Biochemical and Immunological Characterization of Rat Spleen Prostaglandin D Synthetase

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Rat spleen prostaglandin D synthetase (Christ-Hazeloef, E., and Nugteren, D. H. (1978) Biochim. Biophys. Acta 572, 43-51) is very similar to rat brain prostaglandin D synthetase (Urade, Y., Fujimoto, N., and Hayashi, O. (1985) J. Biol. Chem. 260, 12410-12415) as judged by their pl (4.7-5.2), M, (26,000-27,000), and self-inactivation during the isomerization reaction from prostaglandin Hz to prostaglandin Dz. However, the amino acid compositions of these two enzymes were quite different. Furthermore, the spleen enzyme was associated with the glutathione S-transferase activity, differing from the brain enzyme. The synthetase and transferase activities of the spleen enzyme showed almost identical pH and glutathione dependencies, the optimum pH = 8.0 and Km for glutathione = 300 uM. The Km values for prostaglandin Hz and 1-chloro-2,4-dinitrobenzene (a substrate for the transferase) were about 200 uM and 5 mM, respectively. The synthetase activity was dose-dependently inhibited by 1-chloro-2,4-dinitrobenzene (IC50 = 5 mM) and more strongly by nonsubstrate ligands, such as bilirubin and indocyanine green (IC50 = 150 and 2 uM, respectively). Both the synthetase and transferase activities of the purified enzyme dose-dependently decreased and showed identical immunotitration curves by incubation with antibody against this enzyme, but remained unchanged when treated with antibody against the brain enzyme. The antibody specific for the spleen enzyme absorbed almost all of the synthetase activity and about 10% of the transferase activity in the spleen, but not the transferase activity in the liver, heart, and testis. These results show that the two types of prostaglandin D synthetase are similar but different enzymes and that the spleen enzyme is a unique glutathione S-transferase differing from other isozymes and their subunits reported previously.

Prostaglandin (PG) Dz is a naturally occurring prostanoid that shows various kinds of pharmacological activities. It inhibits platelet aggregation (1-3) and induces bronchoconstriction (4, 5), vasoconstriction, and/or vasodilation (5, 6) with unique species or tissue specificity different from those of other prostaglandins. Besides the peripheral effects, PGDz also shows several central actions such as sleep induction (7), hypothermia (8), analgesia (9), etc. However, the physiological function and the metabolic fate of PGDz have yet to be elucidated. Two types of PGD synthetase (PGH-D isomerase, EC 5.3.99.2) catalyzing the conversion of PGHz to PGDz were reported in rats. One is glutathione (GSH)-requiring PGD synthetase, which was purified from the spleen and partially characterized by Christ-Hazeloef and Nugteren (10, 11). The other is GSH-independent PGD synthetase, namely rat brain PGD synthetase, which was first purified and characterized by Shimizu et al. (12, 13) and is localized in the central nervous system (14). More recently, we reexamined the purification procedure and properties of the brain PGD synthetase [(15)] and showed that the brain enzyme is very similar to the spleen one, i.e. both enzymes are acidic proteins with the same M, of 26,000. To compare these two PGD synthetases in more detail, we investigated further the biochemical and immunological properties of the spleen PGD synthetase.

In this paper, we report that the spleen PGD synthetase is biochemically and immunologically different from the brain synthetase and that the spleen enzyme is a previously unknown anionic form of GSH S-transferase (EC 2.5.1.18).

