_2a$ (prostamide H$_2$, 9,11-endoperoxide reductase activity; see “Results”) as well as that of prostamide D$_2$ to 9a,11β-prostamide F$_2$ (prostamide D$_2$, 11-ketoreductase activity, (23)). The $K_m$ and $V_{max}$ values of PGF synthase for prostamide H$_2$ were about 10 $\mu$m and 60 nmol/min/mg of protein, respectively. The $V_{max}$ value of PGF synthase for prostamide H$_2$ was 5-fold lower than that of prostamide/PGF synthase, suggesting that the latter enzyme contributes more to the synthesis of prostamide F$_2a$. Moreover, the distribution of PGF synthase activity (24) was different from that of prostamide F synthase activity. PGF synthase activity was high in lungs and liver and was low in spinal cord and brain. In contrast, the prostamide/PGF synthase activity was high in the spinal cord and brain and, thus, may contribute to the synthesis of prostamide/PGF$_{2a}$ in these tissues. Prostamide/PGF synthase also synthesized not only prostamide F$_2a$ from prostamide H$_2$ but also PGF$_{2a}$ from PGH$_2$.

This enzyme was immunohistochemically localized in the superficial layer of the dorsal horn of the spinal cord. Muratani et al. (31) reported that PGF$_{2a}$ induces mechanical allodynia via a PGF$_{2a}$ receptor. Thus, prostamide/PGF synthase in the spinal cord may serve in the synthesis of PGF$_{2a}$ related to pain. Moreover, the enzyme was detected in pyramidal cells of the cerebrum and hippocampus of the mouse brain. In the kainate-treated rat brain, there was an increased production of PGE$_2$, PGF$_{2a}$, and PGD$_2$ (32), and kainic acid-induced seizure in rats caused production of large amounts of PGF$_{2a}$ and PGD$_2$ in the hippocampus (33). Equally, prostamide/PGF synthase may contribute to the elevation of PGF$_{2a}$ in kainate-treated brains. The widespread distribution of prostamide/PGF synthase in the central nervous system (CNS) may play a potentially important role of prostamide/PGF$_{2a}$ in modulation of neuronal activity. Besides the CNS, the genital organs also expressed the enzyme. PGF$_{2a}$ is found in genital organs and has been called the primary PG. Many reports concerning the physiological functions of PGF$_{2a}$ in the genital organs have been available until now (34). Not only in the CNS, but also in genital organs, prostamide/PGF synthase would be expected to contribute to the synthesis of PGF$_{2a}$.

Knock-out mice lacking fatty acid amide hydrolase, which catalyzes the hydrolysis of the endocannabinoid/endovanilloid anandamide, have been reported to produce prostamides from anandamide (35). Recently, it was reported that a prostamide F$_{2a}$ receptor distinct from the PGF-sensitive FP receptor seems to exist, based on studies with antagonists (10, 11). More recently, Maione et al. (36) reported that the elevation of anandamide tissue levels seemed to be highly related to both acute and chronic peripheral pain. This result together with the localization of prostamide/PGF synthase in the spinal cord suggests that prostamide F$_{2a}$ may also contribute to the pain. Considering the wide distribution of this enzyme, its product prostamide F$_{2a}$ may play new physiological roles in the CNS and other tissues via the prostamide F$_{2a}$ receptor.

