Immuoaffinity Purification and Characterization of Thromboxane Synthase from Porcine Lung*

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Thromboxane synthase has been purified 620-fold from porcine lung microsomes by a three-step purification procedure including Lubrol-PX solubilization, active blue agarose chromography, and immunoaffinity chromatography. The purified enzyme exhibited a single protein band (53,000 daltons) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Rabbit antiserum raised against the purified enzyme immunoprecipitated thromboxane synthase activity from crude enzyme preparations of porcine lung, cow lung, and human platelets, indicating the existence of structural homology of the enzyme in these species. Immunoblotting experiment identified the same polypeptide (53,000 daltons) in porcine lung and a polypeptide of 50,000 daltons in human platelets, confirming the identity of the enzyme and the specificity of the antiserum. Purified thromboxane synthase is a homoprotein with a Soret-like absorption peak at 418 nm. The enzyme reaction has a $K_m$ for 15-hydroxy-9a,11a-peroxidoprosta-5,13-dienoic acid of 12 $\mu$M, an optimal pH of 7.5, and an optimal temperature of reaction at 30 °C. Purified thromboxane synthase catalyzed the formation of both thromboxane $B_2$ and 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT). The ratios of HHT to thromboxane $B_2$ varied from 1.6 to 2.1 dependent on the reaction conditions. Except that HHT was formed at a greater rate, the formation of HHT and that of thromboxane responded identically to pH, temperature, substrate concentration, kinetics of formation, metal ions, and inhibitors suggesting that the two products are probably formed at the same active site via a common intermediate. Thromboxane synthase was irreversibly inactivated by 15-hydroxy-9a,11a-peroxidoprosta-5,13-dienoic acid during catalysis and by treatment of 15-hydroperoxyicosatetraenoic acid. The irreversible inactivation, however, could be protected by reversible inhibitors such as sodium (E)-3-[4-(1-imidazolylmethyl)phenyl]-2-propenoate and 15-hydroxy-11a,9a-epoxymethano-prosta-5,13-dienoic acid, suggesting that the inactivation occurred at the active site of the enzyme. The catalytic inactivation of thromboxane synthase and the greater rate of formation of HHT in thromboxane-synthesizing system may probably play important regulatory roles in the control of thromboxane synthase.

Hamberg and Samuelsson (1) reported that prostaglandin endoperoxides (15-hydroperoxy-9a,11a-peroxidoprosta-5,13-dienoic acid and PGH$_2$) were transformed by human platelets into a 17-carbon hydroxy fatty acid 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and a hemiacetal compound 8-(1-hydroxy-3-oxo-propyl)-9,12-dihydroxy-5,10-heptadecadienoic acid (TXB$_2$). Subsequent studies on the mechanism of the formation of TXB$_2$ from PGH$_2$ have led to the discovery of an unstable intermediate which induced platelet aggregation and contracted arterial smooth muscle (2). The compound was named TXA$_2$ and was probably the major component of rabbit aorta contracting substance described by Piper and Vane (3). The bicyclic oxane-oxetane ring in TXA$_2$ has been inferred from chemical trapping experiments (2). Although TXA$_2$ per se has not been isolated, the enzyme catalyzing the transformation of PGH$_2$ into TXA$_2$, thromboxane synthase, was first demonstrated in 1976 by Needelman et al. (4) in the microsomal fraction of human and horse platelets. Thromboxane synthase activity was later found to be present in many other tissues (5, 6) and in lung fibroblasts (7, 8). The thromboxane-synthesizing system has been extensively studied in platelet and lung microsomes (9–13). The enzymatic reaction appeared to require no exogenous cofactor or metal ions and was inhibited by a variety of inhibitors (14–19).

