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REACTIONS OF PROSTAGLANDIN ENDOPEROXIDES WITH PROSTAGLANDIN I SYNTHETASE SOLUBILIZED FROM RABBIT AORTA MICROSOMES*

Kikuko Watanabe, Shozo Yamamoto§ and Osamu Hayaishi

Department of Medical Chemistry, Kyoto University
Faculty of Medicine, Sakyo-ku, Kyoto 606, Japan

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SUMMARY Prostaglandin I (prostacyclin) synthetase was solubilized from rabbit aorta microsomes. When the solubilized enzyme was reacted with prostaglandin H_2 and the reaction mixture was extracted under acidic conditions, the major product was 6-keto-prostaglandin $F_{1\alpha}$. The formation of prostaglandin I_2 as the primary reaction product was demonstrated by the treatment of the reaction mixture with diazomethane under non-acidic conditions. In contrast, the reaction of prostaglandin H_1 with the enzyme resulted in the production of 12L-hydroxy-8,10-heptadecadienoic acid rather than prostaglandin I_1.

PG[1] I_2, originally referred to as prostacyclin (1), is a bioregulator with biological activities including vasodilation and inhibition of platelet aggregation (2, 3). The synthetase of this compound was reported to be localized in aorta microsomes of various mammals (4). As will be reported in this communication, the enzyme is now solubilized from rabbit aorta microsomes, and is shown to catalyze different types of reaction with two PG endoperoxides (PGH_2 and PGH_1).

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§ Present address, Department of Biochemistry, School of Medicine, Tokushima University, Tokushima 770, Japan.

1/ Abbreviations used: PG, prostaglandin; HHT, 12L-hydroxy-5,8,10-heptadecatrienoic acid; HHD, 12L-hydroxy-8,10-heptadecadienoic acid.

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EXPERIMENTAL PROCEDURES

[1-^{14}C]Arachidonic acid (60.2 mCi/mmol) and 8,11,14-[1-^{14}C]-eicosatrienoic acid (58 mCi/mmol) were purchased from Radiochemical Centre (Amersham) and New England Nuclear (Boston), respectively. Arachidonic acid was a product of P-L Biochemicals (Milwaukee). 8,11,14-Eicosatrienoic acid, 6-keto-$PGF_{1\alpha}$ and PGI_2-methyl ester were kindly provided by Mr. M. Hayashi of the Ono Central Research Institute. Precoated Silica Gel 60 F254 glass plates were purchased from E. Merck (Darmstadt), and DEAE-cellulose (DE52) from Whatman (Kent). PGH_1 and PGH_2 were prepared by the use of acetone powder of sheep vesicular gland microsomes (Ran Biochemicals, Tel-Aviv) as described previously (5). 15-Hydroperoxyarachidonic acid was prepared by incubation of arachidonic acid with soybean lipoxidase (Sigma).

Rabbit aorta microsomes were prepared as follows. Rabbits were killed by bleeding under anesthesia. Aorta (30 g) was dipped into liquid nitrogen and crushed into a fine powder with the aid of a Waring blendor. The resulting powder was suspended in 120 ml of 50 mM Tris-HCl buffer at pH 8.0, and then homogenized using a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 10,000 X *g* for 10 min, and then the supernatant solution was further centrifuged at 105,000 X *g* for 60 min. The precipitate was suspended in 20 mM Tris-HCl buffer at pH 8.0 to a final volume of 7 ml (the microsomes).

The standard assay mixture contained 50 mM Tris-HCl buffer at pH 8.0, 40 μM [1-^{14}C]PGH_2 (120,000 cpm dissolved in 5 μl of diethylene glycol dimethyl ether) and enzyme in a total volume of 100 μl. Reaction was started by the addition of substrate and carried out at 24°C for 30 s. Termination of the reaction and extraction from the reaction mixture were carried out under cold conditions as described previously (6). Thin layer chromatography was performed with the organic phase of ethyl acetate/isooctane/acetic acid/water (90:50:20:100) as Solvent System A. Radioactivity on chromatographic plate was monitored by a Packard radiochromatogram scanner model 7201. For quantitative determination of the enzyme activity, the glass plate was kept on a Kodak X-ray film overnight for autoradiography. The silica gel in the region of each visualized radioactive spot was scraped off, and the radioactivity was measured by a Packard liquid scintillation spectrometer model 3385. Protein concentration was determined according to the method of Lowry *et al.* (7).

