Solubilization and Resolution of Thromboxane Synthesizing System from Microsomes of Bovine Blood Platelets*  

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TANIHIRO YOSHIMOTO, SHOZO YAMAMOTO, MINORU OKUMA,‡ AND OSAMU HAYAISHI  

From the Department of Medical Chemistry and the First Division, Department of Internal Medicine,‡  
Kyoto University Faculty of Medicine, Kyoto, Japan

The thromboxane synthetase system of the microsomes of bovine blood platelets was solubilized by the treatment with Triton X-100. The solubilized preparation was separated into two enzyme fractions by DEAE-cellulose chromatography. One catalyzed the formation of prostaglandin H₂ from arachidonic acid in the presence of heme and tryptophan. The other fraction converted prostaglandin H₂ to thromboxane B₂ and 12l-hydroxy-5,8,10-heptadecatrienoic acid. However, incubation of the latter fraction with prostaglandin H₂ at lower temperature produced an unstable compound with platelet-aggregating activity, which was presumably thromboxane A₂ and which decomposed readily to thromboxane B₂ and 12l-hydroxy-5,8,10-heptadecatrienoic acid.

Thromboxane A₂ is an extremely unstable but biologically potent compound with aorta-contracting and platelet-aggregating activities, whereas thromboxane B₂ is a stable compound without significant biological activity and derived from thromboxane A₂ (1). The formation of thromboxane A₂ from prostaglandin endoperoxide was demonstrated with human blood platelets (1), and the enzyme responsible for this conversion was localized in the microsomal fraction (2-6). This paper reports solubilization and resolution of the enzymes of bovine blood platelets which are involved in the synthesis of prostaglandin endoperoxide and its transformation to thromboxane (see Fig. 4). Properties of the isolated enzymes and their utility as a generating system of the unstable thromboxane A₂ for biological studies are also described.

EXPERIMENTAL PROCEDURES

Chemicals and Enzymes—Arachidonic acid was purchased from P-L Biochemicals (Milwaukee) and [1-14C]arachidonic acid (60 mCi/mmol) from the Radiochemical Centre (Amersham). Prostaglandins E₂, D₂, and thromboxane B₂ were kindly provided by Dr. M. Hayashi and Mr. F. Hirata of the Ono Central Research Institute. Beef blood hemoglobin (type I) was obtained from Sigma. Glutathione was supplied by the Yamanouchi Central Research Institute. Indomethacin was kindly provided by Dr. J. Suzuki of the Takeda Research Laboratories and Dr. A. Kato of Sumitomo Chemical Co. Ltd. D-Tosyl-N-methyl-N-nitrosoamide was purchased from Nakarai Chemicals (Kyoto) and N-(trimethylsilyl)diethylamine from Tokyo Chemical Industry (Tokyo). Silicic acid (100 mesh) for column chromatography was obtained from Mallinckrodt, silica gel for thin layer chromatography from Woelm, precoated Silica Gel 60 F254 glass plates from E. Merck (Darmstadt), and DEAE-cellulose (DE52) from Whatman. Prostaglandin endoperoxide synthetase (7) and E isomerase (8) were donated by Dr. S. Okh and Dr. N. Ogino of this laboratory, respectively.

Preparation of Prostaglandin H₂—A large scale preparation of prostaglandin H₂ was carried out by the use of a purified preparation of prostaglandin endoperoxide synthetase from bovine vesicular gland. Incubation was performed in five tubes each containing the following reaction mixture (2.0 ml): 100 mM Tris/HCl buffer at pH 7.4—200 μM [1-14C]arachidonic acid (6 x 10⁶ cpm), 5 μM hemoglobin, 5 mM L-tryptphan, and enzyme (376 μg of protein). After incubation at 24°C for 2 min, the reaction was terminated by the addition of 0.5 ml of 0.2 M citric acid precooled in an ice bath. The reaction mixture in each tube was extracted twice with 5 ml of ethyl ether precooled to 10°C. Subsequent manipulations were performed in a cold room at 4°C. The ethereal extracts were combined and dehydrated with anhydrous sodium sulfate, and the solvent was evaporated under reduced pressure. The dried material dissolved in 0.5 ml of petroleum ether (b.p. 30–60°C) was applied to a column of silicic acid (0.8 g, 0.6 x 6.8 cm). The column was washed with 40 ml of the same solvent, and then prostaglandin H₂ was eluted with 40 ml of petroleum ether (b.p. 30–60°C) (4-6). After evaporation of the solvent the dried material was dissolved in a small volume of acetone and stored at -70°C.