Purification of thromboxane synthase has been attempted by several groups of investigators, the enzyme has been solubilized by detergent and separated from prostaglandin endoperoxide synthase by DEAE-cellulose column chromatography (20–22). A 17-fold purification of porcine lung thromboxane synthase was described by Hall and Tai (23), while a 37-fold purification of human platelet enzyme was reported by Ullrich and Haurand (24). Partially purified thromboxane synthase appeared to catalyze the formation of both TXB$_2$ and HHT, since formation of both products was inhibited identically by inhibitors or heat-treatment (23, 25).

Despite the intense interest in TXA$_2$ in the past decade, relatively little is known about thromboxane synthase which catalyzes the biosynthesis of TXA$_2$. Characterization of this enzyme has been largely done with microsomes, or, in a few cases, with partially purified enzyme. The biochemical properties of the enzyme per se are lacking, so are the regulatory mechanisms controlling the biosynthesis and activity of this enzyme. Formation of TXA$_2$ from PGH$_2$ catalyzed by thromboxane synthase involves an unusual rearrangement, the molecular mechanism of which remained to be explored.

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1 The abbreviations used are: PGH$_2$, 15-hydroxy-9a,11a-peroxidoprosta-5,13-dienoic acid; TXA$_2$, thromboxane A$_2$; TXB$_2$, thromboxane B$_2$; HHT, 12-hydroxy-5,8,10-heptadecatrienoic acid; 15-HPETE, 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid; OKY-046, sodium (E)-3-[4-(1-imidazolylmethyl)phenyl]-2-propenoate; U-46,619, 15-hydroxy-11a,9a-epoxymethano-prosta-5,13-dienoic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
In order to get a deeper insight into the biochemical and physiological significance of thromboxane synthase it is apparent that more research needs to be done with the purified thromboxane synthase. We have recently obtained two monoclonal cell lines secreting antibodies to porcine thromboxane synthase (26). Here we report the immunoaffinity purification and characterization of porcine lung thromboxane synthase.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine serum albumin, pre-coated Silica Gel G, and stannous chloride were purchased from Sigma. Affi-Gel 10 was purchased from Bio-Rad. Labeled protein A, [1-14C]arachidonic acid, and [5,6,8,9,12,14,15-3H]arachidonic acid were obtained from New England Nuclear. OKY-046 was a gift from the ONO Pharmaceutical Co. TXB2 and U-46,619 were kindly provided by the Upjohn Co. All other reagents used were obtained from the sources as described in Ref. 26. HHT was prepared according to published procedures (27). 15-HETE was prepared according to the procedure of Graff (28). [14C]PGH2 and [5,6,8,9,11,12,14,15-3H]TPH2 were biosynthesized according to the method described by Hambreg et al. (29).

**Methods**—The production and purification of two monoclonal antibodies to porcine lung thromboxane synthase, the preparation of microsomes, and the solubilization and partial purification of thromboxane synthase by reactive blue-agarose column chromatography have been described (26).

**Assay of Thromboxane Synthase Activity**—During the purification of thromboxane synthase, the enzyme activity was assayed by radioimmunoassay of TXB2 transformed from PGH2 as described (26). When both HHT and TXB2 needed to be quantitated, the enzyme activity was assayed using a modified procedure of Sun (12). Thromboxane synthase was incubated with [14C]PGH2 or [14C]PGH2 (4.6 nmol) in 0.2 ml of Tris-HCl buffer (pH 7.5) at room temperature for 0-3 min. The reaction was arrested by the addition of 0.05 ml of SnCl2 (20 mg/ml ethanol), which converted all unreacted PGH2 to PGF2α. The products were extracted with 1.5 ml of ethyl acetate. The ethyl acetate extract was dried under N2 gas, then dissolved in 0.05 ml of acetone and spotted on Silica Gel G plates. Unlabeled TXB2 and HHT were also spotted as internal standards. The plates were developed in ethyl acetate/acidic acid (99:1). After chromatography the plates were placed in an iodine chamber to localize HHT and TXB2, and then scanned in a Berthold scanner. Zones corresponding to HHT and TXB2 were separately scraped into scintillation vials containing 10 ml of scintillation fluid and counted in a Beckman beta-counter. The amount of HHT or TXB2 formed was corrected for extraction efficiency (78% for TXB2 and 91% for HHT) and counting efficiency (90% for 14C and 54% for 3H).

