cDNA Cloning, Expression, and Mutagenesis Study of Liver-type Prostaglandin F Synthase*

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Prostaglandin (PG) F synthase catalyzes the reduction of PGD<sub>2</sub> to 9α,11β-PGF<sub>2</sub> and that of PGH<sub>2</sub> to PGF<sub>2α</sub> on the same molecule. PGF synthase has at least two isoforms, the lung-type enzyme (K<sub>m</sub> value of 120 μM) for PGD<sub>2</sub> (Watanabe, K., Yoshida, R., Shimizu, T., and Hayaishi, O. (1985) J. Biol. Chem. 260, 7035–7041) and the liver-type one (K<sub>m</sub> value of 10 μM) for PGD<sub>2</sub> (Chen, L.-Y., Watanabe, K., and Hayaishi, O. (1992) Arch. Biochem. Biophys. 296, 17–26). The liver-type enzyme was presently found to consist of a 869-base pair open reading frame coding for a 323-amino acid polypeptide with a Mr of 36,742. Sequence analysis indicated that the bovine liver PGF synthase had 87, 79, 77, and 76% identity with the bovine lung PGF synthase and human liver dihydrodiol dehydrogenase (DD) isozymes DD1, DD2, and DD4, respectively. Moreover, the amino acid sequence of the liver-type PGF synthase was identical with that of bovine liver DD3. The liver-type PGF synthase was expressed in COS-7 cells, and its recombinant enzyme had almost the same properties as the native enzyme. Furthermore, to investigate the nature of catalysis and/or substrate binding of PGF synthase, we constructed and characterized various mutant enzymes as follows: R27E, R91Q, H170C, R223L, R225L, S301R, and N306Y. Although PGD<sub>2</sub> 11-ketoreductase activity was competitively inhibited by PQ, PGH<sub>2</sub> 9,11-endoperoxide reductase activity was not inhibited by PGD<sub>2</sub>, PGF<sub>2α</sub>, or PGH<sub>2</sub> by PG 9-ke toreductase, PGD 11-ketoreductase, or PGH 9,11-endoperoxide reductase, respectively. PGF synthase (EC 1.1.1.188) was purified from bovine lung by Watanabe et al. (3). It forms 9α,11β-PGF<sub>2</sub> (4) from PGD<sub>2</sub> (PGD<sub>2</sub> 11-ketoreductase activity) and PGF<sub>2α</sub> from PGH<sub>2</sub> (PGH<sub>2</sub> 9,11-endoperoxide reductase activity) on the same molecule in the presence of NADPH (3, 4). This enzyme catalyzes the reduction of other carbonyl compounds including 9,10-phenanthrenequinone (PQ) as well as that of PGD<sub>2</sub> and PGH<sub>2</sub> but does not catalyze the reduction of PGE<sub>2</sub>. Although PGD<sub>2</sub> 11-ketoreductase activity was competitively inhibited by PQ, PGH<sub>2</sub> 9,11-endoperoxide reductase activity was not inhibited by PGD<sub>2</sub> or PQ (3). PGF synthase belongs to the aldo-keto reductase family. The bovine lung PGF synthase is a monomeric protein with a Mr of 36,666 consisting of 323 amino acids, and its amino acid sequence shows high homology compared with that of other aldo-keto reductase family members (5). PGF synthase has two isozymes, one in the lung (3) and the other one in the liver (6), with different K<sub>m</sub> values for PGD<sub>2</sub> (120 and 10 μM, respectively). The regulation by metals, the sensitivity to chloride ions, the inhibition by CuSO<sub>4</sub> and HgCl<sub>2</sub>, and the profile of immuno-precipitation with anti-bovine lung PGF synthase antibody are different between the two isozymes (6). Although Kuchinke et al. (7) isolated a clone (PGFS II) of PGF synthase from bovine liver and determined its amino acid sequence, the 99% similarity with the amino acid sequence of lung PGF synthase and the high K<sub>m</sub> value for PGD<sub>2</sub> of this recombinant PGFS II indicated that its cDNA was that of the lung-type enzyme even though it had been isolated from liver. Until now, the primary structure of the liver-type PGF synthase and the amino acids related to the affinity for PGD<sub>2</sub>...
have not yet been defined.

