Structural Basis of Hematopoietic Prostaglandin D Synthase Activity Elucidated by Site-directed Mutagenesis*

Elena Pinzar‡, Masashi Miyano§, Yoshihide Kanaoka‡¶, Yoshihiro Urade‡¶, and Osamu Hayaishi‡**

From the ‡Department of Molecular Behavioral Biology and §Core Research for Evolutional Science and Technology, Japan Science Technology Corporation, Osaka Bioscience Institute, 6-2 Furuedai, Suita, Osaka 565-0874, Japan and the ¶Central Pharmaceutical Research Institute, Japan Tobacco Incorporated, 1-1 Murasaki-cho, Takatsuki, Osaka 569-1125, Japan

Hematopoietic prostaglandin (PG) D synthase (PGDS) is the first identified vertebrate ortholog in the Sigma class of the glutathione S-transferase (GST) family and catalyzes both isomerization of PGH2 to PGD2 and conjugation of glutathione to 1-chloro-2,4-dinitrobenzene. We introduced site-directed mutations of Tyr8, Arg14, Trp104, Lys112, Tyr152, Cys156, Lys198, and Leu199, which are presumed to participate in catalysis or PGH2 substrate binding based on the crystallographic structure. Mutants were analyzed in terms of structure, GST and PGDS activities, and activation of the glutathione thiol group. Of all the mutants, only Y8F, W104I, K112E, and L199F showed minor but substantial differences in their far-UV circular dichroism spectra from the wild-type enzyme. Y8F, R14K/E, and W104I were completely inactive. C156L/Y selectively lost only PGDS activity. K112E reduced GST activity slightly and PGDS activity markedly, whereas K198E caused a selective decrease in PGDS activity and Km for glutathione in the PGDS reaction. No significant changes were observed in the catalytic activities of Y152F and L199F, although their Km for glutathione was increased. Using 5,5′-dithiobis(2-nitrobenzoic acid) as an SH-selective agent, we found that only Y8F and R14K/E did not accelerate the reactivity of the glutathione thiol group under the low reactivity condition of pH 5.0. These results indicate that Lys112, Cys156, and Lys198 are involved in the binding of PGH2, Trp104 is critical for structural integrity of the catalytic center for GST and PGDS activities, and Tyr8 and Arg14 are essential for activation of the thiol group of glutathione.

* This work was supported in part by Grants-in-aid for Scientific Research 07585108 and 07457033 from the Ministry of Education, Science, and Culture of Japan (to Y. U.) and by grants from the Uehara Memorial Foundation, the Takeda Science Foundation, and the Science and Technology Agency (to E. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Dept. of Medicine, Harvard Medical School, Div. of Rheumatology, Immunology, and Allergy, Brigham and Women’s Hospital, One Jimmy Fund Way, Boston, MA 02115.

** To whom correspondence should be addressed. Tel.: 81-6-6872-4833; Fax: 81-6-6872-4818; E-mail: hayaishi@obisun1.obi.or.jp.

† The abbreviations used are: PG, prostaglandin; PGD2, prostaglandin D synthase; GST, glutathione S-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

© 2000 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.
EXPERIMENTAL PROCEDURES

Materials—The Gene Amp kit (Stratagene, La Jolla, CA), restriction endonucleases and a ligation kit (Takara Shuzo, Kyoto, Japan), and the SequiTHERM Long-Read Cycle DNA LC sequencing kit (Epiconcept Technologies, Madison, WI) were purchased from the sources indicated. 1-[2,4,6-Tris(2-nitrophenyl)phosphinyl]adamantine (NEN Life Science Products). Primers designed for polymerase chain reaction were obtained from Life Technologies, Inc.