One unit of enzyme activity is defined as the amount of enzyme required to produce 1 nmol of TXB2/min under the assay conditions.

**Preparation of Anti-thromboxane Synthase Immunoaffinity Column**—TS1 antibody (65 mg in 0.1 M citrate buffer, pH 6.0) purified by protein A-Sepharose column chromatography was dialyzed against 1 liter of 0.1 M NaHCO3 overnight at 4 °C. About 5 ml of Affi-Gel 10 was washed first with 10 ml of isopropyl alcohol, then with 10 ml of cold (4 °C) distilled water by gentle suction through a sintered glass funnel. The gel cake was transferred into a large test tube 15 × 150 mm) to which the antibody solution was added. The gel was suspended in solution by gentle agitation and the tube was sealed with paraffin, mounted on a tube rotator, and rotated for 2 h at 4 °C. The gel was separated from the solution by filtration, washed 3 times with PBSE buffer (10 mM sodium phosphate, 0.15 M NaCl, and 0.1 mM EDTA, pH 7.5), and suspended in 10 ml of 1 M ethanolamine/HCl buffer (pH 8.0) for 1 h at room temperature to block all the binding sites remaining on the gel. The gel was again washed three times with PBSE buffer, packed into a column, and stored at 4 °C.

**Spectrophotometric Analysis**—The visible spectrum of the purified enzyme was recorded in 1 ml of buffer (Tris-HCl, 25 mM; glycerol, 2.5%; DTT, 0.055 mM; EDTA, 0.25 mM; and Lubrol-PX, 0.025%, pH 7.5) in an Aminco spectrophotometer.

**Formation of TS1 and HHT by Thromboxane Synthase**—Purified thromboxane synthase in 0.2 ml of 50 mM Tris-HCl buffer (pH 7.5) was assayed by the addition of [14C]PGH2 (4.6 nmol, 169,000 cpm) for 0-3 min at room temperature. Control experiments were carried out similarly except that the enzyme was inactivated by boiling for 1 min before the addition of the substrate. The amounts of HHT and TXB2 formed were analyzed by TLC.

**Cathecol Inactivation of Thromboxane Synthase by PGH2**—In one set of test tubes 1 μg of purified thromboxane synthase (0.2 ml of 50 mM Tris-HCl buffer incubated with [14C]PGH2 (4.6 nmol, 169,000 cpm) for 30, 60, or 90 s, respectively. In another set of test tubes the enzyme reaction was run similarly but with the addition of fresh [H]PGH2 (4.6 nmol, 169,000 cpm) at 30, 60, or 90 s. The enzyme reaction was terminated 30 s after the addition of each substrate, the products were isolated and quantitated by TLC as described above.

**Protection of Thromboxane Synthase from Cathecol Inactivation by OKY-046 or U-46,619**—In a series of test tubes 1 μg of TS1 antibody was incubated with 0.1 ml of 10% Staphylococcus aureus cells in 1 ml of PBSE buffer for 30 min at room temperature. The tubes were centrifuged at 1,800 × g for 4 min. The pellets were washed with 1 ml of PBSE buffer and then incubated with partially purified thromboxane synthase (1.2 units in OKY-046 experiment; 2.4 units in U-46,619 experiment) in 0.1 ml of PBSE buffer containing 0.5% Tween 20 for 2 h at 4 °C. The tubes were centrifuged at 1,800 × g for 4 min. The pellets which contained immobilized thromboxane synthase were washed 3 times with 1 ml of PBSE buffer containing 0.5% Tween 20 and then resuspended in 1 ml of 50 mM Tris-HCl buffer (pH 7.5). One set of the tubes was assayed for thromboxane synthase activity in the presence of OKY-046 (0-100 nm) or U-46,619 (0-100 nm). The other set of tubes was incubated with 20 μg PGH2 for 2 min in the absence or presence of OKY-046 (0-100 nm) or U-46,619 (0-100 nm). The immobilized enzyme was then pelleted by centrifugation, washed twice with 1 ml of 50 mM Tris-HCl buffer (pH 7.5), resuspended in 1 ml of the same buffer, and assayed for activity. All activity assay was initiated by the addition of PGH2 (10 nmol in 5 μl of ethyl ether) for 2 min and terminated by 44 μM TXB2. TXB2 formed was determined by radioimmunoassay as described (26).

