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Site-directed mutagenesis of human prostacyclin synthase: Alteration of Cys^{441} of the Cys-pocket, and Glu^{347} and Arg^{350} of the EXXR motif

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Abstract **The possible active site Cys^{441} in the Cys-pocket and Glu^{347} and Arg^{350} of the EXXR motif of the human prostacyclin synthase, which catalyzes the conversion of prostaglandin H_2 to prostacyclin, were subjected to site-directed mutagenesis in order to understand the role of these residues in expressing the enzymatic activity. Five expression vectors encoding the mutant enzymes with a single replacement, Cys^{441}Ala, Cys^{441}Ser, Cys^{441}His, Glu^{347}Ala and Arg^{350}Ala, as well as the wild-type enzyme were expressed in 293 cells. The microsomal fraction of the cells expressing the wild-type enzyme showed a specific activity of 96 nmol 6-keto-$PGF_{1\alpha}$/min per mg protein. All of the mutant enzymes examined showed no detectable enzyme activity, although immunoblot analysis demonstrated that levels of all the expressed mutant enzymes were similar to that of the wild-type enzyme. These results indicated that the Cys^{441} in the Cys-pocket, and Glu^{347} and Arg^{350} of the EXXR motif of human prostacyclin synthase are important for expressing the enzymatic activity.**

Key words: Prostacyclin synthase; Prostaglandin I_2; Site-directed mutagenesis; Cytochrome P450

1. Introduction

Prostacyclin (prostaglandin I_2: PGI_2) has strong vasodilation and antithrombogenic effects and is thought to be a physiological antagonist of thromboxane A_2. Thus, the balance between the production of PGI_2 and thromboxane A_2 is postulated to be important for the circulatory system, and the imbalance of these products causes diseases such as myocardial infarction, stroke, and atherosclerosis [1]. PGI_2 is formed via isomerization of PGH_2 by PGI_2 synthase (EC 5.3.99.4) in vascular endothelial cells and vascular smooth muscle cells [2]. We have suggested that PGI_2 synthase is a hemoprotein of the cytochrome P450 type [3–5], and demonstrated by molecular cloning of PGI_2 synthase cDNAs from bovine and human vascular endothelial cells that the enzyme belongs to a new family (CYP8) in the P450 superfamily [6–8]. As shown in Fig. 1, the cysteine residue constituting the 5th ligand of the heme in the Cys-pocket (the β-bulge region) near the helix L, and the glutamic acid and arginine residues of EXXR motif in the helix K are highly conserved among P450s [9,10]. These amino acid residues of P450s are also well conserved in the deduced primary structure of PGI_2 synthase [7,9]. On the other hand, many cytochrome P450s generally catalyze the oxidation of a variety of hydrophobic substrates, but PGI_2 synthase catalyzes the isomerization of PGH_2. Therefore, it is of interest to study the difference between the active site structures of PGI_2 synthase and other P450s.

Here we performed the site-directed mutagenesis of human PGI_2 synthase. The possible active site cysteine residue Cys^{441} was replaced by alanine, serine and histidine, and Glu^{347} or Arg^{350} of the EXXR motif was mutagenized to alanine. It was found that all the mutations examined entirely abolished the enzyme activity, suggesting the importance of these residues in expression of the enzymatic activity of PGI_2 synthase.

2. Materials and methods

2.1. Materials

[1-^{14}C]Arachidonic acid (1.85 GBq/mmol) was purchased from Amersham (Bucks, UK). pCMV-7 and pVA-1 [11] were obtained from Dr. David W. Russell (Department of Molecular Genetics, University of Texas Southwestern Medical Center). 293 cells (ATCC CRL 1573) were obtained from American Type Culture Collection (Rockville). Restriction endonuclease and other modifying enzymes were from Toyobo. Polymerase chain reaction (PCR) primers were synthesized on a DNA synthesizer (model 391, Perkin-Elmer).