Dihydrodiol dehydrogenase (DD, EC 1.3.1.20) catalyzes the NAPD-linked oxidation of trans-dihydrodiols of aromatic hydrocarbons to the corresponding catechols and is distributed in various mammalian tissues. DD, also belonging to the aldehyde reductase superfamily, has been purified from various animal tissues, i.e., human liver (8), rat liver (9, 10), rabbit liver (11), mouse liver (12), bovine liver (13), guinea pig testis (14), and so forth. Human liver DD exists in at least four multiple forms (DD1–DD4) with similar mass of about 36 kDa (8). Human DD3 was identified as an aldehyde reductase, and the other three forms exhibited 3α-hydroxydehydrogenase (HSD, EC 1.1.1.213) activity. Bovine liver DD also has three multiple forms (DD1–DD3), namely DD1 (3α-HSD), DD2 (high Km aldehyde reductase), and DD3 (dihydrodiol-specific enzyme) (15). Human liver DD1, DD2, and DD3 are not identical with bovine liver DD1, DD2, and DD3, respectively, on the basis of their enzymatic properties including the substrate specificity. The amino acid sequence of the bovine lung-type PGF synthase (3, 7) showed an identity of 81, 79, 78, and 87% with that of human liver DD1, DD2, and DD4 (15, 16) and bovine liver DD3 (17). Among human liver DDs, DD1 and DD2 exhibited PGF synthase activity with Km values of 12 and 79 μM, respectively, for PGD2, but this activity of DD4 was not detected (16). Moreover, PGF synthase activity of bovine liver DD3 has not yet been reported.

In the present study, we describe the primary structure of the bovine liver-type PGF synthase and the enzymatic properties of the recombinant enzyme in COS-7 cells. Based on the comparison of the amino acid sequences among the liver-type and the lung-type PGF synthases and human liver DDs, several mutants were constructed, and their enzymatic properties were examined. The results of mutagenesis indicated the amino acid residues related to the binding sites of PGD2, PGH2, and PQ.

**EXPERIMENTAL PROCEDURES**

Materials—5,6,8,9,12,14,15-7H)PGD2 (3.7 TBq/mmol) was obtained from New England Nuclear Products. [1-3H]PGH2 was prepared as described previously (18), with acetone powder of sheep vesicular gland microsomes (Rao Biochemicals, Tel Aviv, Israel) used as a source of PG endoperoxide synthase. Authentic PGs were kindly donated by Ono Pharmaceutical Co. pEF-BOS mammalian expression vector was a generous gift from Dr. S. Nagata. Other materials and commercial sources were as follows: NAPD, NADPH, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase from bakers’ yeast (type IX), from Sigma (Japan); Red Sepharose and NAP10, from Amersham Pharmacia Biotech, UK; glucose-6-phosphate dehydrogenase from bakers’ yeast (type IX), from NEN Life Science Products. [1-14C]PGH2 was prepared as described previously (5).

**cDNA Cloning of Liver-type Prostaglandin F Synthase**—Total RNA was prepared from bovine liver with a total RNA purification kit (Amersham Pharmacia Biotech, UK) according to the manufacturer’s manual. The resulting purities were determined to be 1.8 by spectrophotometry. The resulting RNAs were divided into two equal portions and were subjected to ammonium sulfate fractionation between 40 and 75% saturation. The precipitate containing the internal cDNA and were used as two different probes for screening of the library.

The bovine liver cDNA library was constructed from 5 μg of poly(A)+ RNA with SuperScript™ Plasmid System (Life Technologies, Inc.) for cDNA synthesis and a Plasmid Cloning Kit (Life Technologies, Inc.) for cloning. The resulting plasmid was transformed into Electromax DH12S competent cells (Life Technologies, Inc.) by the electroporation method using a GenePulser (Bio-Rad). The library yielded 2.0 × 106 independent clones. Full-length cDNA clones were obtained by colony hybridization method. All clones were spread on 20 sheets of nylon filters for the master filter, and then two filters were replicated from each master filter. The replicate filters were alkaline-denatured and fixed by baking at 80 °C for 2 h. Two reverse transcriptase-PCR products, 720 and 477 base pairs described above, were randomly labeled by [α-32P]dCTP (111 TBq/mmol, Amer- sham Pharmacia Biotech, UK) with a Megaprime random primer labeling kit (Amersham Pharmacia Biotech, UK) and used as two probes for hybridization. After hybridization in 5× SSC, 0.1% SDS, 100 μg salmon sperm DNA, and 10× Denhardt’s solution at 65 °C for overnight, each filter was washed extensively twice in 2× SSC, 0.1% SDS at room temperature for 5 min and twice in 0.5× SSC, 0.1% SDS at 60 °C for 30 min. Thirty two double-positive clones against the two different probes were obtained from 2.0 × 107 independent clones. Six clones were picked up and sequenced with an Applied Biosystems Inc. automated DNA sequencer 373A (Perkin-Elmer). All clones coded for full-length cDNAs of full-length liver PGF synthase. One of these six clones was named pSPORT-BLIFS27.