For the isolation of 6-keto-$PGF_{1\alpha}$, the reaction mixture (0.3 ml) contained 50 mM Tris-HCl buffer at pH 8.0, 40 μM [1-^{14}C]PGH_2 (300,000 cpm) and enzyme (4 mg protein). For the isolation of HHD, the reaction mixture (7.5 ml) contained 50 mM Tris-HCl buffer at pH 8.0, 33 μM [1-^{14}C]PGH_1 and enzyme (17 mg protein). Incubation was carried out at 24°C for 1 min. The etherial extract was placed on a silica gel plate which was developed in Solvent System A. The major product with R_F value corresponding to 6-keto-$PGF_{1\alpha}$ was extracted from silica gel with ethyl acetate, and that corresponding to HHD with ethyl ether.

The identification of HHD by combined gas chromatography-mass spectroscopy was performed as follows. The reaction product from PGH_1 was converted to a methyl ester using diazomethane prepared

from N-methyl-N-nitroso-p-toluenesulfonamide. The methyl ester was purified by silica gel thin layer chromatography and further converted to a trimethylsilyl derivative using N-trimethylsilyl-imidazole. The derivative was analyzed by a JEOL combined gas chromatograph (model 20K)-mass spectrometer (model JMS D-100) in collaboration with Dr. M. Kurono, Mr. M. Sawada, and Dr. S.Takaoka of the Ono Central Research Institute.

For the isolation of the reaction product from PGH_2 as a methyl ester, the reaction mixture (0.2 ml) was mixed with ice-cold acetone (0.4 ml) followed by the addition of diazomethane dissolved in ethyl ether (0.4 ml). The etherial layer was dried over sodium sulfate and subjected to silica gel thin layer chromatography using Solvent System B consisting of ethyl ether/acetone/triethylamine (100:10:0.1). The material with an R_F value identical with that of authentic PGI_2 methyl ester was extracted from silica gel with ethyl ether.

Platelet aggregation was followed by the increase in light transmission as described previously (5).

RESULTS AND DISCUSSION

As shown in Fig. 1A, when the microsomes were incubated with [1-^{14}C]PGH_2, the major radioactive product co-migrated with authentic 6-keto-$PGF_{1\alpha}$ (peak K). There was another small but significant peak (peak T) ahead of the unchanged PGH_2 (peak H_2), which was presumably HHT, as judged from its R_F value.

The microsomes suspended in 20 mM Tris-HCl buffer at pH 8.0, were mixed with one ninth the volume of 10% Triton X-100 solution. The mixture was stirred for 90 min in an ice bath and centrifuged at 105,000 X g for 60 min. The supernatant solution was concentrated by 5-fold with the aid of Centriflo CF25. The concentrate was applied on a DE52 column (1 X 5 cm) equilibrated with 20 mM Tris-HCl buffer at pH 7.4 containing 0.2% Triton X-100. The column was washed with 150 ml of the same buffer, and then the enzyme was eluted with a linear concentration gradient of NaCl from 0 to 1 M (50 ml of each solution in the above-mentioned buffer). Fractions of 3 ml were collected, and the enzyme activity was detectable between 10th and 20th fractions after the start of the gradient elu-