Oligonucleotide Primer Design and Polymerase Chain Reaction Mutagenesis—Two external primers were designed for polymerase chain reaction mutagenesis. Primer I (TGTCTATGCCCACAATCAGAATGCTG) is the 5′-end forward sequence of the cDNA for rat hematopoietic PGDS and contains a NdeI site. Primer II (CGGCAATTCATTAGGTTTGTGTA) is the 3′-end inverse sequence with an EcoRI site. The internal primers were designed to preserve both antisense mutations and a conserved 3′-end GSH-binding site. The mutagenic primers were amplified in Escherichia coli DH5α. All mutations were confirmed by DNA sequencing.

Expression and Purification of Mutant PGDSs—E. coli BL21(DE3) cells were transformed with the prepared plasmids. Mutant enzyme was expressed and purified as described previously (22). In brief, cells were collected and disrupted by sonication. After removal of cell debris by centrifugation, the supernatant was suspended in phosphate-buffered saline (PBS) and purified to homogeneity by one-step GSH affinity chromatography. The purified mutant enzymes migrated to the same position as the wild-type enzyme in SDS-polyacrylamide gel electrophoresis (M r, 26,000). Protein concentration was determined by the Bradford method (29) with bovine γ-globulin as a standard.

Immunoblot Analysis—For Western blot analysis, the soluble fraction of each transformant was subjected to 14% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Bio-Rad). The membrane was immunostained with rabbit polyclonal antisera against rat hematopoietic PGDS and anti-rabbit IgG (Dako; Los Angeles, CA). The membrane was immunostained with rabbit polyclonal antisera against rat hematopoietic PGDS and anti-rabbit IgG (Dako; Los Angeles, CA). The membrane was immunostained with rabbit polyclonal antisera against rat hematopoietic PGDS and anti-rabbit IgG (Dako; Los Angeles, CA).

UV Circular Dichroism Spectroscopy—CD spectra of the wild-type and mutant enzymes were recorded on a J-600 spectropolarimeter (Jasco International Co. Ltd., Tokyo) at 10 °C in the presence of 1 mM GSH except when otherwise indicated.

Enzyme Assays—GST activity was measured by the spectrophotometric method (15) with 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 1 mM GSH in 0.1 M potassium phosphate (pH 6.5). PGDS activity was assayed in 50 μl of reaction mixture containing 100 mM Tris-HCl (pH 8.0), 40 μM [1-C]PGH2, 1 mM GSH, 1 mg/ml IgG, and an aliquot of the enzyme (30). The rates of all enzymatic reactions were calculated after subtracting the blank experiment without the enzyme. In the PGDS reaction, kinetic constants for PGH2 and GSH were measured with 1 mM GSH or 40 μM PGH2 and varying concentrations of the other substrate ranging from 0.1 to 10 mM; in the GST reaction, kinetic constants for PGH2 and GSH were measured with 1 mM GSH or 1 mM CDNB and varying concentrations of the other substrate ranging from 0.1 to 10 mM.

5,5′-Dithiobis(2-nitrobenzoic Acid) (DTNB) Reactivity Assay—The activated thiol group of GSH bound to the enzyme was measured spectrophotometrically at 410 nm after incubation of the enzyme with DTNB, a thiol-specific reacting agent, under a low reaction condition in 0.5 M sodium acetate (pH 5.0) (31).

RESULTS

Expression, Purification, and Characterization of Recombinant Mutant PGDSs—Based on the crystal structure of rat hematopoietic PGDS (15), the Y8F, R14E/K, W104I, K112E, Y152F, C156LY, K198E, and L199F mutants were prepared to determine the catalytic mechanisms of PGDS and GST activities. Tyr9 and Arg14 were proposed to activate the thiol group of GSH, whereas the other residues are located in the putative PGH2-binding site (Fig. 1).