2.2. Expression vector and mutagenesis

A 1.6-kb fragment of the full-length human PGI_2 synthase cDNA constructed by ligation of pHPGIS36 and pHPGIS135 [7] was inserted into the *Sal*I and *Bam*HI sites of the expression vector pCMV-7 to generate the plasmid pCMV/PGISWT. The mutants were prepared by 'overlap extension' mutagenesis [12] using three-step PCR amplification of the cDNA for human PGI_2 synthase. The primers for the first amplification of the cDNAs harboring the carboxyl terminal region of the mutant enzymes were (bases underlined in the primer sequences described below indicate the changes introduced in the cDNA sequences): Cys^{441}Ala, 5′-AATCACGCGCTGGGGAGGAGTTATGCGGTCAACAGC-3′ (P6, with a TGC to GCG substitution of the sense-strand sequence at nucleotide 1321–1323), and 5′-GGGATCCAGATCTTCATGGGCGGATGCGGTA-3′ (P48, the antisense-strand complimentary to the 3′ region of the coding sequence); Cys^{441}Ser, 5′-AATCACTCCCTGGGGAGGAGTTATGCGGTCAACAGC-3′ (P50, with a G to C substitution of the sense-strand sequence at nucleotide 1322) and P48; Cys^{441}His, 5′-AATCACCACCTGGGGAGGAGTTATGCGGTCAACAGC-3′ (P49, with a TG to CA substitution of the sense-strand sequence at nucleotide 1321–1322) and P48; Glu^{347}Ala, 5′-TTGATAGCGTGCTGAGTGCGAGCCTCAGGC-3′ (P79, with an A to C substitution of the sense-strand of the sequence at nucleotide 1040) and P48; Arg^{350}Ala, 5′-CTGAGTGAGAGCCTCGCGCTTACAGCTGC-3′ (P61, with an AG to GC substitution of the sense-strand sequence at nucleotide 1048–1049) and P48. The PCR reactions were run for 25 cycles in the following cycle profile: 94°C for 1 min, 55°C for 45 s and 72°C for 2 min. The primers for the second amplification of the cDNAs covering the amino terminal region of the mutant enzymes were: Cys^{441}Ala, 5′-GCTGTTGACCGCA-

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Abbreviations: PG, prostaglandin; PGI_2, prostacyclin; PCR, polymerase chain reaction; DTT, dithiothreitol; BHT, butylated hydroxytoluene; PVDF, polyvinylidene difluoride; KPB, potassium phosphate buffer

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TAACTCCTCCCCAGCGCGTGATT-3′ (P7) which is complementary to P6 and 5′-CGTCGACCCACCATGGCTTGGGCCGCG-CTC-3′ (P57) which corresponds to the 5′-coding region of the cDNA; $Cys^{441}Ser$, 5′-GCTGTTGACCGCATAACTCCTCCCCA-GGGAGTGATT-3′ (P74) which is complementary to P50 and P57; $Cys^{441}His$, 5′-GCTGTTGACCGCATAACTCCTCCCCAGGTGGT-GATT-3′ (P73) which is complementary to P49 and P57; $Glu^{347}Ala$, 5′-GCCTGAGGCTCGCACTCAGCACGCTATCAA-3′ (P60) which is complementary to P79 and P57; $Arg^{350}Ala$, 5′-GCAGCTGTA-AGCGCGAGGCTCTCACTCAG-3′ (P62) which is complementary to P61 and P57. The second amplifications were performed under the same PCR conditions used for the 1st PCR. Finally, the full-length cDNAs with a mutation were prepared by the third PCR using the overlap extension technique [12]. The 1st and 2nd PCR products were purified by agarose gel electrophoresis, reannealed and reamplified using P48 and P57 as primers. The PCR was run for 10 cycles in the following cycle profile: 94°C for 1 min, 55°C for 45 s and 72°C for 2 min. The products were purified, digested with *Xho*I and *Bam*HI and introduced into the *Xho*I-*Bam*HI site of the expression vector encoding the wild-type enzyme, pCMV/PGISWT. All the mutant vectors pCMV/PGIS$Cys^{441}Ala$, pCMV/PGIS$Cys^{441}Ser$, pCMV/PGIS$Cys^{441}His$, pCMV/PGIS$Glu^{347}Ala$ and pCMV/PGIS$Arg^{350}Ala$ were completely sequenced as described in [6] to verify that no other mutations had been introduced by the PCR amplification.