**Expression of Bovine Liver PGF Synthase in COS-7 Cells and Purification of Its Expressed Protein**—The bovine liver cDNA insert was removed from pSPORT-BLIFS27 by digestion with EcoRI and AflIII, and its ends were blunted. The blunt-ended insert containing the complete coding region was subcloned into the blunt-ended pBluescript KS(−) vector of a pBluescript mammalian expression vector (20). Monkey COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium (Nissui Co., Tokyo) containing 10% fetal calf serum. COS-7 cells (5 × 105 cells) were transfected with 20 μg of plasmid DNA by the electroporation method using a Gene Pulser (Bio-Rad). These cells were incubated in a 5% CO2 containing medium at 37 °C for 72 h. The transfected cells and COS-7 cells containing the empty vector pSPORT-BLIFS27 were plated into a 24-well culture plate in 2 ml of 10% fetal calf serum growth medium (KPB) (pH 7.0). The recombinant enzyme was purified by the method of Chen et al. (6) with a minor modification. The cytosol fraction of the homogenated cells, which was centrifuged at 100,000 × g, was subjected to ammonium sulfate fractionation between 40 and 75% saturation. The precipitate formed was suspended in 500 μl of 10 mM KPB (pH 7.0) and desalted by passage through a NAP-10 column. The desalted sample was applied to...
a Red Sepharose column, and the enzyme was eluted with 10 mM KPB (pH 7.0) containing 1 mM KCl and 1 mM NADP. About 2.8-fold purification of the recombinant protein was achieved, and the apparent homogeneity was concluded following SDS-polyacrylamide gel electrophoresis (PAGE) and staining with Two-dimensional Silver Stain II (Daichii Pure Chemicals Co., Ltd., Japan). A polyclonal antibody against PGF synthase was raised in a rabbit by the same procedure as described previously (3), with the enzyme purified from bovine liver used as the immunogen (6). For Western blot analysis, the purified enzyme was subjected to SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride membrane (Ammersham Pharmacia Biotech, UK). Protein bands were immunostained with the anti-bovine liver PGF synthase antibody and reagents from a Vectastain ABC kit (Vector Laboratories) and visualized with an Enhanced Chemiluminescence Kit (ECL•, Amersham Pharmacia Biotech, UK).

**Enzyme Assay**—The PGD₄₁-ketoreductase, PGH₂, 9,11-endoperoxide reductase, and PQ reductase activities of the recombinant protein were measured as described previously (3). The standard assay mixture for PGD₄₁-ketoreductase contained 0.1 mM KPB (pH 6.5), 0.5 mM NADP, 5 mM glucose 6-phosphate, glucose-6-phosphate dehydrogenase (1 unit), 1.5 mM [³H]PGD₂ (3.7 KBq), and enzyme in a total volume of 50 μl. Incubation was carried out at 37 °C for 30 min. The PGH₂, 9,11-endoperoxide reductase activity was assayed under the same conditions as those of the PGD₄₁-ketoreductase enzyme activity except that 40 μM [¹⁴C]PGH₂ (4 MBq) was used as a substrate in place of 1.5 mM [³H]PGD₂. The PGD₂ 11-ketoreductase activity was measured spectrophotometrically at 37 °C by following a decrease in absorbance at 340 nm in the assay mixture consisting of 0.1 mM KPB (pH 6.5), 80 μM NADPH, 10 μM PQ, and enzyme in a total volume of 0.5 ml. One unit of enzyme activity was defined as the amount that produced 1 μmol of PGF₃, per min at 37 °C. Specific activity was expressed as the number of units/mg of protein. Protein was determined according to the method of Lowry et al. (21).

**Site-directed Mutagenesis**—A mutagenesis study was performed by the method of Jones et al. (22). The mutagenesis primers for the mutant R27E were designed as follows: LiFSN1, 5′-CTCCCTTGCGGCTTCTG-3′; LuFSN1, 5′-TTGCGGCTTCTGAGCC-3′; LuFSP1, 5′-TTGCGGCTTCTGAGCC-3′; LuFSN2, 5′-CTCCTCTCGGGGCTACAGGTT-3′; LiFSPC, 5′-GAATCTCAGGGCAGGTCG-3′; and LiFSFP2, the wild type and 3′UTR of DNA polymerase I after recovery. These products were mixed at the ratio of 1 to 1, and the second PCR was conducted using LiFSNP and LiFSFP2 as described above. The product of the second PCR was blunt-ended and then was ligated to the blunt BamHI sites of the pEF-BOS expression vector. Consequently, the mutant of R27E was formed. The other mutant enzymes were formed by the same procedure as used for the R27E mutant.