Mutants were expressed in E. coli, purified, and examined for retention of native structure. The expression level of the W104I and C156LY mutants was comparable to that of the wild-type enzyme (Fig. 2). The expression level of the Y8F, R14E/K, K198E, and L199F mutants varied from 70 to 40% of that of the wild-type enzyme. The lowest expression was observed for the K112E, C156LY, and Y152F mutants, being 12, 17, and 16% of the wild-type enzyme, respectively. Each mutant showed only one immunoreactive band at a position of the wild-type enzyme. All mutants could be purified by GSH affinity chromatography, indicating that the GSH-binding site was not structurally or functionally disrupted. The far-UV CD spectra of most of the mutants were similar to the wild-type spectrum and were characteristic of an α-helical dominant protein (Fig. 3). However, the [α]222 nm value decreased by 8% for W104I and L199F and increased by 20% for K112E as compared with the value for the wild-type enzyme. The CD spectrum of Y8F appeared to be intermediate between those spectra of the wild-type and K112E enzymes. The spectra of R14E/K, Y152F, C156LY, and K198E were the same as the wild-type spectrum. The deviations in the CD spectra of Y8F, K112E, W104I, and L199F suggest that these residues are structurally important.

PGDS and GST Activities of Mutants—To examine the effect of the amino acid substitution on the enzymatic catalysis, we measured both PGDS and GST activities of the purified mutant enzymes and compared them with those of the wild-type enzyme (Fig. 4). There were three types of effects: retention of both activities, as in K112E, Y152F, K198E, and L199F; complete loss of only the PGDS activity, as in C156LY; and complete loss of both activities, as seen in Y8F, R14E/K, and W104I. The mutants associated with PGDS activity isomerized PGH2 to PGD2 specifically without enzymatic production of PGE2 and PFG2α, similar to the wild-type enzyme.

Mutations of Lys112 and Cys156 in the PGH2-binding pocket severely affected PGDS activity, but the mutants retained some level of GST activity. As compared with the wild-type enzyme, K112E showed 10% of the PGDS activity and 50% of the GST activity, whereas C156LY completely lost the PGDS activity and had 27 and 11% of the GST activity, respectively. On the other hand, as observed for mutations of Tyr8, Arg14, and Trp14, complete loss of GST activity was accompanied by complete loss of PGDS activity.

Kinetic Characterization of Mutants—To further characterize the effect of each mutation, we determined the kinetic properties of the mutant proteins. When the kinetic parameters of the mutant and wild-type enzymes were examined, the PGDS and GST activities were saturable with increasing GSH concentration; alternatively, the PGDS and GST activities increased almost linearly up to 200 μM PGH2 and 2 mM CDNB. Therefore, only the apparent K m could be estimated with respect to the PGH2 and CDNB concentration (Table I).

In the wild-type enzyme, PGH2 binding apparently increased the affinity for GSH as evidenced by the lower K m for GSH in the PGDS activity assay (K m(GSH) = 0.1 mM) than in the GST activity assay (K m(GSH) = 0.5 mM). This effect was also observed in all the mutants; and it is notable that for K112E, the K m value for GSH increased in both assays, although more significantly so in the GST assay. In contrast, Y152F, K198E, and L199F showed slightly lower K m(GSH) values (0.05–0.09 mM) than the wild-type enzyme (0.1 mM) in the PGDS assay and a relatively small increase in K m(GSH) in the GST assay (0.7–0.8 mM) as compared with wild-type PGDS (0.5 mM).

The apparent K m values for PGH2 and CDNB for the wild-
type enzyme were calculated to be \( \sim 500 \, \mu M \) and 3 mM, respectively. For almost all mutants, the \( K_m \) values for PGH \(_2\) and CDNB were comparable to those obtained for the wild-type enzyme, with the exceptions of L199F, with a 2-fold decrease in affinity for PGH\(_2\), and C156L, with a 5-fold decrease in \( K_m \) for CDNB.

Among the mutants with both PGDS and GST activities, K112E and K198E showed the most remarkable changes in the \( k_{cat} \) values for PGDS activity determined with varying concentrations of either of the substrates. The efficiency of the PGDS activity of K112E (\( k_{cat(GSH)} = 3.42 \, s^{-1} \) and \( k_{cat(PGH)} = 4.30 \, s^{-1} \)) was 9- and 5-fold reduced as compared with the wild-type enzyme, with the exceptions of L199F, with a 2-fold decrease in affinity for PGH\(_2\), and C156L, with a 5-fold decrease in \( K_m \) for CDNB.