**Inhibition of TXB2 and HHT Formation by 15-HETE and the Protection by U-46,619**—Thromboxane synthase was immobilized on S. aureus cells by 1 μg of TS1 antibody as described above. The immobilized enzyme was suspended in 1 ml of 50 mM Tris-HCl buffer (pH 7.5). To one set of test tubes 10 μl of U-46,619 (5 mM in ethanol) was added, while the other set of tubes received 10 μl of ethanol. To each tube 15-HETE in 12.5 μl of ethanol was added to make final concentrations ranging from 10 to 60 μM. The tubes were incubated at room temperature for 2 min and centrifuged at 1,800 × g for 2 min. The pellets were washed twice with 1 ml of 50 mM Tris-HCl buffer (pH 7.5) and resuspended in 0.5 ml of the same buffer. The enzyme activity was assayed by TLC method using [14C]PGH2 as a substrate.

**Preparation of Polyclonal Antiserum to Thromboxane Synthase**—Purified thromboxane synthase (100 μg) in 0.2 ml of PBSE buffer was added to an equal volume of complete Freund’s adjuvant and the mixture was vigorously vortexed for 15 min. The emulsion was injected to a rabbit subcutaneously in the back at multiple sites. The rabbit was boosted monthly with 25 μg of the same antigen. Blood was withdrawn from the ear vein was centrifuged at 1800 x g for 20 min. The resultant antisera were removed and stored at -80 °C.

**Polyacrylamide Gel Electrophoresis and Immunoblotting of Thromboxane Synthase**—Proteins are fractionated by SDS-PAGE according to the method of Laemmli (30). The gel was stained with Coomassie Blue R in CH3OH/H2O/CH3COOH (45:45:10) and destained in the same solvent. Electrophoretic transfer of proteins from polyacrylamide gel to nitrocellulose paper was performed according to the method of Towbin et al. (31). After 1 h incubation in 3% bovine serum albumin (w/v) in phosphate-buffered saline, the nitrocellulose blot was incubated with rabbit antiserum to thromboxane synthase (1:30 dilution in phosphate-buffered saline) overnight at 4 °C. The blot was washed 3 × 10 min with phosphate-buffered saline and then incubated with 125I-labeled protein A (0.5 μCi/ml) for 1 h at room temperature. After thorough washing in phosphate-buffered saline, the blot was dried and exposed to Kodak X-Omat AR x-ray film with Cronex intensifying screen.

**Gas Chromatography/Mass Spectrometry**—This analysis of HHT was performed as described by Liu et al. (27).

**Immunoprecipitation of Thromboxane Synthase by Rabbit Antiserum**—The immunoprecipitation of thromboxane synthase by the antiserum was performed similar to that described before for the immunoprecipitation of thromboxane synthase by monoclonal antibodies (26).