## RESULTS

cDNA Cloning of Bovine Liver PGF Synthase—Screening of 2.0 × 10⁶ clones with the two probes (P1/P2 and P3/P4) products described under “Experimental Procedures” gave 32 double-positive clones, and six independent clones of these 32 clones were picked up. DNA sequencing confirmed that these clones coded for full-length cDNAs of bovine liver PGF synthase. The deduced amino acid sequences of bovine liver PGF synthase contained all the amino acid sequences of the nine peptide fragments obtained from the native bovine liver enzyme (Fig. 1). The bovine liver PGF synthase cDNA clone BLiFS27 contained a polyadenylation signal after the stop codon (Fig. 1), showing that it coded for a full-length PGF synthase. BLiFS27 contained an open reading frame of 969 base pairs coding for 323 amino acids. The calculated Mr of the bovine liver enzyme was 36,742, a value similar to that of the native enzyme, which is about 36 kDa (6). The identity between the liver and lung enzymes was 87% at the amino acid level and 90% at the nucleotide level. As shown in Fig. 2, this enzyme showed a high identity in amino acid sequence with not only bovine lung PGF synthase (87%) but also human liver DD1 (79%), DD2 (77%), and DD4 (76%). Moreover, its sequence was identical with that of bovine liver DD3.

Identification and Size Determination of Bovine Liver PGF Synthase mRNA by Blot Hybridization Analysis—Fig. 3A shows the result of the Northern blot analysis of bovine liver mRNA with the P3/P4 PCR product (477 base pairs) used as a probe. From its migration in a denaturing gel system, the sequence of PGF synthase mRNA from bovine liver was estimated to be 1400 nucleotides. Therefore, assuming a length for the poly(A) tail of 100–150 nucleotides, the insert cDNA sequence of 1139 nucleotides extended nearly the full length of the mRNA.

Expression of Bovine Liver PGF Synthase in COS-7 Cells and Purification of Its Expressed Protein—pBOS-BLiFS27 carrying the full-length bovine liver PGF synthase in the mammalian expression vector pEF-BOS was prepared by use of the strategy described under “Experimental Procedures.” COS-7 cells were transfected with pBOS-BLiFS27. The recombinant protein was expressed transiently in COS-7 cells and was located in the cytosol fraction. The recombinant protein was purified to an apparent homogeneity by the following purification steps: ammonium sulfate fractionation between 40 and 75% saturation and Red Sepharose column chromatography, as described under “Experimental Procedures.” About 2.8-fold purification of the PGD₄₁-ketoreductase activity was achieved from the cytosol of COS-7 cells with a yield of 44% (Table I). A sample of each of the purification steps was subjected to SDS-PAGE. Silver staining of the gel indicated that an approximately 36.7-kDa protein was produced in the cells harboring pBOS-BLiFS27, and this protein was purified to an apparent homogeneity (Fig. 3B). Western blot analysis of each sample revealed that the 36.7-kDa protein was recognized by an antilovine liver PGF synthase antibody (Fig. 3C). The molecular weight of the expressed enzyme was the same as that of the native enzyme, as shown in Fig. 3, B and C. No protein from the control COS-7 cells bearing pEF-BOS without the insert DNA interacted with this antibody (data not shown).

The purified recombinant protein exhibited enzymatic properties similar to those of the native enzyme. The Km values for PGD₂, PGH₂, and PQ were 15, 25, and 1.1 μM, respectively (Table I). The Km value for PGD₂ was essentially identical to that of the native liver-type enzyme and was different from the high Km value (120 μM) of the lung-type one. The specific activities of the recombinant protein for PGD₂, PGH₂, and PQ were 23, 9, and 186 milliunits/mg of protein, respectively. However, PGE₂ was not reduced. Moreover, the IC₅₀ value for inhibition of PGD₂ 11-ketoreductase activity of the expressed enzyme by CuSO₄ was 0.5 mM (data not shown), which was almost the same as that for inhibition of the native liver-type enzyme (0.4 mM) but unlike that for the lung-type enzyme (0.003 mM) (6). These results indicate that the cloned cDNA coded for the liver-type PGF synthase and not for the lung-type synthase.