The CD spectra of Y8F (dashed line), K112E (dashed and dotted line), and W104I (dotted line) are shown as those that deviated most from the wild-type spectrum (solid line). The CD spectrum of L199F resembled that of W104I. deg, degrees.

Reactivity of Bound GSH in Mutant Enzymes—Since all mu-
tient enzymes bound to GSH-Sepharose, the loss of PGDS and GST activities in some of the mutants was not considered to be attributable to the loss of GSH binding. Therefore, we examined the reactivity of the thiol group of GSH bound to the mutants with a thiol-modifying agent, DTNB (Fig. 5).

When wild-type PGDS was incubated with DTNB in the absence of GSH, no reaction was observed at pH 5.0, indicating that the two free cysteine residues in the wild-type protein were not titrated with DTNB. In solution with 0.5 mM GSH, DTNB was converted to 5-thio-2-benzoic acid at an initial rate of 15 mM/min (Fig. 5, inset). The addition of the wild-type enzyme or the W104I, K112E, Y152F, C156L/Y, K198E, or L199F mutant increased the reaction rate exactly 2-fold, indicating that GSH had been activated. Interestingly, the W104I mutant, devoid of both PGDS and GST activities, could still bind GSH and apparently activate it. However, no enhancement of thiol activity was observed with Y8F and R14E/K, even though these mutants bound GSH as revealed by their purification on GSH-Sepharose.

**DISCUSSION**

In the present mutational analysis of hematopoietic PGDS, we defined the role of Tyr and Arg in the activation of the thiol group of GSH bound to PGDS, identified amino acid residues contributing to the PGH₂-binding site, showed the requirement for Trp in sustaining the catalytic cleft in an active form, and indicated the importance of the C-terminal domain eave-like structure for catalysis. Tyr and Arg are absolutely critical for catalysis as evidenced by the fact that Y8F and R14E/K lost both PGDS and GST activities (Fig. 4 and Table I). However, these mutants bound GSH, yet the K112E mutant could be expressed in *E. coli* at a level comparable to that of the wild-type protein (Fig. 2) and could bind to GSH-Sepharose during purification. Furthermore, the thiol group of GSH bound to W104I was activated, as judged by the increased reactivity with DTNB (Fig. 5), most likely due to the hydrogen bonding with the Tyr hydroxy group. Proper folding of the inactive W104I protein was also indicated by the far-UV CD spectrum, which was similar in shape to that of the wild-type enzyme (Fig. 3). However, the deeper [θ]222nm value of W104I compared with that of the wild-type enzyme indicates an increase in the α-helix content of the mutant. This increased content agrees with a role for Trp in forming a kinked backbone in the connecting loop between α-helices 4 and 5 within the eave-like structure of the enzyme (Fig. 1A). Thus, a point mutation of Trp may expand α-helix 4 in hematopoietic PGDS, which is shorter than that found in other GSTs (22). In addition, due to the localization of Trp in proximity to the cyclopentane head of PGH₂ and the unique direction of the indole ring of Trp parallel to α-helix 4 and the GSH backbone in hematopoietic PGDS, Trp is presumed to be making van der Waals contact with the hydrophobic substrate(s) of hematopoietic PGDS, being responsible for forming the remarkably deep and wide cleft, to accommodate lipophilic PGH₂ inserted into the catalytic center (Fig. 1). Substitution of Trp is considered to result not only in the loss of an indole ring interacting with PGH₂ but also in disruption of the overall structure of the active site and thereby PGDS and GST activities (Fig. 1B). These findings imply the importance of the eave-like structure in hematopoietic PGDS for catalysis and of the role of Trp in maintaining the active eave-like structure and thus proper functioning of the enzyme.