EXPERIMENTAL PROCEDURES AND RESULTS

Catalytic Properties of Rat Spleen PGD Synthetase—As shown in Fig. 4, when the purified enzyme was incubated with PGHz, the enzymatic formation of PGDz continued linearly for 30 s, gradually slowed down, and almost stopped after 90 s, at which time about 30% of the substrate still remained in this experiment. The remaining PGHz was converted to PGDz by a further addition of the enzyme (data not shown). However, if another 2 nmol of PGHz were added to the reaction mixture instead of enzyme, further enzymic formation of PGDz was not observed (data not shown). When the enzyme was incubated with PGHz in the absence of GSH, no significant enzymatic formation of PGDz was observed. However, after the addition of GSH, the enzyme reaction commenced and proceeded for a further 60 s. These results both confirm the GSH dependency and indicate that the enzyme is inactivated during the isomerization reaction from PGHz to PGDz, similar to rat brain PGD synthetase (15). Under our standard assay conditions using a 1-min incubation, the enzymic formation of

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** Portions of this paper (including "Experimental Procedures," part of "Results," Figs. 1-3 and 5-9, and Tables 1-III) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-2835, cite the authors, and include a check or money order for $6.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverley Press.
PGD<sub>2</sub> was dependent on the enzyme concentration. No other PGs such as PGE<sub>2</sub> and PGF<sub>2α</sub> were formed enzymatically (data not shown).

As shown in Fig. 1, the synthetase activity was co-purified with part of GSH S-transferase. The transferase reaction proceeded linearly up to 15 min under the standard assay conditions. When substrate specificity of the transferase activity was determined with CDNB (1,2-dichloro-4-nitrobenzene) p-nitrobenzyl chloride, ethacrynic acid, 1,2-epoxy-3(p-nitrophenoxy)propane, and 4-phenyl-3-buten-2-one as substrates, no significant activity was observed with substrates other than CDNB up to 2 μg of the purified enzyme.

pH, GSH, and substrate dependencies of the PGD synthetase and GSH S-transferase activities are shown in Figs. 5-7, respectively. Both activities showed the same pH dependence and had pH optima at pH 8.0. No significant activities were found at pH values lower than 5 or higher than 11. The enzyme absolutely required GSH for these two reactions and was inactive with other sulfhydryl compounds such as β-mercaptoethanol, dithiothreitol, and cysteine. The GSH dependence of both activities showed the same Michaelis-Menten saturation kinetics, and the K<sub>m</sub> values for GSH were calculated to be about 300 μM in both reactions. When we determined the concentration of GSH during the PGD synthetase and GSH S-transferase reactions, almost stoichiometric consumption of GSH was observed in the transferase reaction; but no net change of GSH was detected in the synthetase reaction (data not shown). The enzyme activities increased almost linearly as a function of the substrate concentrations up to 80 μM PGH<sub>2</sub> in the PGD synthetase reaction and to 2 mM CDNB in the GSH S-transferase reaction. From the Lineweaver-Burk plots, the apparent K<sub>m</sub> values for PGH<sub>2</sub> and CDNB were calculated to be about 200 μM and 5 mM, respectively (data not shown).

When we performed the reaction with PGH<sub>2</sub> in the presence of various concentrations of CDNB, the PGD synthetase activity was dose-dependently inhibited and decreased by about 30% with 4 mM CDNB (Fig. 8). The IC<sub>50</sub> value of CDNB was extrapolated to be about 5 mM, which is almost the same as the K<sub>m</sub> value for the transferase activity of the enzyme. Since several nonsubstrate ligands of GSH S-transferase, such as bilirubin and indocyanine green, are known to inhibit the enzyme activity (25), we examined the inhibition of PGD synthetase activity with these ligands (Fig. 8). These two compounds also inhibited the PGD synthetase activity and even more strongly than CDNB. The IC<sub>50</sub> values of bilirubin and indocyanine green were determined to be 150 and 2 μM, respectively.

**Immunological Characterization of Rat Spleen PGD Synthetase**—In Ouchterlony immunodiffusion test (Fig. 9), the anti-rat spleen PGD synthetase antibody showed a linear immunoprecipitin line against crude extracts of the spleen and the purified enzyme, but not against rat brain PGD synthetase. In contrast, the anti-rat brain PGD synthetase antibody formed a precipitin line against the brain PGD synthetase but not against the spleen enzyme. By immunotitration analysis using purified rat spleen PGD synthetase and the antibodies (Fig. 10A), both activities of PGD synthetase and GSH S-transferase were dose-dependently decreased by incubation with anti-rat spleen PGD synthetase antibody and showed almost identical titration curves. However, neither enzyme activity was affected by the incubation with antibody against rat brain PGD synthetase. These results, together with those of the Ouchterlony test, show that rat spleen PGD synthetase is an enzyme immunologically different from rat brain PGD synthetase and also suggest that both PGD synthetase and GSH S-transferase activities are exerted by the immunologically same protein.