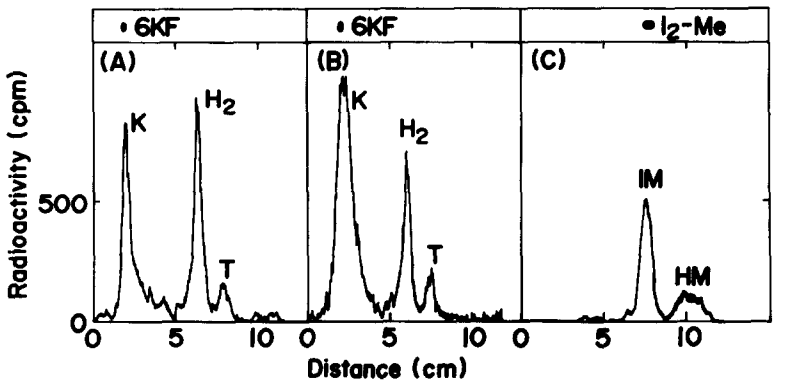

Fig. 1. Reaction of PGH_2 with PGI synthetase of rabbit aorta. Reactions with the microsomes (A) and the DE52 eluate (B) and thin layer chromatography using Solvent System A were performed under the standard assay conditions. (C) Reaction of PGH_2 with the DE52 eluate was also carried out, but terminated under non-acidic conditions, followed by the addition of diazomethane, as described under Experimental Procedures. Solvent B was used for thin layer chromatography. Symbols used are: 6KF; 6-keto-$PGF_{1\alpha}$, I_2-Me; PGI_2 methyl ester.

tion. These fractions were pooled and concentrated to about 3 ml. The DE52 eluate thus prepared gave a specific activity of approximately 15 nmol/min/mg protein (2-3 fold purification over the microsomes), and could be stored at -70°C without appreciable inactivation for at least 1 month.

Upon the reaction of the DE52 eluate with PGH_2, as shown in Fig. 1B, the PGH_2 (peak H_2) was converted predominantly to 6-keto-$PGF_{1\alpha}$ (peak K) with a concomitant production of HHT (peak T) in a small quantity as in the microsomal incubation. This small amount of HHT was significantly above the amount due to a non-enzymatic degradation of PGH_2. The radioactive material was extracted from the silica gel in the region of 6-keto-$PGF_{1\alpha}$, and subjected to silica gel thin layer chromatography in three different solvent systems as follows: ethyl ether/petroleum ether/acetic acid (85:15:0.1); benzene/dioxane/acetic acid (20:20:1); ethyl ether/acetone/acetic acid (100:10:0.1). In all the systems the radioactive product co-chromatographed with 6-keto-$PGF_{1\alpha}$. When the

radioactive product was treated with sodium borohydride and examined by thin layer chromatography with Solvent System A, the product was converted to a more polar compound. The result was consistent with the interpretation that the 6-keto group was reduced to a hydroxyl group.

Since PGI_2 was known to be unstable particularly under acidic conditions, the reaction of the DE52 eluate with [1-^{14}C]PGH_2 was terminated by the addition of acetone (not citric acid). The reaction mixture was immediately treated with diazomethane dissolved in ethyl ether. When the etherial extract was examined by thin layer chromatography under basic conditions, the radioactive product (peak IM) co-migrated with PGI_2 methyl ester (Fig. 1C). The product was extracted from silica gel. When the human platelet-rich plasma was preincubated with the compound, there was no platelet aggregation upon the addition of 0.3 μM epinephrine. The epinephrine-induced-platelet aggregation was inhibited essentially to the same extent by the same concentration of the diazomethane-treated reaction product and the authentic PGI_2 methyl ester. When both compounds were pretreated under acidic conditions, there was no inhibition of the platelet aggregation. These results indicated that the primary reaction product was PGI_2 which was isolated as a methyl ester under non-acidic conditions but decomposed to 6-keto-$PGF_{1\alpha}$ methyl ester in the presence of acid.