The drastically reduced catalytic efficiency of the K112E mutant (Fig. 4 and Table I) also supports the idea that residues in the eave-like structure in the C-terminal domain of PGDS are important for the catalysis of the enzyme. Lys is conserved throughout the members of the Sigma class of GSTs (39). This residue is located within α-helix 5 in hematopoietic PGDS (Fig. 1A) and has been proposed to make a salt bridge with the C-1 carboxylate of PGH₂ (Fig. 1B) (22). The selective decrease in PGDS activity with only a slight change in the GST activity of the K112E mutant supports the model in which Lys is involved only in PGH₂ activity (Fig. 4). Although the expression level of the K112E mutant was much lower than that of the wild-type protein (Fig. 2) and the α-helix content of K112E was significantly decreased (Fig. 3), the maintenance of GST activity suggests that the disruptions in the structure are primarily localized at the PGH₂-binding site. Because the Lys mutation significantly affected the catalytic activity of the enzyme, we also mutated Lys, which is located in the vicinity of Lys, on the other side of the carboxyl anion of PGH₂ (Fig. 1A). The K198E mutation decreased the PGH₂ isomerization efficiency with *k*ₐ and *k*ᵣ/Kₐ values 4- and 3-fold decreased as compared with those of the wild-type enzyme (Table I) and increased the affinity for GSH with a *Kₐ* value half that of wild-type PGDS. The K198E mutation had only a moderate effect on the GST activity (Fig. 4 and Table I) and no effect on the overall protein structure as judged by the absence of GSH, no reaction was observed at pH 5.0, indicating that the two free cysteine residues in the wild-type protein were not titrated with DTNB. In solution with 0.5 mM GSH, DTNB was converted to 5-thio-2-benzoic acid at an initial rate of 15 mM/min (Fig. 5, inset). The addition of the wild-type enzyme or the W104I, K112E, Y152F, C156L/Y, K198E, or L199F mutant increased the reaction rate exactly 2-fold, indicating that GSH had been activated. Interestingly, the W104I mutant, devoid of both PGDS and GST activities, could still bind GSH and apparently activate it. However, no enhancement of thiol activity was observed with Y8F and R14E/K, even though these mutants bound GSH as revealed by their purification on GSH-Sepharose. The drastically reduced catalytic efficiency of the K112E mutant (Fig. 4 and Table I) also supports the idea that residues in the eave-like structure in the C-terminal domain of PGDS are important for the catalysis of the enzyme. Lys is conserved throughout the members of the Sigma class of GSTs (39). This residue is located within α-helix 5 in hematopoietic PGDS (Fig. 1A) and has been proposed to make a salt bridge with the C-1 carboxylate of PGH₂ (Fig. 1B) (22). The selective decrease in PGDS activity with only a slight change in the GST activity of the K112E mutant supports the model in which Lys is involved only in PGH₂ activity (Fig. 4). Although the expression level of the K112E mutant was much lower than that of the wild-type protein (Fig. 2) and the α-helix content of K112E was significantly decreased (Fig. 3), the maintenance of GST activity suggests that the disruptions in the structure are primarily localized at the PGH₂-binding site. Because the Lys mutation significantly affected the catalytic activity of the enzyme, we also mutated Lys, which is located in the vicinity of Lys, on the other side of the carboxyl anion of PGH₂ (Fig. 1A). The K198E mutation decreased the PGH₂ isomerization efficiency with *k*ₐ and *k*ᵣ/Kₐ values 4- and 3-fold decreased as compared with those of the wild-type enzyme (Table I) and increased the affinity for GSH with a *Kₐ* value half that of wild-type PGDS. The K198E mutation had only a moderate effect on the GST activity (Fig. 4 and Table I) and no effect on the overall protein structure as judged by the absence of GSH, no reaction was observed at pH 5.0, indicating that the two free cysteine residues in the wild-type protein were not titrated with DTNB. In solution with 0.5 mM GSH, DTNB was converted to 5-thio-2-benzoic acid at an initial rate of 15 mM/min (Fig. 5, inset). The addition of the wild-type enzyme or the W104I, K112E, Y152F, C156L/Y, K198E, or L199F mutant increased the reaction rate exactly 2-fold, indicating that GSH had been activated. Interestingly, the W104I mutant, devoid of both PGDS and GST activities, could still bind GSH and apparently activate it. However, no enhancement of thiol activity was observed with Y8F and R14E/K, even though these mutants bound GSH as revealed by their purification on GSH-Sepharose. The drastically reduced catalytic efficiency of the K112E mutant (Fig. 4 and Table I) also supports the idea that residues in the eave-like structure in the C-terminal domain of PGDS are important for the catalysis of the enzyme. Lys is conserved throughout the members of the Sigma class of GSTs (39). This residue is located within α-helix 5 in hematopoietic PGDS (Fig. 1A) and has been proposed to make a salt bridge with the C-1 carboxylate of PGH₂ (Fig. 1B) (22). The selective decrease in PGDS activity with only a slight change in the GST activity of the K112E mutant supports the model in which Lys is involved only in PGH₂ activity (Fig. 4). Although the expression level of the K112E mutant was much lower than that of the wild-type protein (Fig. 2) and the α-helix content of K112E was significantly decreased (Fig. 3), the maintenance of GST activity suggests that the disruptions in the structure are primarily localized at the PGH₂-binding site. Because the Lys mutation significantly affected the catalytic activity of the enzyme, we also mutated Lys, which is located in the vicinity of Lys, on the other side of the carboxyl anion of PGH₂ (Fig. 1A). The K198E mutation decreased the PGH₂ isomerization efficiency with *k*ₐ and *k*ᵣ/Kₐ values 4- and 3-fold decreased as compared with those of the wild-type enzyme (Table I) and increased the affinity for GSH with a *Kₐ* value half that of wild-type PGDS. The K198E mutation had only a moderate effect on the GST activity (Fig. 4 and Table I) and no effect on the overall protein structure as judged by
Mutagenesis of Hematopoietic Prostaglandin D Synthase