The constructed expression vectors were transfected into 293 cells. Monolayers of 293 cells were seeded (3×10^5 cells per 60 mm dish) in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. After incubation for 24 h, the cells were transfected with 3 μg of either an expression vector coding for the wild-type or the mutant enzyme, or the control vector pCMV-7 plus 0.3 μg of pVA-1 (a plasmid encoding adenovirus-associated RNA_1) [13] using LipofectAMINE (Gibco BRL). After 36 h, cells washed once with ice-cold phosphate-buffered saline (PBS) were harvested with PBS and stored at −80°C [14].

2.3. Characterization of expressed proteins

The cells (2×10^6 cells) were suspended in 1 ml of 100 mM potassium phosphate buffer (KPB), pH 7.5, 1 mM EDTA, 0.1 mM dithiothreitol (DTT), 0.1 mM butylated hydroxytoluene (BHT) and 44 μg/ml phenylmethylsulfonyl fluoride, and sonicated 4 times at 150 W for 30 s (Astrason, model XL2020). The homogenate was centrifuged at $10000\times g$ for 10 min, and the supernatant was centrifuged at $100000\times g$ for 1 h. Pellets were suspended in 10 mM KPB (pH 7.5) containing 20% (v/v) glycerol and 0.1 mM EDTA, 0.1 mM DTT, 0.1 mM BHT (buffer A), and the protein concentration was adjusted to 2 mg/ml with buffer A. The expressed proteins separated by SDS-polyacrylamide gel electrophoresis on 10% (w/v) gels were stained with silver nitrate (Silver stain II kit, Wako) and subjected to immunoblot analysis. The proteins were transferred electrophoretically onto a polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore). After treatment with 5% skim milk in Tris-HCl-buffered saline (TBS), pH 7.4 at 24°C for 12 h, the blotted membrane was incubated with the polyclonal antibody against bovine PGI_2 synthase [15] in TBS containing 3% skim milk. After rinsing with TBS containing 0.05% Tween 20, the membrane was incubated with alkaline phosphatase-conjugated anti-rabbit immunoglobulin G in TBS containing 3% skim milk at 37°C for 1 h. After successive washings with TBS containing 0.05% Tween 20 and then with TBS, immunoreactive bands were stained by the Immun-Lite II kit (Bio-Rad). Gels were calibrated with the protein markers (low range SDS-polyacrylamide gels electrophoresis standards, Bio-Rad or Rainbow colored protein molecular weight markers, Amersham). Protein was determined with bicinchoninic acid protein assay reagent (Pierce, Rockford) and bovine serum albumin as a standard.

2.4. PGI_2 synthase assay

The activity of PGI_2 synthase was determined as described previously [6]. Briefly, the reaction mixture for PGI_2 synthase assay contained 0.1 M Tris-HCl buffer, pH 7.4, [1-^{14}C]PGH_2 (5 nmol), and an enzyme preparation in a final volume of 100 μl. The reaction was carried out at 24°C for the specified time (0–30 s) and terminated by the addition of 0.3 ml of diethyl ether/methanol/0.2 M citric acid (30:4:1). The organic phase (100 μl) was directly applied on a silica gel plate at 4°C, and the plate was developed at room temperature using an organic layer of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (110:50:20:100). The amounts of ^{14}C-labeled PGH_2 and 6-keto-$PGF_{1\alpha}$, a stable degradation product of PGI_2, were visualized and calculated using an imaging analyzer (BAS2000, Fuji Photo Film Co.).