**Site-directed Mutagenesis**—The comparison of amino acid sequences among PGF synthases of bovine liver and lung, and human DD1, DD2, and DD4 is shown in Fig. 2. The Km values of the lung-type (3) and the liver-type (6) PGF synthases and the human liver DD1 and DD2 for PGD₂ (16) were 120, 10, 12, and 79 μM, respectively. DD4 does not catalyze the reduction of PGD₂ (16). To determine which amino acid residues were related to PGF synthase activity, especially to PGD₂ 11-ketoreductase activity, we conducted a site-directed mutagenesis study. Arg²⁷, Arg⁹¹, Arg¹²³, Lys¹²⁵, Ser²⁰¹ or Asn³⁰⁶ of the liver-type PGF synthase with a low Km value for PGD₂ was changed to Glu, Gin, Leu, Ser, Arg, or Tyr, respectively, the latter of which are the residues of the lung-type PGF synthase.
with a high $K_m$ value for PGD$_2$. In addition to these mutations, His$^{170}$ was changed to the Cys of DD4 (Fig. 2), which has no PGD$_2$ 11-ketoreductase activity. The mutant enzyme, R27E, R91Q, H170C, R223L, K225S, S301R, or N306Y, was expressed in COS-7 cells and was purified to an apparent homogeneity (Fig. 4) as described under “Experimental Procedures.” The results of their final purification step are shown in Table I. The $k_{cat}$, $K_m$, and $k_{cat}/K_m$ values for three representative substrates, i.e., PGD$_2$, PGH$_2$, and PQ, of the purified mutant enzymes are shown in Table III.

Although the $k_{cat}/K_m$ values of almost all mutants for PGH$_2$ and PQ were retained above 50% of the wild-type value, these values of all mutants for PGD$_2$ decreased. The $k_{cat}/K_m$ values of R27E, R91Q, H170C, R223L, and N306Y for PGD$_2$ were only about 10% that of the wild type, and the $K_m$ values of R27E, R91Q, H170C, R223L, and N306Y were 110, 145, 75, 180, and 100 μM, respectively. These $K_m$ values were 5–10-fold higher than the wild-type value and were almost the same as that of the lung-type PGF synthase. Considering the amino acid residues of these mutants were changed from the liver-type PGF synthase to the lung-type synthase for R27E, R91Q, R223L, and N306Y or to DD4 for H170C, these results suggest that Arg27, Arg91, His170, Arg223, and Asn306 are essential to give a low $K_m$ value for PGD$_2$ and that these amino acid residues play an important role on the binding for PGD$_2$ to PGF synthase. In addition, the R223L mutant increased $k_{cat}$ for PGD$_2$ 5-fold, indicating that the amino acid residue at 223 has a profound effect on $k_{cat}$ for PGD$_2$ reduction. The $k_{cat}/K_m$ values of R27E, R91Q, and N306Y mutants for PQ were 50–80% of that value of the wild type, indicating that Arg27, Arg91, and...
Asn306 have little effect on the catalytic efficiency for PQ, less than that of these residues on the catalytic efficiency for PGD2. Moreover, the $K_m$ values of R223L, K225S, and S301R for PQ were about 2–10-fold lower than the wild-type value, and the $k_{cat}/K_m$ values of these mutants for PQ were 3–15-fold higher than the value of the wild type. These results suggest that the amino acid residues at 223, 225, and 301 are related to the binding for PQ to the enzyme and that the binding to the carbonyl group is different between PG and quinone compounds. On the other hand, the $K_m$ value of H170C for PGH2 was 8-fold lower than that of the wild type, and the $k_{cat}/K_m$ values of H170C and N306Y for PGH2 were 5–6-fold higher than that of the wild type. These results indicate that the amino acid residue at 170 is related to the binding for PGH2 and that Cys170 seems to confer greater affinity for PGH2 than His. Moreover, the amino acid residue at 306 plays an important role in catalytic efficiency for PGH2.

### DISCUSSION

We isolated a clone of the liver-type PGF synthase with a low $K_m$ value for PGD2 from the cDNA library of bovine liver, expressed the enzyme in COS-7 cells, and constructed seven mutants. Moreover, we examined the enzymatic properties of the wild-type enzyme and of the mutant enzymes, and we investigated the amino acid residue(s) related to the affinity of PGF synthase for the substrates.