When crude extracts of the spleen were incubated with the anti-rat spleen PGD synthetase antibody, the PGD synthetase and GSH S-transferase activities were also dose-dependently decreased (Fig. 10B). After incubation with an excess amount of the antibody, the PGD synthetase activity almost completely disappeared while about 90% of the transferase activity remained, indicating that almost all of the PGD synthetase activity and about 10% of the GSH S-transferase activity in the spleen are catalyzed by this enzyme. When

![Fig. 4. Time course of PGD synthetase reaction catalyzed by purified rat spleen PGD synthetase. Assay method is described under "Experimental Procedures." After the preincubation at 25 °C for 10 min, 2 nmol of PGH<sub>2</sub> were incubated in the presence of the enzyme (15 ng) (O, Δ, □) or its absence (■, ▲, ■) for various intervals. Circles indicate values in the presence of GSH. In the case of the reaction started with GSH (triangles), 0.05 μmol of GSH dissolved in 1 μl of distilled water was injected into the mixture (arrow) after a 1-min incubation of PGH<sub>2</sub> in the absence of GSH, and the reaction was continued. At this time, PGH<sub>2</sub> concentration had decreased to about 80% due to autodegradation. Squares represent the formation of PGD<sub>2</sub> in the incubation without GSH. When boiled enzyme (100 °C, 5 min, pH 7.0) was used, PGD<sub>2</sub> formation was almost the same as that without the enzyme.](image1)

![Fig. 10. Immunotitration of PGD synthetase and GSH S-transferase activities of purified enzyme (A) and crude extracts of the spleen (B). Various amounts of anti-rat spleen PGD synthetase antibody (C, ∗), anti-rat brain PGD synthetase antibody (Δ, △), or IgG fraction from nonimmunized rabbits (□, ■) were incubated overnight at 4 °C with a constant amount of the purified enzyme (0.2 μg of protein; PGD synthetase activity, 11 nmol/min; GSH S-transferase activity, 0.3 nmol/min) or crude extracts of the spleen (150 μg of protein; PGD synthetase activity, 12 nmol/min; GSH S-transferase activity, 1.9 nmol/min). The purified enzyme was incubated with the antibodies in the presence of nonimmunized rabbit IgG (1 mg/ml) to prevent the inactivation of the enzyme during the incubation. The residual activities of PGD synthetase (open symbols) and GSH S-transferase (closed symbols) are expressed as percentages of those before the incubation.](image2)
nonimmunized rabbit IgG or anti-rat brain PGD synthetase antibody was incubated with the crude extract, no significant decrease in either activity was found, suggesting also that PGD synthetase of the brain type does not contribute to the biosynthesis of PGD₂ in the spleen.

Since acidic isozymes of GSH S-transferase are reportedly distributed in the rat liver (26), heart (27), and testis (28), the immunotitration analysis was also carried out with anti-rat spleen PGD synthetase antibody and crude extracts of these tissues. However, no decrease in the transferase activity was observed under the condition of antibody excess. Furthermore, the PGD synthetase activity of these tissues was not absorbed by the antibody, indicating that the spleen PGD synthetase is immunologically different from GSH S-transferase in those tissues (data not shown).

**DISCUSSION**

As summarized in Table IV, two types of PGD synthetases purified from the spleen and brain showed similar yet different molecular and catalytic properties. Although these two PGD synthetases are acidic and monomeric proteins with the same Mr, 26,000, the amino acid compositions are quite different (Table III). It is, therefore, unlikely that each enzyme is a product of the other by some minor modifications, which conclusion is also supported by the immunological study using two kinds of polyclonal antibodies specific for each PGD synthetase. That is, in the Ouchterlony test (Fig. 9) and by immunotitration analysis (Fig. 10), neither PGD synthetase bound to the heterologous antibody. Therefore, these PGD synthetases do not show any common antigenic sites.