3. Results and discussion

Previously, we expressed bovine PGI_2 synthase with an expression vector pcDL-SRα296 in COS-7 cells but the level of

consensus	--E--R--	---C-g---a--e--------l--
	* *	*
PGIS	345-LSESLRLT-352	438-HNHCLGRSYAVNSIKQFVFLVLVH-461
P450ch7α	350-IKESLRLS-357	441-ATICPGRLFAIHEIKQFLILMLSY-464
TXS	397-IAETLRMY-404	476-PRSCLGVRLGLLEVKLTLLHVLHK-499
P450d	370-ILEIYRYT-377	453-KRRCIGEIPAKWEVFLFLAILLHQ-476
P450M1	353-VHEIQRYI-360	432-KRICAGEALARTELFLFFTTILQN-455
P450cam	285-CEELLRRF-292	354-SHLCLGQHLARREIIVTLKEWLTR-377
P450trp	304-VDEAVRWT-311	374-AHMCLGQHLAKLEMKIFFEELLPK-397
P450BM-3	318-IYESMRYQ-325	397-QRACIGQQFALHEATLVLGMMLKH-420
P450nor	276-VEELCRYH-283	349-DHRCIAEHLAKAELTTVFSTLYQK-372
	Helix K	Helix L

Fig. 1. Alignment of amino-acid sequences in the heme proximal region of PGI_2 synthase and P450s. The trivial name, standard nomenclature, and source organism for the sequences presented are: PGI_2 synthase (PGIS) (CYP8, human), P450ch7α (CYP7, human) [26], TXS (CYP5, human) [27], P450d (CYP1A2, rat) [28], P450M1 (CYP2C11, rat) [29], P450cam (CYP101, *Pseudomonas putida*) [30,31], P450trp (CYP108, *Pseudomonas* spp.) [32], P450BM-3 (CYP102, *Bacillus megaterium*) [33], P450nor (CYP55, *Fusarium oxysporum*) [34]. The asterisks indicate Cys^{441}, Glu^{347} and Arg^{350} of PGI_2 synthase, which are conserved in each of these P450s. The consensus sequence is presented as follows: upper-case letter, 100% conservation; lower-case letter, most frequently occurring amino acid in ≥85% of the sequences. The approximate location of helices K and L in the known three crystal structures of P450cam, P450terp and P450BM-3 is shown below the sequences. The standard single-letter code is used.