The amino acid sequence of the liver-type PGF synthase consisted of 323 amino acid residues with a $M_r$ of 36,742 (Fig. 1) and showed 87% identity with that of the lung-type synthase (Fig. 2). When the liver-type enzyme was expressed in COS-7 cells (Fig. 3), the recombinant purified protein (Fig. 3, B and C, and Table I) was essentially identical to the native liver-type enzyme and not to the lung-type enzyme, based on the enzymatic properties (Table II) including the low $K_m$ value (15 $\mu$M) for PGD2 and the results of Western blot analysis (Fig. 3).
TABLE I
Purification of recombinant wild-type PGF synthase and R27E, R91Q, H170C, R223L, K225S, S301R, and N306Y mutants

| Recombinant bovine liver PGF synthase wild type | Total protein | Total activity | Specific activity | Fold Yield |
|------------------------------------------------|--------------|---------------|------------------|------------|
| Cytosol (step 1)                                | 1 ml         | 5.1 mg        | 41.7 milliunits  | 8.2        |
| (NH4)2SO4(40–75%) (step 2)                      | 1 ml         | 0.8 mg        | 40.6 milliunits  | 20.3       |
| Red Sepharose (step 3)                          | 0.5 ml       | 0.8 mg        | 28.4 milliunits  | 23.0       |
| Mutants (step 3)                                |              |               |                  |            |
| R27E                                            | 0.8 mg       | 0.6 mg        | 13.1 milliunits  | 21.8       |
| R91Q                                            | 0.5 mg       | 0.5 mg        | 9.2 milliunits   | 18.3       |
| H170C                                           | 0.5 mg       | 0.5 mg        | 9.0 milliunits   | 18.0       |
| R223L                                           | 0.7 mg       | 0.3 mg        | 33.7 milliunits  | 112.3      |
| K225S                                           | 0.6 mg       | 0.5 mg        | 22.9 milliunits  | 45.8       |
| S301R                                           | 0.7 mg       | 0.4 mg        | 22.0 milliunits  | 55.0       |
| N306Y                                           | 0.9 mg       | 1.2 mg        | 16.3 milliunits  | 13.6       |

TABLE II
Comparison of kinetic constants for PGD2, PGH2, and phenanthrenequinone among expressed liver PGF synthase, native liver PGF synthase, and native lung PGF synthase

| Substrate           | Expressed liver PGFS | Native liver PGFSa | Native lung PGFSb |
|---------------------|----------------------|--------------------|-------------------|
|                     | K_m (µM) | Specific activity (milliunits/mg protein) | K_m (µM) | Specific activity (milliunits/mg protein) | K_m (µM) | Specific activity (milliunits/mg protein) |
| PGD2                | 15       | 23                  | 10              | 95                 | 10       | 95                 |
| PGH2                | 25       | 9                   | 25              | 3                  | 25       | 3                  |
| 9,10-Phenanthrenequinone | 1.1       | 186                | 2               | 339                | 0.7      | 378                |

a Reported by Chen et al. (6).

b Reported by Watanabe et al. (3).

FIG. 4. SDS-PAGE (A) and Western blot analysis (B) of PGF synthase mutants. Wild-type and the mutant PGF synthases were purified in an identical manner and analyzed by SDS-PAGE silver stain (0.1 µg for each lane) (A) and Western blot analysis (0.01 µg for each lane) (B) using the antiserum against the purified native bovine liver PGF synthase: the native enzyme purified from bovine liver (lane 1), the purified wild-type recombinant enzyme (lane 2), and the purified R27E (lane 3), R91Q (lane 4), H170C (lane 5), R223L (lane 6), K225S (lane 7), S301R (lane 8), and N306Y (lane 9) mutants cells were loaded into the indicated lanes. The positions of the molecular mass standards are shown in Fig. 3.

The results of the study confirm that this liver-type PGF synthase is distinct from the lung-type PGF synthase isolated from the cDNA library of bovine lung (5) or liver (7). Recently, Jez et al. (23) reported on a structure/function analysis of the aldo-keto reductase superfamily. They reported that five amino acid residues, i.e. Asp50, Asn167, Gln190, Ser271, and Arg276, and three residues, i.e. Asp30, Tyr35, and Lys84, function in cofactor binding and in the active site, respectively. Based on the locations of the cofactor-binding pocket and the active site, a putative substrate-binding site was also proposed. However, the binding site for PGs has not yet been reported. The amino acid sequence of the liver-type PGF synthase was highly homologous with those sequences of DD1, DD2, and DD4 of human liver (Fig. 2), and DD1 and DD2 exhibited PGD2 11-ketoreductase activity (16). Based on the comparison among the amino acid sequences of human liver DDs (15, 16) and the lung-type and the liver-type PGF synthases, we studied the site-directed mutagenesis to change seven amino acid residues as follows: R27E, R91Q, H170C, R223L, K225S, S301R, and N306Y. All mutants expressed the proteins in COS-7 cells to almost the same extent as the wild-type protein, suggesting that the tertiary structures of these mutants were not drastically changed. Moreover, all mutants retained the activity with above 50% of K_cat/K_m values of the wild type for PGH2 and PQ, indicating that the structures of all mutants were conserved. Therefore, the change in the enzymatic properties of the mutants reflected the mutation of the amino acid residue and not a change in the structure. Judging from the results of this study, Arg27, Arg91, His170, Arg223, and Asn306 are essential to give a low K_cat value for PGD2 and play an important role in the binding of PGD2 and the amino acid residue at 223 has a significant effect on K_cat for PGD2 reduction. Moreover, the results of K_cat of the R223L, K225S, and S301R mutants for PQ and PGD2 show that R223L, K225S, and S301R mutations acquired high binding for PQ and low for PGD2. Leu223, Ser225, and Arg301 of the lung-type PGF synthase have a positive effect.
The enzyme activities for PGD₂, PGH₂, and phenanthrenequinone were measured in the presence of wild-type PGF synthase and seven mutants (0.06–15 μg for PGD₂, 0.3–19 μg for PGH₂, and 0.3–10 μg for PQ) by the methods shown in Table II.