**Other Methods**—Lung fibroblast (WI-38) cells were cultured in Dulbecco’s modified Eagle’s medium and 10% fetal bovine serum. The microsomes were prepared and solubilized as described (26). Preparative cell-free translations were performed using bovine serum albumin as a standard. Absorbance at 280 nm was used to monitor the elution profile of protein during chromatog-
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RESULTS

Immunoaffinity Purification of Thromboxane Synthase—An antibody column was prepared for immunoaffinity chromatography of thromboxane synthase by coupling TS2 antibody to Affi-Gel 10. The column (5-ml bed volume) contained about 55 mg of TS2 antibody and was equilibrated in PBSE buffer. Three ml of porcine lung thromboxane synthase (330 units, 55 mg of protein) partially purified from 250 g of lung tissue by reactive blue-agarose column chromatography was diluted with 3 ml of PBSE buffer. The enzyme solution was applied to the antibody column by circulating through the gel overnight at 4 °C. The column was washed with 75 ml of washing buffer (50 mM potassium phosphate, 10% glycerol, 1 M KCl, and 1% Lubrol-PX, pH 7.5) to remove nonspecifically bound proteins. Thromboxane synthase was then eluted by 0.1 M acetic acid containing 20% glycerol and 0.1% Lubrol-PX. The eluate was collected into test tubes containing 2 ml of 1 M Tris-HCl buffer (pH 8.0) for neutralization. Fig. 1 shows the elution profile of thromboxane synthase from the antibody column. The proteins applied were almost washed out by the washing buffer while 90% of the applied activity was retained. About 11% of the applied activity was finally eluted from the column. Fractions containing the enzyme activity were collected, concentrated, and dialyzed against 25 mM Tris-HCl buffer (pH 7.5) containing 2.5% glycerol, 0.025% Lubrol-PX, 0.25 mM EDTA, and 0.025 mM DTT.

The purity of the enzyme from different stages of purification was assessed by SDS-PAGE, as shown in Fig. 2. The sample from reactive blue-agarose column shows dozens of polypeptide bands while that from the antibody column shows only a polypeptide band having a subunit molecular weight of 53,000. A summary of the overall purification is presented in Table I. The enzyme was purified 624-fold by this three-step purification procedure. The recovery of the activity from microsomes was about 5%, while the enzyme protein obtained from 250 g of porcine lung was 160 μg.

Immunoprecipitation and Immunoblotting of Thromboxane Synthase—As reported earlier, monoclonal antibodies TS1 and TS2 were species-specific and did not cross-react with thromboxane synthase from other species tested (26). We also found that these antibodies failed to bind porcine thromboxane synthase electrophoretically to nitrocellulose paper after SDS-PAGE. We therefore raised rabbit antiserum to purified porcine lung thromboxane synthase and tested the cross-reactivity of the antiserum to thromboxane synthase from other species. As shown in Fig. 3, the rabbit antiserum not only immunoprecipitated thromboxane synthase of porcine lung but also immunoprecipitated thromboxane synthase
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TABLE I

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Protein Activity</th>
<th>Specific Activity</th>
<th>Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude microsomes</td>
<td>1929 mg</td>
<td>716 units</td>
<td>0.37</td>
<td>100 %</td>
</tr>
<tr>
<td>Solubilized microsomes</td>
<td>1169 mg</td>
<td>641 units</td>
<td>0.54</td>
<td>89 %</td>
</tr>
<tr>
<td>Reactive blue-agarose</td>
<td>55 mg</td>
<td>333 units</td>
<td>6.0</td>
<td>47 ×16</td>
</tr>
<tr>
<td>Antibody (TS), Affi-Gel 10</td>
<td>0.16 mg</td>
<td>37 units</td>
<td>231</td>
<td>5 ×624</td>
</tr>
</tbody>
</table>

* Assayed by radioimmunoassay of TXB₂ with 10 μM PGH₂ as substrate.

Fig. 3. Immunoprecipitation of thromboxane synthase by rabbit antiserum. Rabbit antiserum was generated against purified porcine lung thromboxane synthase as described under “Experimental Procedures.” Various amounts of the antiserum were incubated with 0.1 ml of S. aureus cell suspension (10%) in PBSE buffer. The S. aureus cells were pelleted, washed, and incubated with 2.0 units of thromboxane synthase partially purified by reactive blue-agarose column from porcine or bovine lung, or human platelets. The immunoprecipitated enzyme was assayed by radioimmunoassay of TXB₂ formed as described under “Experimental Procedures.”