Preparation of Bovine Blood Platelet Microsomes—All the following manipulations were performed at 0-4°C. Bovine blood (8 liters) containing 7.7 mM EDTA as an anticoagulant, was centrifuged at 200 x g for 15 min to remove red and white blood cells. The upper layer was further centrifuged at 4000 x g for 20 min. The resulting platelet pellet was washed twice with 0.15 M NaCl containing 10 mM EDTA. The washed platelets (about 5 g, wet weight) were suspended in 100 mM potassium phosphate buffer at pH 7.4, and the platelet suspension was sonicated twice at 20,000 Hz for 1½ min with a Branson sonifier model W185D. The sonicate was centrifuged at 10,000 x g for 10 min, and the supernatant was further centrifuged at 105,000 x g for 60 min. The resulting precipitate was washed once and suspended in 10 mM potassium phosphate buffer at pH 7.4 to a final volume of 18.5 ml and a protein concentration of 6.9 mg/ml ("microsomal fraction"). Protein concentration was determined by the method of Lowry et al. (9).

Enzyme Reactions with Arachidonic Acid and Prostaglandin H₂—Reaction with arachidonic acid was carried out in the following reaction mixture (0.1 ml): 100 mM Tris/HCl buffer at pH 7.4, 30 μM [1-14C]arachidonic acid (1 x 10⁶ cpm), 2 μM hemoglobin, 5 mM L-tryptphan, and enzyme. Reaction with prostaglandin H₂ was performed in a reaction mixture (0.1 ml) containing 100 mM Tris/HCl buffer at pH 7.4, 50 μM [1-14C]prostaglandin H₂ (7 x 10⁶ cpm) and enzyme. In either case, the incubation was performed at 24°C for 1 to 5 min. Termination of reaction, extraction from the reaction mixture and thin layer chromatography were carried out under cold conditions as described previously (7). The following solvent systems were used for thin layer chromatography: I. ethyl ether/petroleum
ether:glacial acetic acid (85:15:0:1) (7); and II, ethyl ether:acetone: glacial acetic acid (80:15:0:5). Radioactivity on chromatographic plates was monitored by a Packard radiochromatogram scanner model 7201.

Identification of Product Formed from Arachidonic Acid by Fraction A—The reaction mixture described above was scaled up by 5-fold. After a 3-min incubation with 365 μg of Fraction A (see below) at 24°C, the reaction product was isolated and identified to be prostaglandin H₂ by the methods described previously for prostaglandin H₂ (5, 9, 11).

Identification of Products Formed from Prostaglandin H₂ by Fraction B—The incubation of Fraction B (see below) with prostaglandin H₂ of a lower specific radioactivity was performed in eight tubes, each containing the following reaction mixture (1.5 ml): 100 mM Tris/ HCl buffer at pH 7.4, 100 μM 1-CIC prostaglandin H₂ (1.35 × 10⁶ cpm) and Fraction B (1.35 μg of protein). After a 5-min incubation at 24°C, the reaction mixture was treated as described above. The dried material dissolved in 0.5 ml of petroleum ether:ethyl ether (8:2) was applied to a silicic acid column (1.0 g, 0.63 mm). After washing the column with 25 ml of a mixture of 9 parts human blood and 1 part 3.8% sodium citrate was centrifuged at 190,000 × g for 10 min, the supernatant was further centrifuged at 17,000 × g for 10 min, and the pellet was suspended in a 100-fold volume by the use of an Amicon centriflo CF 50A, and Fraction A was five times concentrated with the aid of a Diaflo membrane PM 10. Fig. 1c shows that both Compounds I and II were obtained by the simultaneous presence of both Fractions A and B.