### Table III

**Comparison of kinetics for PGD₂, PGH₂, and phenanthrenequinone among expressed liver PGF synthase and mutants**

| PGD₂ 11-ketoreductase | PGH₂ 9,11-endoperoxide reductase | Phenanthrenequinone reductase |
|-----------------------|----------------------------------|------------------------------|
|                       | \( k_{\text{cat}} \) | \( K_m \) | \( k_{\text{cat}}/K_m \) | \( q^a \) | \( k_{\text{cat}} \) | \( K_m \) | \( k_{\text{cat}}/K_m \) | \( q^a \) | \( k_{\text{cat}} \) | \( K_m \) | \( k_{\text{cat}}/K_m \) | \( q^a \) |
| **Expressed liver PGFS** | | | | | | | | | | | | |
| Mutants | min⁻¹ | μM | min⁻¹/μM | | | min⁻¹ | μM | min⁻¹/μM | | min⁻¹ | μM | min⁻¹/μM | |
| R27E | 0.81 | 110 | 0.007 | 12 | 0.21 | 14 | 0.015 | 107 | 3.31 | 1.07 | 3.09 | 50 |
| R91Q | 0.66 | 145 | 0.005 | 9 | 0.17 | 10 | 0.017 | 121 | 2.54 | 0.80 | 3.18 | 51 |
| H170C | 0.66 | 75 | 0.009 | 16 | 0.26 | 3 | 0.087 | 621 | 3.78 | 0.51 | 7.41 | 119 |
| R223L | 4.12 | 180 | 0.023 | 40 | 0.11 | 10 | 0.011 | 79 | 10.80 | 0.12 | 90.00 | 1450 |
| R225S | 1.69 | 50 | 0.034 | 60 | 0.25 | 28 | 0.009 | 64 | 9.66 | 0.36 | 26.83 | 432 |
| S301R | 2.02 | 40 | 0.051 | 89 | 0.21 | 30 | 0.007 | 50 | 7.42 | 0.46 | 16.13 | 260 |
| N306Y | 0.51 | 100 | 0.005 | 9 | 0.73 | 10 | 0.073 | 521 | 3.31 | 0.67 | 4.94 | 80 |

*a* The data show the ratio of \( k_{\text{cat}}/K_m \) value of the mutant to that of the wild type for each substrate.

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Twelve amino acid residues among the 42 amino acid residues of the liver-type enzyme that differed from those of the lung type were located in the α-helix or β-sheet, and the other 30 amino acid residues were located in loop structures of the tertiary structure of the liver-type PGF synthase, as inferred from data on human aldose reductase (24, 25) and rat 3α-HSD (26), which showed 46 and 70% identity, respectively, in terms of amino acid sequence with bovine liver-type PGF synthase. As a general rule, an amino acid(s) located in an α-helix or β-sheet is involved in supporting the tertiary structure and that in loop structures of aldo-keto reductases is critical for the affinity and specificity for the substrate. The C-terminal loops of aldo-keto reductase superfamily are in part distinguished by unique C-terminal loops (28–30). C-terminal loops of aldo-keto reductase are unique for each member and differ drastically in length and amino acid composition; and the C-terminal loop is critical for catalytic efficiency and for substrate and inhibitor specificity (28–30). In the case of PGF synthases, the C-terminal region may be also critical for the affinity and specificity for the substrate.