Table I

Kinetic parameters of wild-type and mutant PGDSs

| Enzyme | $K_m$ (PGH$_2$) | $k_{cat}$ (PGH$_2$) | $K_m$ (GSH) | $k_{cat}$ (GSH) | $K_m$ (CDNB) | $k_{cat}$ (CDNB) |
|--------|----------------|-------------------|-------------|----------------|-------------|----------------|
| WT     | 0.10           | 31.07             | 0.50        | 21.70          | 0.50        | 2.00           |
| Y8F    | ND             | ND                | ND          | ND             | ND          | ND             |
| R14E   | ND             | ND                | ND          | ND             | ND          | ND             |
| R14K   | ND             | ND                | ND          | ND             | ND          | ND             |
| W104I  | ND             | ND                | ND          | ND             | ND          | ND             |
| K112E  | 0.18           | 3.42              | 0.49        | 4.30           | 2.23        | 1.92           |
| Y152F  | 0.08           | 29.50             | 0.55        | 15.55          | 0.80        | 1.76           |
| C156L  | ND             | ND                | ND          | ND             | ND          | ND             |
| C156Y  | ND             | ND                | ND          | ND             | ND          | ND             |
| K199E  | 0.05           | 8.40              | 0.70        | 7.18           | 0.70        | 2.16           |
| L199F  | 0.09           | 29.60             | 1.01        | 20.30          | 0.70        | 1.50           |

WT $k_{cat}$ (GSH) = 31.07 s$^{-1}$, $K_m$ (GSH) = 0.50 mM, $k_{cat}$ (CDNB) = 2.00 s$^{-1}$, $K_m$ (CDNB) = 3.00 mM.