Synthase—The visible spectrum of purified thromboxane synthase is shown in Fig. 5. The enzyme exhibited a Soret-like absorbance band at 418 nm, suggesting that the enzyme is a hemoprotein. No absorption peak beyond 500 nm was detectable at the enzyme concentration used (198 μg/ml).

Previous studies using partially purified thromboxane synthase have indicated that the formation of TXA₂ and HHT might be catalyzed by the same enzyme (23, 25). To verify this hypothesis, radiolabeled PGH₂ was biosynthesized and used as substrate for the purified thromboxane synthase. The reaction products were analyzed by TLC. Fig. 6 shows the radiochromatograms obtained from experiments using native or heat-denatured thromboxane synthase. It was clear that thromboxane synthase catalyzed the formation of both TXB₂ and HHT in a time-dependent manner. As a control, the heat-denatured enzyme did not produce HHT or TXB₂. It was also apparent that the amount of TXB₂ formed was not equal to that of HHT. A quantitative analysis of the products formed in a 3-min experiment is shown in Fig. 7. The formation of
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**Fig. 5.** The visible spectrum of purified thromboxane synthase of porcine lung. The spectrum was recorded in 1 ml of buffer (25 mM Tris-HCl, 2.5% glycerol, 0.025 mM DTT, 0.25 mM EDTA, and 0.025% Lubrol-Px) containing 198 μg of the enzyme against the blank buffer.

**Fig. 6.** Formation of TXB₂ and HHT with native and heat-denatured thromboxane synthase. Purified thromboxane synthase (1 μg) was assayed in the presence of [3H]PGH₂ (23 nM). The heat-denatured enzyme was prepared by boiling the enzyme for 1 min. Products were extracted and separated by TLC as described under "Experimental Procedures." The silica plates were scanned in a Berthold radioscanner.

Both products was linear up to 30 s and reached their maximal amounts at 2 min. To confirm that the compound co-migrated with biosynthesized HHT was actually HHT, it was extracted from silica gel, derivatized, and subjected to gas chromatography/mass spectrometry analysis. The mass spectrum of the trimethyl derivative of the methyl ester of the isolated compound showed ions at m/z 366 (M⁻), 335, 295, 276, and 255, consistent with the reported results (1).

The enzymic reaction catalyzed by thromboxane synthase showed a pH maximum at 7.5. Significant enzyme activity was lost at pH below 6.0 or above 8.5 (data not shown). The temperature dependence of the enzyme reaction (Arrhenius plot) is shown in Fig. 8. The optimum temperature for both HHT and TXB₂ formation was around 30 °C. Calculation of the activation energy from slopes of the linear portion of the curves gave 1.8 X 10⁴ and 1.7 X 10⁴ cal/mol for HHT and TXB₂, respectively. The enzyme activity was not affected by exogenous phospholipids or heme-containing compounds. At 1 mM, Cu²⁺ ion almost completely blocked the enzymic reaction while Zn²⁺ ion gave 50% inhibition. Other metals such as Ca²⁺, Mg²⁺, Mn²⁺, Co³⁺, and Fe²⁺ had little or no effect on the enzyme reaction, although Fe²⁺ ion prevented the formation of TXB₂ by non-enzymic conversion of PGH₂ to HHT (1). The effect of PGH₂ concentration on thromboxane synthase activity is shown in Fig. 9. Similar substrate-dependent curves were observed for both HHT and TXB₂ formation. The ratio of HHT to TXB₂ increased with increas-
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Fig. 9. Effect of \( \text{PGH}_2 \) concentration on thromboxane synthase activity. Thromboxane synthase (1 \( \mu \)g) was assayed under various concentrations of \( \text{PGH}_2 \) as described under "Experimental Procedures." Each point is the mean of duplicate assays. HHT, ●; TXB\(_2\), ○; HHT/TXB\(_2\), □.