Considering the one binding site for NADPH in the inferred tertiary structure of the liver-type PGF synthase, the catalytic site for the various substrates of aldo-keto reductase including PGF synthase seems to be the same. Tyr55 of aldo-keto reductase is favored as the catalytic acid in the reaction mechanism (23). Tyr55 of PGF synthase also plays the same role as that of the corresponding position in the other aldo-keto reductases.

The reductions of PGD₂, PGH₂, and PQ by the mutants at 3α-HSD near the active site may have roles in substrate binding. They proposed that Trp⁵⁵ is important in binding to a steroid ligand, whose A-ring lies between this Trp and the cofactor, and that Trp⁵⁵ interacts with the C- and/or D-rings of steroid ligands. The effect of R91Q on PGD₂ taken together with the report on Trp⁵⁵ of the 3α-HSD suggests that Arg²²₃ is an essential amino acid residue to give a low \( K_m \) value for PGD₂ of the liver-type PGF synthase and that the amino acid residue at 223 plays an important role in \( k_{\text{cat}} \) of PGD₂ reduction. The results of the R223L and R225S mutants for PQ suggest that Leu²²⁵ and Ser²²⁵ have a significant effect on the binding of PQ. PGD₂ and PQ seem to bind to the same apolar pocket of PGF synthase differently, like steroids, non-steroidal anti-inflammatory drugs, and aldose reductase inhibitors of 3α-HSD (27). Moreover, the effect of S301R on PQ suggests that Ser⁴⁰¹ also has a role in the binding of PQ to the enzyme. Asn³⁰⁶, located in the C-terminal region, may have structural importance for the binding of the substrate. Members of the NADPH-dependent aldo-keto reductase superfamily are distinguished by unique C-terminal loops (28–30). C-terminal loops of aldo-keto reductase are unique for each member and differ drastically in length and amino acid composition; and the C-terminal loop is critical for catalytic efficiency and for substrate and inhibitor specificity (28–30). In the case of PGF synthases, the C-terminal region may be also critical for the affinity and specificity for the substrate. The effect on the binding of the N306Y mutant for PGD₂ and that on \( k_{\text{cat}}/K_m \) of this mutant for PGH₂ also suggest that Asn³⁰⁶ is a critical determinant of PGFs.

The data show that the ratio of \( k_{\text{cat}}/K_m \) value of the mutant to that of the wild type for each substrate.

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*a* M. Sato, M. Tanaka, K. Ikehara, T. Suzuki, and K. Watanabe, manuscript in preparation.
tion between PGH$_2$ and PGD$_2$/PG (3, 6), indicate that the reduction mechanism for PGD$_2$ may be the same as that for PG but not the same as that for PGH$_2$.

3α-HSD, which catalyzes the NADP-dependent reversible oxidation of the 3α-hydroxy group of various steroids, interacts extensively with bile acids (31); and it and human liver high affinity bile acid-binding protein with minimal 3α-HSD activity are multifunctional proteins in bile acid transport and xenobiotic metabolism (32). The 3α amino acid sequences of HSD and high affinity bile acid-binding protein are similar to the sequence of bovine liver PGF synthase, and DD2 and DD4 exhibited binding activity for bile acids (33). Therefore, bovine liver PGF synthase may also be expected to exhibit the ability to bind bile acids. In the liver, PGF$_{2α}$, PGE$_2$, and PGD$_2$ reduce bile flow and bile acid secretion, and especially the effect of PGF$_{2α}$ is more potent than that of PGE$_2$ or PGD$_2$ (34). PGF synthase may be multifunctionally involved in the biosynthesis of PGF and in the binding of bile acids, and PGF synthase in the liver may reduce bile flow. Moreover, PGF$_{2α}$ stimulates hepatocyte DNA synthesis and may have a role in promoting hepatocyte proliferation (35, 36). Furthermore, PGF$_{2α}$ and PGD$_2$ are released from primary Ito cell cultures after stimulation by noradrenaline and ATP (37). PGF$_{2α}$ plays an important physiological role in the liver, and the liver-type PGF synthase mainly contributes to the biosynthesis of PGF$_{2α}$ there.

Recently, the sequence of the bovine liver DD3 (17) was reported. The sequence of the liver-type PGF synthase reported in this paper was found to be identical to that of the bovine liver DD3. The liver-type PGF synthase showed the enzyme activity for $(S)$-$(+)$-indanol (6.3 μmol/min/mg) which is a typical substrate of DD3 (13, 17). However, PG(s) is the naturally occurring substrate(s) for this enzyme, as indanol is a xenobiotic compound.

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$^3$T. Suzuki, and K. Watanabe, unpublished results.