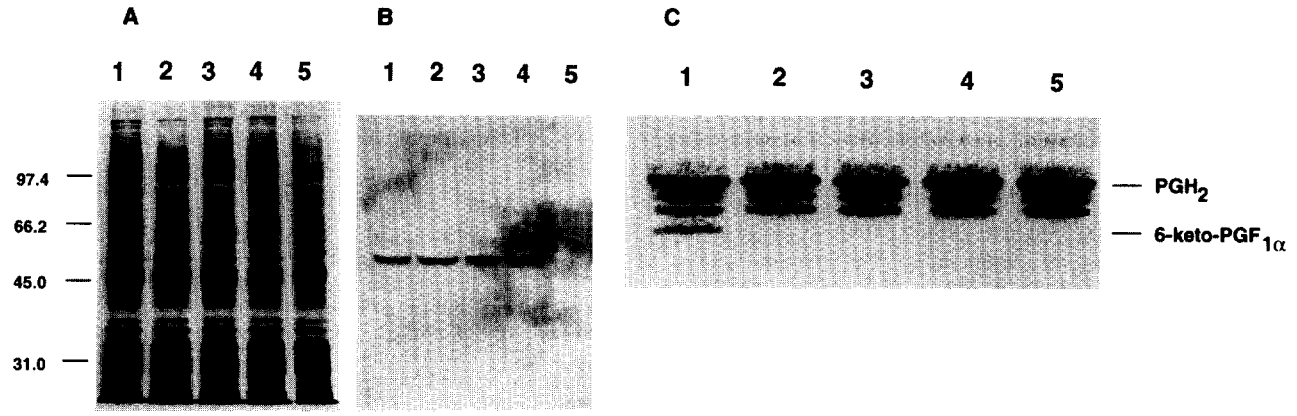

Fig. 2. SDS-polyacrylamide gel electrophoresis, immunoblot analysis and determination of enzymatic activity of the mutant PGI_2 synthase expressed in the microsomes from expression-vector transfected 293 cells. (A) Electrophoresis of microsomal fraction (10 μg protein) was carried out on 10% (w/v) SDS-polyacrylamide gel, and proteins were stained with silver nitrate. Protein size markers are indicated in kDa on the left side. (B) Microsomal proteins (40 μg) separated on 10% (w/v) SDS-polyacrylamide gel were transfered onto PVDF membrane, which was treated with rabbit polyclonal anti-bovine PGI_2 synthase antibody. (C) Microsomal fractions (20 μg) were incubated with ^{14}C-labeled PGH_2 (5.0 nmol) at 24°C for 30 s. The reaction products were separated by thin-layer chromatography and radioactive products were detected using a BAS2000 image analyzer. Migrations of PGH_2 and 6-keto-$PGF_{1\alpha}$ are indicated by bars. Lanes: 1, human wild-type PGI_2 synthase; 2, Cys^{441}-Ala mutant; 3, Cys^{441}His mutant; 4, Cys^{441}Ser mutant; 5, mock (pCMV-7).

the expressed enzyme was too low to characterize the enzyme [6]. Thus, we first attempted to establish an efficient system for expressing the wild-type human PGI_2 synthase with our cloned cDNA [7]. An expression vector (pCMV-7) with an insert of the entire protein-coding region of the cDNA for the enzyme (pCMV/PGISWT) was constructed and transfected into 293 cells. As shown in Fig. 2A, a 51 kDa protein was expressed in the microsomal fractions of the transfected cells (lane 1); this band was not found in the mock (pCMV-7)-transfected cells (lane 5). Furthermore, the same microsomal fractions were subjected to immunoblot analysis using the polyclonal antibody against bovine PGI_2 synthase [15]. As shown in Fig. 2B, a specific immunoreactive band corresponding to the 51 kDa protein was observed only with the cells transfected with pCMV/PGISWT (lane 1). In order to examine the PGI_2 synthase activity of the expressed protein, the microsomal fractions were incubated with ^{14}C-labeled PGH_2 as a substrate. Fig. 3 demonstrates that the substrate was converted to 6-keto-$PGF_{1\alpha}$, a stable degradation product of PGI_2 (panel A, lanes 2–4). The formation of 6-keto-$PGF_{1\alpha}$ was linear at least for the initial 30 s with a concomitant decrease of PGH_2 (Fig. 3B) and the activity of the preparation was 96 nmol 6-keto-$PGF_{1\alpha}$/min per mg protein. This activity was about 8 times as much as that of bovine aorta microsomes [16]. The microsomal fractions transfected with the control vector without insert showed no detectable PGI_2 synthase activity (Fig. 3A, lane 5). These results indicated that the wild-type human PGI_2 synthase could be expressed at a sufficient level for characterization of the enzyme using pCMV-7 inserted with the human PGI_2 synthase cDNA in 293 cells.

We applied this expression system for the site-directed mutagenesis study of the human PGI_2 synthase. According to the three-dimensional structure of $P450_{cam}$, the heme disc embedded between helices I and L constituting the active site. Such an arrangement is a common feature in many heme proteins [17]. As shown in Fig. 1, comparison of the amino acid sequence of human PGI_2 synthase with P450s shows that the PGI_2 synthase has a significant sequence similarity to other P450s in the C-terminal region including the Cys-pocket