Incubation of Arachidonic Acid with Fraction A or B—Incubation of arachidonic acid with Fraction A alone gave a product migrating slightly ahead of prostaglandin D₂ (Fig. 1d). It was converted to prostaglandin P₄₅ by the addition of stannous chloride and to prostaglandin E₂ by the action of prostaglandin endoperoxide E₅ isomerase. Based on these findings the reaction product was identified as prostaglandin H₂. On the other hand, Fraction B did not produce any product from arachidonic acid (Fig. 1e).

Incubation of Prostaglandin H₂ with Fraction B or A—The results described above indicate that Fraction A converted arachidonic acid to prostaglandin H₂, which may be further converted to Compounds I and II by the action of Fraction B. In order to test this possibility, prostaglandin H₂ was incubated with Fraction B. As presented in Fig. 2a, in addition to unchanged prostaglandin H₂ appearing slightly ahead of prostaglandin B₂, Compounds I and II were obtained. Prostaglandin H₂ remained essentially unchanged essentially when incubation with Fraction A (Fig. 2b).

Compound I which migrated close to prostaglandin E₂ was not converted to prostaglandin B₂ by alkaline treatment, thereby being distinguishable from prostaglandin E₂.
Compound I was treated with sodium borohydride, it was converted to a product migrating behind prostaglandin \( \text{F}_2 \). These observations were identical with those described by Hamberg et al. of thromboxane \( \text{B}_2 \). Compound I was treated with diazomethane and then with \( \text{N}-(\text{trimethylsilyl}) \) diethylamine and was subjected to gas chromatography-mass spectrometry as described under "Experimental Procedures." The mass spectrum gave ions essentially identical with those obtained with a trimethylsilyl derivative of the methyl ester of authentic thromboxane \( \text{B}_2 \) and contained the following ions reported in previous papers by other investigators (6, 13): 600 (M), 585, 510, 495, 439, 420, 366, 323, 301, 295, 256, 225, 217, 199, and 173.

As shown in Fig. 2c, when thin layer chromatography was performed with Solvent System II, authentic thromboxane \( \text{B}_2 \) appeared in two spots and there were two radioactive peaks (Compounds Ia and Ib) corresponding to the two spots, although their \( R_f \) values and relative heights varied from experiment to experiment. When each of these two compounds was extracted from silica gel and rechromatographed, in each case there were two peaks corresponding to Compounds Ia and Ib. The borohydride treatment of either Compound Ia or Ib produced a more polar compound with an identical \( R_f \) value migrating behind prostaglandin \( \text{F}_2 \). These findings suggest that Compounds Ia and Ib are closely related compounds. The two compounds may be in an equilibrium between \( \alpha \) and \( \beta \) configurations of the hemiacetal hydroxyl group of thromboxane \( \text{B}_2 \). Alternatively, either Compound Ia or Ib may be an acetylated derivative (6) produced during the development in Solvent System II which contained acetic acid.

Compound II was treated with diazomethane and then with \( \text{N}-(\text{trimethylsilyl}) \) diethylamine as described under "Experimental Procedures." When this derivative was analyzed by gas chromatography-mass spectrometry, the mass spectrum was consistent with that of the derivative of 12\( \text{t}-\)hydroxy-5,8,10-heptadecatrienoic acid reported by Hamberg et al. (10, 14) and contained the following ion peaks: 396 (M), 381, 333, 295, 276, 225, and 173.

The findings described above indicate that Fraction A contains an enzyme producing prostaglandin \( \text{H}_2 \) from arachidonic acid and Fraction B contains another enzyme catalyzing the conversion of prostaglandin \( \text{H}_2 \) to thromboxane \( \text{B}_2 \) and 12\( \text{t}-\)hydroxy-5,8,10-heptadecatrienoic acid (see Fig. 4).

Properties of Fractions A and B — Table I presents requirements for reactions catalyzed by Fractions A and B. The addition of both hemoglobin and tryptophan markedly stimulated the production of prostaglandin \( \text{H}_2 \) by Fraction A. The conversion of prostaglandin \( \text{H}_2 \) by Fraction B was scarcely stimulated by the addition of glutathione. A boiled preparation of Fraction A or B was almost inactive. Both Fractions A and B could be stored at \(-70^\circ\) without an appreciable loss of enzyme activities for several months.