The purified rat spleen PGD synthetase was associated with significant GSH S-transferase activity. In immunotitration with the antibody against the spleen PGD synthetase, the GSH S-transferase activity showed a titration curve identical with that of the PGD synthetase activity (Fig. 10). We therefore conclude that the spleen enzyme is an isozyme of GSH S-transferase. This enzyme is a unique GSH S-transferase in that it is anionic (pl 5.2) and monomeric, because most GSH S-transferases reported so far are cationic and dimeric in form. When we determined the transferase activity in each fraction of DE52 chromatography (1st step of the purification), most of the GSH S-transferase activity in the spleen was of the cationic type and was recovered in the flow-through fraction. Anionic GSH S-transferases were eluted in three peaks at concentrations of 40, 80, and 100 mM NaCl. The 2nd peak of anionic GSH S-transferase activity was copurified with the PGD synthetase activity. The recovery of the anionic GSH S-transferase after DE52 chromatography was 13% of the total transferase activity of the crude extract (Table I). This value is consistent with the 10% of the GSH S-transferase activity in the crude extract found to be absorbed by incubation with an excess amount of antibody specific for spleen PGD synthetase (Fig. 10B). The spleen PGD synthetase was incubated with a high concentration (5 mM) of sulfhydryl compounds during the purification, which might result in the cleavage of intermolecular disulfide linkages. However, when crude extracts of the spleen were directly subjected to the gel filtration chromatography with Sephadex G-190 in the presence of a lower concentration (0.5 mM) of β-mercaptoethanol, the PGD synthetase activity was eluted at the same position as that of the purified enzyme. Thus, the enzyme is thought to be naturally monomeric. As judged by the Mr, pl value, amino acid composition (Table III), partial amino acid sequence from the N terminus (Table II), and immunotitration analysis, the spleen enzyme appears to be a new isozyme of GSH S-transferase different from all other isozymes and their subunits reported previously.

The spleen enzyme showed unique substrate specificity. This enzyme was more active with PGH₂ than with CDNB and almost inactive with other synthetic compounds used as the substrates for alkylation, alkene epoxidation and epoxide transferase. Christ-Hazelhof et al. (29) reported that an isozyme of GSH S-transferase from sheep lung catalyzed the isomerase reaction of PGH₂ to PGD₂. Recently, Ogorochi et al. (30) found that PGH-E isomersases purified from the cytosol of human brain were associated with active GSH S-transferase. These enzymes belong to a new category of GSH S-transferases.

As shown in Fig. 4, the spleen PGD synthetase is readily inactivated by the isomerase reaction of PGH₂ to PGD₂. However, the GSH S-transferase reaction continued linearly up to 15 min under our standard assay conditions, during which time the turnover number of the transferase reaction catalyzed by this enzyme (70 min⁻¹) is almost comparable with that of the PGD synthetase reaction for 1 min (1250 min⁻¹) (Table IV). Therefore, the inactivation of the enzyme is thought to be specific for the reaction with PGH₂. Since the enzyme was not inactivated by the incubation with PGH₂ in the absence of GSH (Fig. 4), it appears that coexistence of PGH₂ and GSH is essential for the inactivation. Similar inactivation has been observed in the reaction catalyzed by rat brain PGD synthetase (15) and also in the PGH-E isomerase reaction by the human brain enzyme (30). Therefore, such an inactivation is a common phenomenon among these isomerases.