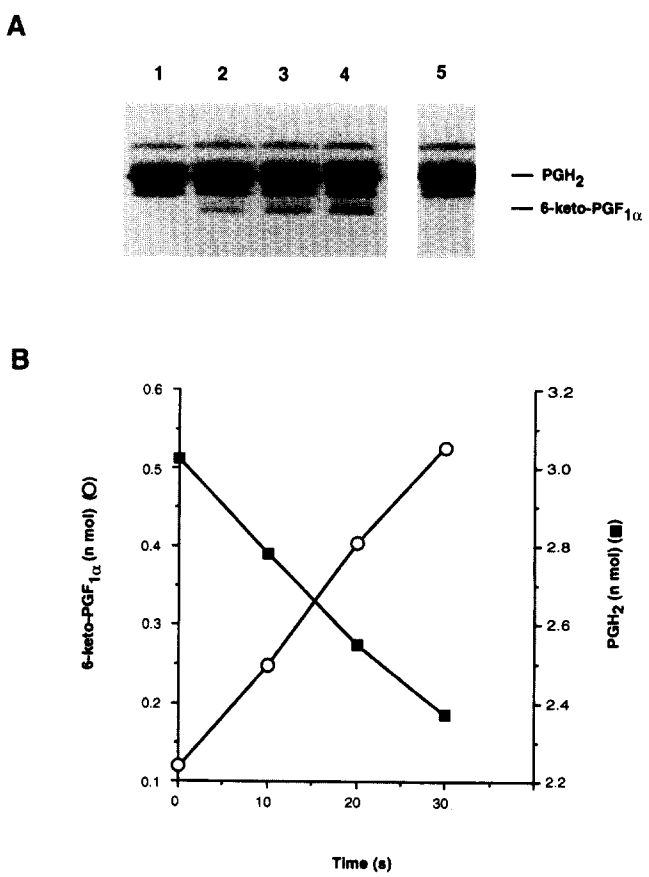

Fig. 3. Time course of isomerization of PGH_2 by recombinant human PGI_2 synthase. Microsomal fractions (8.9 μg) from cells transfected with the expression vector coding for the wild-type enzyme were incubated with ^{14}C-labeled PGH_2 (5.0 nmol) at 24°C for specified times. (A) The reaction products were separated by thin-layer chromatography, and radioactive products were detected using a BAS2000 image analyzer. Migrations of PGH_2 and 6-keto-$PGF_{1\alpha}$ are indicated by bars. (B) Amounts of 6-keto-$PGF_{1\alpha}$ and PGH_2 in the reaction mixture. Lanes 1–4 indicate the reaction mixtures incubated for 0, 10, 20 and 30 s, respectively. Lane 5, products formed by incubation for 30 s with microsome fraction of control cells transfected with the expression vector pCMV-7.

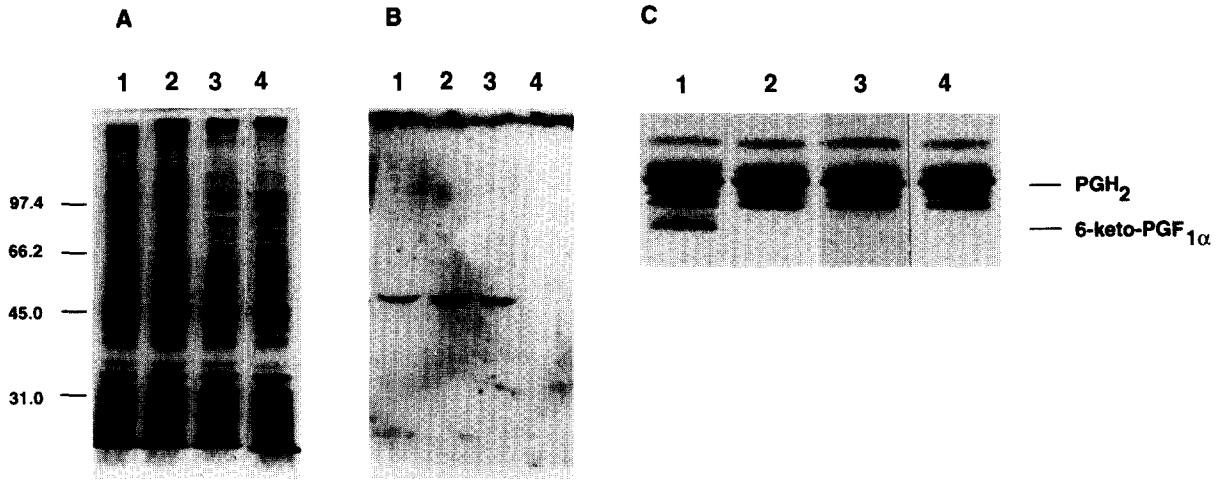

Fig. 4. SDS-polyacrylamide gel electrophoresis, immunoblot analysis and PGI_2-synthetic activity of the microsomes from expression-vector transfected 293 cells. (A) Microsomal proteins (10 μg) were analyzed by SDS-polyacrylamide gel electrophoresis as described in the legend to Fig. 2A. Protein size markers are indicated in kDa on the left. (B) Microsomal fractions (40 μg) were subjected to immunoblot analysis as described in Fig. 2B. (C) Determination of enzyme activities of microsomal fractions (20 μg) were performed as described in Fig. 2C. Lanes: 1, wild-type enzyme; 2, $Glu^{347}Ala$; 3, $Arg^{350}Ala$; 4, mock. Migrations of PGH_2 and 6-keto-$PGF_{1\alpha}$ are indicated. Lanes: 1, human wild-type PGI_2 synthase; 2, $Glu^{347}Ala$; 3, $Arg^{350}Ala$; 4, mock (vector pCMV-7).