Fig. 5. Time course for the excess reaction of GSH bound to the wild-type and mutant enzymes with DTNB at pH 5.0. The excess SH-selective reaction of the mutants W104I (●), C156L (■), Y152F (○), R14E/K (△), and Y8F (▲) including the wild-type enzyme (□) with DTNB is shown after subtraction of the control reaction with no enzyme. The reaction rates for the K112E, C156Y, K199E, and L199F mutants with DTNB were identical to the rate of the wild-type enzyme. The R14E and Y8F mutants showed no excess reactivity with DTNB. The inset represents the raw reaction curves during 5 min of reaction of the wild-type enzyme (WT; □) and 1 mM GSH + 0.05 mM DTNB as the control reaction (■). The concentrations used were as follows: enzymes, 0.1 mM; GSH, 0.5 mM; and DTNB, 0.05 mM.

protein expression level (Fig. 2) and far-UV CD analysis (Fig. 3). Taken together, these results indicate that introduction of a positively charged residue on both sides of the eave-like structure of the catalytic cleft is important for PGH$_2$ function, probably by interacting with the carboxyl anion of PGH$_2$.

On the other side of the proposed PGH$_2$-binding site, Cys$^{156}$ at the boundary between the hydrophilic and hydrophobic domains of the catalytic cleft was proposed to interact with the PGH$_2$ aliphatic chain. Mutation of Cys$^{156}$ to either Leu or Tyr selectively abolished the PGDS activity, but the GST activity was still retained, supporting this model for PGH$_2$ binding. The bulky side chains of Leu and Tyr most likely fill the center of the wide catalytic cleft between the N- and C-terminal domains of PGDS (Fig. 1) and sterically block the insertion of PGH$_2$, preventing the interaction of the peroxidase group of PGH$_2$ with the activated thiolate of bound GSH. On the other hand, the GSH-binding region of the mutants is still open and allows the conjugation of GSH to CDNB, although with a lesser efficiency due to the partial blockage of the catalytic cleft. The expression level of C156Y was very low (Fig. 2), although there were no changes in its overall structure as judged by CD spectroscopy (Fig. 3). Therefore, the replacement of Cys$^{156}$ by an amino acid residue with an aromatic side chain may affect the folding process.

The Y152F and L199F mutations had the least effect on the enzymatic catalysis of hematopoietic PGDS. Tyr$^{152}$, together with Arg$^{14}$, Glu$^{106}$, and two water molecules bound at the bottom of the catalytic cleft (Fig. 1C), is thought to form the hydrogen bonding network involved in the binding of GSH, as reported in the Pi class GSTs (40). The partial decrease in both PGDS and GST activities of Y152F (Fig. 4 and Table I) as well as its low expression level (Fig. 2) indicate that Tyr$^{152}$ primarily contributes to sustaining an active architecture for the hydrogen bonding network within the catalytic cleft. C-terminal Leu$^{199}$ underneath the eave-like structure of the catalytic cleft is assumed to play an important role in PGH$_2$ binding because of its proximity to the cyclopentane ring of PGH$_2$ (Fig. 1, A and B). However, the kinetic parameters of the L199F mutant were not significantly changed with respect to PGDS catalysis and were only moderately altered in the case of GST activity (Table I). The L199F mutation caused a slight structural alteration of the protein since the CD spectrum analysis revealed that the α-helix content of L199F was higher than that of the wild-type enzyme (Fig. 3). The absence of a significant change in PGDS activity does not agree with the location of Tyr$^{152}$ and Leu$^{199}$ in the proposed PGH$_2$-binding pocket. It is possible that the mutation of these residues, which was designed to disrupt PGDS function, was in fact accommodated by subtle changes in the structure.