Both PGD synthetase and GSH S-transferase activities of the purified enzyme showed almost identical pH and GSH dependencies (Figs. 5 and 6). CDNB, bilirubin and indocyanine green, a substrate and nonsubstrate ligands of GSH
S-transferase, dose-dependently inhibited the PGD synthetase activity (Fig. 8). It is, therefore, likely that the isomerase reaction from PGH₂ to PGD₂ is catalyzed by this enzyme by the same mechanism which underlies the transferase reaction. Similar isomerase reactions are reportedly catalyzed by certain GSH S-transferase isozymes termed steroid isomerase or Δ₅-3-ketosteroid isomerase (25, 31). In the case of those isomerases, no net change of the GSH concentration was observed during the reactions, as is also the case for spleen PGD synthetase.

Active biosynthesis of PGD₂ has been reported to occur in various rat tissues (32–34) and cells (35). Although several researchers have tried to determine separately the activities of GSH-dependent and -independent PGD synthetases (14, 36), their assay conditions seem to have been insufficient to separate the activities of these two PGD synthetases. For example, 1 mM CDNB has been commonly used to inhibit the GSH-dependent PGD synthetase activity, although the compound at this concentration inhibits only 10% or less of the spleen PGD synthetase activity (Fig. 8) and almost all of the brain PGD synthetase activity. In some cases, GSH-independent PGD synthetase activity was determined without any sulfhydryl compounds, which are essential for the reaction of this enzyme. The catalytic properties of the two types of PGD synthetase reported in this and a previous study (15) provide useful information, for evaluation of the contribution of each enzyme to the biosynthesis of PGD₂ in those tissues or cells. Furthermore, the polyclonal antibodies specific for these two PGD synthetases are valuable probes to determine the tissue distribution and cellular localization of each PGD synthetase by immunoabsorption analysis and immunohistochemical staining, respectively. Studies along these lines are now in progress.

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REFERENCES


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The purified enzyme (about 300 μl) was applied to a Sephadex G-100 column equilibrated with 0.1 M Tris-Cl (pH 6.0) and 5 μM epinephrine. The column was eluted at a flow rate of 0.5 ml/min. The flow was absorbed at 280 nm, and peaks were identified by bioassays (Fig. 1). The purity of the purified enzyme was determined as described under "Experimental Procedures". The Mn and pl were determined using a Pharmacia Low Molecular Weight Calibration Kit and a Pharmacia pl Calibration Kit, respectively.

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The pH dependence of PGD synthetase (α) and GSH S-transferase (β) activities of purified rat spleen PGD synthetase was studied. The activities were measured in the presence of 40 μM PGG₂ and 1 mM GSH. The values represent those obtained after subtraction of the activity values in the absence of the enzyme. The buffers used were as follows (final 0.1 M): pH 4.0, 4.8, 5.5, sodium acetate; pH 6.2, 7.0, potassium phosphate; pH 8.0, 8.8, 9.5, tri-HCl; pH 9.5, 11.0, glycine-NaOH.

The GSH dependence of PGD synthetase (α) and GSH S-transferase (β) activities of purified rat spleen PGD synthetase was also investigated. The enzyme activities were determined in the presence of 40 μM PGG₂ and 1 mU CDNB for the reactions of PGD synthetase and GSH S-transferase, respectively.

The inhibition of PGD synthetase activity by various concentrations of CDNB, bilirubin (β), and indocyanine green (β) was also examined. Assays were carried out in the presence of 45 μM PGG₂ and 1 mM GSH as described under "Experimental Procedures". The enzyme amount was chosen to convert PGG₂ to 0.6 μmol assay mixture (upper limit - 4 mg CDNB was dissolved in ethanol and various volumes of the solution were dried up in the assay tube. None of the inhibitors themselves accelerated the autodegradation of PGD₂. The remaining activities are expressed as percentages of those without inhibitors.

Immunoaffinity chromatography was used to purify PGD synthetase from rat spleen and brain. The enzyme activities were determined as described under "Experimental Procedures".

The amino acid composition of purified rat spleen PGD synthetase was determined by sequence analysis with 400 pmol of purified rat spleen PGD synthetase. Values of mole% of the amino acids represent the average of three determinations (α, β, and β-hydroxypropyl).