near the helix L. The cysteine residue in the pocket has been shown to constitute the fifth ligand of the heme iron [17–21]. Thus, it is very possible that this invariant cysteine residue with a polar side chain at position 441 of the PGI_2 synthase plays an important role in the catalytic activity of the enzyme. For confirmation of this possibility we substituted alanine and serine for Cys^{441} by site-directed mutagenesis. The $Cys^{441}Ala$ and $Cys^{441}Ser$ mutants were expressed in 293 cells under the same conditions as used for the expression of the wild-type enzyme. Both mutant proteins were expressed at similar levels to that of the wild-type enzyme, as revealed by SDS-polyacrylamide gel electrophoresis and immunoblot analysis (Fig. 2A,B, lanes 2,4), but no PGI_2 synthase activities could be detected (< 0.2 nmol 6-keto-$PGF_{1\alpha}$/min per mg protein) in the microsome fractions from the 293 cells expressing $Cys^{441}Ala$ or $Cys^{441}Ser$ (Fig. 2C, lanes 2,4). Similarly, it was reported that the substitution of histidine for this cysteine in rat $P450_d$ caused a loss of catalytic activity of the enzyme [20]. In contrast, Tomura et al. [22] reported that the substitution of histidine for this cysteine in *Fusarium oxysporum* $P450_{nor}$ retained a weak but significant level of the enzymatic activity. In order to examine the effect of this substitution in human PGI_2 synthase, we replaced the Cys^{441} by histidine. An undetectable level of the PGI_2 synthase activity (< 0.2 nmol 6-keto-$PGF_{1\alpha}$/min per mg protein) was observed with the microsome fractions from the cells expressing $Cys^{441}His$ (Fig. 2C, lane 3), although the mutant protein was expressed at a similar level to the wild-type (Fig. 2A,B, lane 3). These results suggest that the Cys^{441} of human PGI_2 synthase is a critical residue for the catalytic activity of the enzyme as shown by $P450_d$ [20] and thromboxane synthase [21].

As shown in Fig. 1, the EXXR motif in helix K, which is highly invariant among sequences of P450s, is also conserved in that of the PGI_2 synthase [8,9]. In addition, it is considered that the EXXR motif of $P450_{cam}$ located on helix K stabilizes the helix by forming two hydrogen bonds between the side chains of the Glu and Arg residues, and these hydrogen bonds are structurally important [23]. To assess the importance of these residues in the catalytic function of the PGI_2 synthase, we changed Glu^{347} or Arg^{350} of the possible EXXR motif of the enzyme to alanine ($Glu^{347}Ala$ or $Arg^{350}Ala$) as shown in Fig. 4A,B (lanes 2,3). Low enzymatic activities of these mutants (< 0.2 nmol 6-keto-$PGF_{1\alpha}$/min per mg protein) could be detected with the microsome fractions from the cells expressing $Glu^{347}Ala$ or $Arg^{350}Ala$ (Fig. 4C, lanes 2,3). Our results indicate that Glu^{347} and Arg^{350} of human PGI_2 synthase play a critical role in the enzyme catalysis. Similarly, Chen and Zhou [24] reported that mutation of Arg^{365} in helix K of aromatase ($P450_{arom}$) led to the production of inactive protein. Shimizu et al. [25] reported that mutants of the corresponding arginine residue of $P450_d$ were inactive, and that these mutants did not bind the heme in the active site of $P450_d$. The results of the present study suggest that the highly conserved EXXR motif among P450s is essential for the expression of the PGI_2 synthase activity, although the PGI_2 synthase has a unique isomerase activity unlike many other cytochrome P450s.

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