Primary Production by Fraction B of Platelet-aggregating Compound — Fraction B was incubated with 5 \( \mu \text{M} \) prostaglandin \( \text{H}_2 \) in an ice bath. Aliquots of the mixture were removed at different time intervals and added to platelet-rich plasma. As shown in Fig. 3, the platelet-aggregating activity increased depending on the incubation time, reaching a maximum after 2 to 4 min and then rapidly decreasing. When the sample was taken after 3 min and kept at 50° for 30 s, the aggregating activity was lost. On the other hand, when the incubation of Fraction B with 5 \( \mu \text{M} \) prostaglandin \( \text{H}_2 \) was performed under identical conditions and the extracts of samples were subjected to thin layer chromatography to determine the formation of thromboxane \( \text{B}_2 \) and 12\( \text{t}-\)hydroxy-5,8,10-heptadecatrienoic acid, the accumulation of these two products also increased up to 4 min and then reached a plateau with a total concentration of the two products at nearly 5 \( \mu \text{M} \). These observations indicate that Fraction B catalyzed primarily the conversion of prostaglandin \( \text{H}_2 \) to a platelet-aggregating compound, which was unstable and decomposed readily to the two degradation products during incubation and isolation.
DISCUSSION

With a microsomal fraction of bovine blood platelets as an enzyme source we were able to solubilize and isolate two enzyme components involved in thromboxane biosynthesis (Fig. 4). The first component (Fraction A) is prostaglandin endoperoxide synthetase producing prostaglandin $H_2$ from arachidonic acid. Properties of the enzyme are very similar to those of a highly purified enzyme from bovine vesicular gland microsomes (7). Formation of prostaglandin $H_2$ catalyzed by the platelet enzyme also requires heme and tryptophan as activators. Such a requirement was also described with an enzyme of rabbit kidney medulla (15), indicate that such a requirement of heme and tryptophan is a general phenomenon regardless of the kind of enzyme source, although the function of these activators remains to be elucidated.

The activity of an isomerase which produced thromboxanes from prostaglandin endoperoxides has been demonstrated with whole cells or microsomes of human blood platelets (1-6). An enzyme converting prostaglandin $H_2$ to thromboxane $B_2$ and 12l-hydroxy-5,8,10-heptadecatrienoic acid was now solubilized as Fraction B from the microsomes of bovine blood platelets. The incubation of this enzyme and prostaglandin $H_2$, produces a compound with platelet-aggregating activity. This activity is easily lost by warming the preincubation mixture. As judged from the time course shown in Fig. 3, the activity is lost rapidly as soon as the compound is produced. Although it is difficult to quantify the platelet aggregation, the comparison of the two time courses presented in Fig. 3 suggests that the unstable platelet-aggregating compound decomposes to thromboxane $B_2$ and 12l-hydroxy-5,8,10-heptadecatrienoic acid, neither of which causes platelet aggregation. Thus, the primary product is presumed to be thromboxane $A_2$, based on its extreme instability and platelet-aggregating activity (1). However, it cannot be ruled out rigorously that the platelet-aggregating compound produced by Fraction B is a compound other than thromboxane $A_2$.

It is not conclusive whether the further degradation of thromboxane $A_2$ is an enzymatic process or a nonenzymatic reaction. The enzyme can be referred to as thromboxane synthetase (9, 16) or prostaglandin endoperoxide thromboxane isomerase. This isomerase should be compared with other isomerases transforming prostaglandin endoperoxides to various prostaglandins or related compounds. Both prostaglandin E and D isomerases require glutathione (8,17,18). In contrast, isomerases producing prostacyclin (19) and thromboxane (Ref. 6 and this paper) do not require glutathione. Although the role of glutathione in the former isomerases has not yet been elucidated (8,17), a certain built-in functional group might be working in place of glutathione in the latter isomerases.

The use of a purified enzyme preparation rather than crude preparations such as whole cells and microsomes is preferable as a thromboxane $A_2$ generating system in biological studies. In view of the biological significance of thromboxane $A_2$, purification of the isomerase, which is fortunately fairly stable, and attempts to isolate thromboxane $A_2$ using this enzyme are currently under investigation in this laboratory.

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