As examined with the initial reaction rate, the enzyme was most active around pH 7.4, and almost saturated with 40 μM PGH_2, Km for which was about 20 μM. With a saturating concentration of PGH_2 as substrate, the enzyme reaction proceeded linearly only for initial 30 s, and then slowed down to a complete cease. The cessation of the reaction was attributed to an inactivation of the enzyme since further addition of PGH_2 did not resume the re-

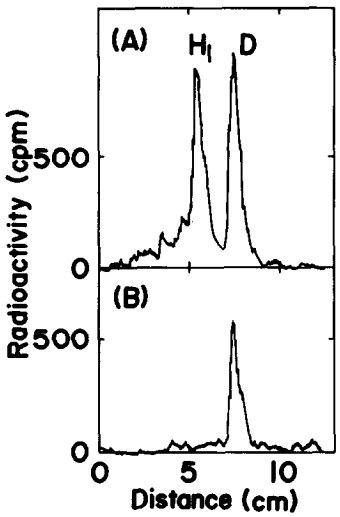

Fig. 2. Comparison of enzymatic (A) and non-enzymatic (B) reaction products from PGH_1. Enzymatic reaction was carried out under the standard conditions with PGH_1 as substrate. PGH_1 was treated with ferrous chloride as described for PGH_2 by Hamberg and Samuelsson (9). Thin layer chromatography in Solvent System A was performed as described under Experimental Procedures.

action but a fresh enzyme added to the reaction mixture was almost fully active. Glutathione, a coenzyme for PGE synthesis (8), did not stimulate the PGI synthetase reaction nor protected the enzyme from inactivation.

When the enzyme was incubated with PGH_1 instead of PGH_2, the reaction product (peak D) was less polar than PGH_1 (peak H_1) (Fig. 2A). This was in contrast to the result obtained with PGH_2 (Fig. 1B). The product was indistinguishable from HHD which was produced from PGH_1 upon the addition of ferrous chloride (Fig. 2B). The identity of the product with HHD was further confirmed by combined gas chromatography-mass spectrometry. The maximal rate of the HHD formation from PGH_1 was about 70% of that of the 6-keto-$PGF_{1\alpha}$ production from PGH_2. The enzyme was saturated with approximately 40 μM PGH_1, and the Km value for PGH_1 was about 20 μM. The inhibitory effect of 15-hydroperoxy-arachidonic acid was

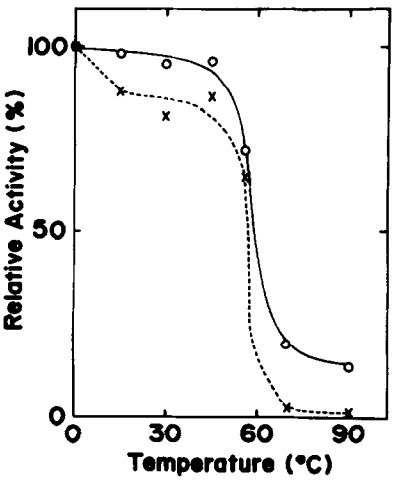

Fig. 3. Heat stability of the enzyme activities toward PGH_2 and PGH_1. The DE52 eluate dissolved in 20 mM Tris-HCl buffer at pH 7.4 was kept for 5 min at various temperatures as indicated, and then an aliquot (0.1 mg protein) was removed for assay in the presence of either PGH_2 (X-----X) or PGH_1 (o———o).

tested on the enzymatic production of HHD from PGH_1. This compound was reported to be a potent inhibitor of PGI synthetase (10). Both activities were inhibited essentially in a parallel fashion. As shown in Fig. 3, both enzyme activities showed similar stabilities upon preincubation of the enzyme at various temperatures. These observations suggested that the HHD formation was also catalyzed by PGI synthetase. Since the enzyme remains still impure, it is not certain whether two reactions are catalyzed by a single enzyme or by two separate enzymes. However, PGH_1 as a substrate analogue may interact with but misfit the active site of PGI synthetase, thus resulting in a side reaction to produce HHD rather than PGI_1. A similar finding has been reported previously for the reaction of PGH_1 with another enzyme, thromboxane synthetase (11).

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