In conclusion, our results agree with the proposed mechanism for PGH$_2$ isomerization in hematopoietic PGDS, where Tyr$^a$ and Arg$^{14}$ are directly involved in activating the thiol group of GSH for nucleophilic attack on the peroxidase group of the PGH$_2$ substrate. Furthermore, Lys$^{112}$, Lys$^{198}$, and Cys$^{156}$ appear to delineate the PGH$_2$-binding pocket, and mutational analysis of Trp$^{104}$ and Lys$^{112}$ established the importance of the unique eave-like structure in forming an extraordinarily wide active cleft in hematopoietic PGDS to accommodate PGH$_2$, resulting in the highly specific and efficient catalysis.

Acknowledgments—We are grateful to Prof. K. F. Austen (Harvard Medical School) and Dr. R. Kikuno (Kazusa DNA Institute) for valuable discussions pertaining to the preparation of the manuscript; to Y. Sakaguchi, D. Irikura, and N. Udoke for technical assistance; and to N. Ueda and S. Sakne for secretarial assistance.
REFERENCES

1. Ito, S., Narumiya, S., and Hayashi, O. (1989) Prostaglandins Leukotrienes Essen. Fatty Acids 37, 219–234

2. Dumitrascu, D. (1996) Rom. J. Intern. Med. 34, 159–172

3. Alving, K., Matran, R., and Lundberg, J. M. (1991) Acta Physiol. Scand. 143, 93–103

4. Darius, H., Michael-Hepp, J. Thierauch, K. H., and Fisch, A. (1994) Eur. J. Pharmacol. 258, 297–313

5. Ram, A., Pandey, H. P., Matsumura, H., Kasahara-Orita, K., Nakajima, T., Takahata, R., Satoh, S., Terao, A., and Hayashi, O. (1997) Brain Res. 75, 81–89

6. Ueno, R., Narumiya, S., Ogorochi, T., Nakayama, T., Ishikawa, Y., and Hayashi, O. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 6093–6097

7. Terao, A., Kitamura, H., Asano, A., Kobayashi, M., and Saito, M. J. (1995) J. Neurochem. 65, 2742–2747

8. Minami, T., Okuda-Ashitaka, E., Mori, H., Ito, S., and Hayashi, O. (1996) J. Pharmacol. Exp. Ther. 278, 1146–1152

9. Urade, Y., Fujimoto, N., Ujihara, M., and Hayashi, O. (1987) J. Biol. Chem. 262, 3820–3825

10. Christ-Hazelhof, E., and Nugteren, D. H. (1979) Biochim. Biophys. Acta 572, 43–51

11. Urade, Y., Fujimoto, N., Ujihara, M., and Hayashi, O. (1985) J. Biol. Chem. 260, 12410–12415

12. Urade, Y., Ujihara, M., Horiguchi, Y., Ikai, K., and Hayashi, O. (1989) J. Immunol. 143, 2982–2989

13. Mahmud, I., Ueda, N., Yamaguchi, H., Yamashita, R., Yamamoto, S., Kanoaka, Y., Urade, Y., and Hayashi, O. (1997) J. Biol. Chem. 272, 28263–28266

14. Urade, Y., Ujihara, M., Horiguchi, Y., Igarashi, M., Nagata, A., Ikai, K., and Hayashi, O. (1990) J. Biol. Chem. 265, 371–375

15. Dirr, H., Reinemer, P., and Huber, R. (1994) Eur. J. Biochem. 220, 645–661

16. Meyer, D. J., and Thomas, M. R. (1995) Biochem. J. 311, 739–742

17. Jacoby, W. B., and Ziegler, D. M. (1990) J. Biol. Chem. 265, 20715–20718

18. Kanoaka, Y., Akiyama, Y., Fujimoto, N., and Hayashi, O. (1992) Biochim. Biophys. Acta 312, 549–555

19. Wang, R., Newton, D. J., Huskely, S. E., Pickett, C. B., and Lu, A. Y. H. (1992) J. Biol. Chem. 267, 19866–19871

20. Prade, L., Huber, R., Mangharam, T. H., Fahl, W. E., and Reuter, W. (1997) Curr. Biol. 7, 1287–1295