Prostamides/PGF synthase required the thioredoxin system for its enzymatic activity. The thioredoxin system (thioredoxin, thioredoxin reductase, and NADPH) is ubiquitous from Archaea to humans. Thioredoxins are critical for redox regulation of protein function and signaling via thiol redox control (37). Proteins in the extracellular environment or on the cell surface are rich in stabilizing disulfides, reflecting oxidizing conditions there. In contrast, the inside of the cell is kept in the reduced state, and so proteins contain many free sulfhydryl groups, and disulfides are rare. The major ubiquitous disulfide reductase responsible for maintaining proteins in their reduced state is thioredoxin, which is reduced by electrons from NADPH via thioredoxin reductase (37). According to the scheme of oxidoreductase activities of the thioredoxin system proposed by Arner and Holmgren (37), prostamide/PGH$_2$ is reduced to prostamide/PGF$_{2a}$ by prostamide/PGF synthase using the thioredoxin system (Fig. 7A). On the other hand, considering that prostamide/PGF synthase has also a CXXC motif like thioredoxin, we speculate another mechanism for the reduction of prostamide/PGH$_2$ to prostamide/PGF$_{2a}$ (Fig. 7B); prostamide/PGH$_2$ is reduced to prostamide/PGF$_{2a}$ by the SH group of Cys$^{44}$ in $^{44}$CXXC$^{47}$, and then Cys$^{44}$ and Cys$^{47}$ are oxidized to form a disulfide. The disulfide is reduced by thioredoxin-(SH)$_2$, and thioredoxin-(SH)$_2$ is oxidized to thioredoxin-S$_2$. The oxidized thioredoxin-S$_2$ is then reduced by NADPH and thioredoxin reductase. Arner and Holmgren (37) reported that thioredoxin reduces disulfides with the rates for proteins being orders of magnitude faster than those for dithiothreitol or GSH, that thiol-disulfide exchange reactions, which are rapid and readily reversible, are also ideally suited to control via the redox state of structural or catalytic SH groups, and that the mechanism of thiol redox control emerges as a major regulatory mechanism in signal transduction. The thioredoxin system in the synthesis of prostamide/PGF$_{2a}$ from prostamide/PGH$_2$ may play physiological and pathological roles in various tissues, especially in the CNS and genital organs. Moreover, prostamide F synthase activity was also detected in swine eyes (data not shown). The thioredoxin system is induced in culture swine

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4 K. Watanabe, unpublished observations.
lenses under oxidative stress (38). In the eye, prostamide/PGF$_{2\alpha}$ may be synthesized by prostamide/PGF synthase using the thioredoxin system.

An unrooted phylogenetic tree of prostamide/PGF synthase and its close relatives is shown in supplemental Fig. 4. The orthologous proteins seem to be widely present in vertebrates, and such a distribution is consistent with the distribution of cyclooxygenase. In addition to these proteins, several proteins derived from other eukaryotes, such as an echinoderm and a platyhelminth, were detected. These were all hypothetical proteins, so that the actual functions of them have not been identified yet. Prostamide/PGF synthase and its relatives show sequence similarity to a one-Cys peroxiredoxin derived from Mycobacterium tuberculosis (39), which is included in the phylogenetic tree of supplemental Fig. 4 as an outgroup. The one-Cys peroxiredoxin belongs to the thioredoxin-like superfamily.

Until today, the enzymes related to the synthesis of PGF$_2\alpha$ have been reported to require NAD(P)H or GSH (28). Those requiring the former belong to the aldo-keto reductase family, whereas those utilizing the latter have not yet been identified, since it has not been revealed what types of GSH-dependent enzymes are related in this activity at this stage. As described above, however, prostamide/PGF synthase belongs to the thioredoxin-like superfamily, and neither NAD(P)H nor GSH was the best reducing equivalent donor for this enzyme. Both the sequence similarity and the enzymatic properties suggest that the evolutionary origin of prostamide/PGF synthase is different from that of the other reported PGF syntheses. With respect to the arachidonic acid cascade, this is the first report of a reduction system using thioredoxin. As an enzyme having the CXXC motif, mPGE synthase-2 has been reported (40). mPGE synthase-2 is an isomerase, unlike prostamide/PGF synthase, which is a reductase, and the enzyme requires di-thiothreitol or dihydrolipoic acid as a cofactor. Although the reaction mechanism of these enzymes is different, we presently found prostamide/PGF synthase to be, like mPGE synthase-2, an enzyme with the CXXC motif. In the near future, other enzymes with the CXXC motif and involvement in eicosanoid metabolism may be found.

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