Using arachidonic acid as substrate, Ho et al. (10) reported that the duration of TXB\(_2\) synthesis by human platelet microsomes was short and addition of fresh enzyme protein to an enzyme reaction mixture that had lost the capacity to synthesize TXB\(_2\) further increased the formation of the product. Since the formation of TXB\(_2\) relies on both prostaglandin endoperoxide synthase and thromboxane synthase, it is not clear whether the short duration of TXB\(_2\) synthesis is due to the inactivation of prostaglandin endoperoxide synthase or that of thromboxane synthase. An experiment was therefore designed to examine if thromboxane synthase would be self-inactivated during catalysis. The result in Fig. 10 shows that the addition of fresh PGH\(_2\) at different time points during an enzymatic reaction leads only to a slight increase in TXB\(_2\) or HHT formation as compared to the ones having the same duration of reaction but without PGH\(_2\) addition. This suggests that the enzyme might have been irreversibly inactivated and the inactivation is a rapid process. Imidazole derivatives and PGH\(_2\) analogs were potent inhibitors of thromboxane synthase (17, 19). We have examined the inhibitory effects of OKY-046 and U-46,619 on purified thromboxane synthase and found that the formation of TXB\(_2\) and HHT was inhibited identically (data not shown). We further examined the effects of these inhibitors on the protection of thromboxane synthase from inactivation by PGH\(_2\). As shown in Table II, pretreatment of thromboxane synthase (immobilized on \( S. \) aureus) in the absence of inhibitor resulted in a considerable loss of the enzyme activity. However, the inactivation of the enzyme could be protected by increasing amounts of the inhibitors, suggesting that the irreversible inactivation by PGH\(_2\) probably occurred at the active site of the enzyme. It was also apparent that OKY-046 and U-46,619 were reversible inhibitors since removal of the inhibitor restored part of the enzyme activity. The incomplete recovery of the enzyme activity in these samples probably was due to the loss of activity during the additional washing procedure to remove the inhibitors before assaying the enzyme activity.

Hydroperoxy fatty acid has been shown to inhibit thromboxane synthase in platelet microsomes (21), but the mechanism of this inhibition was not known. 15-HPETE, like PGH\(_2\), is an oxidant and may inactivate the enzyme by oxidation. Fig. 11 shows that pretreatment of thromboxane synthase with various concentrations of 15-HPETE results in dose-dependent, irreversible inactivation of the enzyme. However, at low concentration of 15-HPETE, the enzyme could be partially protected by 100 \( \mu \)M U-46,619, suggesting

**TABLE II**

<table>
<thead>
<tr>
<th>Concentration of inhibitor</th>
<th>No pretreatment</th>
<th>Thromboxane synthase activity* (pretreatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+PGH(_2) (20 ( \mu )M)</td>
</tr>
<tr>
<td>---------------------------</td>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td>U-46,619 (( \mu )M)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.335 (100)</td>
<td>0.168 (50)</td>
</tr>
<tr>
<td>10</td>
<td>0.284 (88)</td>
<td>0.238 (71)</td>
</tr>
<tr>
<td>50</td>
<td>0.215 (64)</td>
<td>0.287 (86)</td>
</tr>
<tr>
<td>100</td>
<td>0.124 (37)</td>
<td>0.285 (85)</td>
</tr>
<tr>
<td>OKY-046 (nM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.231 (100)</td>
<td>0.073 (31)</td>
</tr>
<tr>
<td>10</td>
<td>0.115 (50)</td>
<td>0.165 (45)</td>
</tr>
<tr>
<td>50</td>
<td>0.048 (20)</td>
<td>0.133 (58)</td>
</tr>
<tr>
<td>100</td>
<td>0.023 (14)</td>
<td>0.127 (55)</td>
</tr>
</tbody>
</table>

*Numbers in parentheses represent percent activity of the control.

**FIG. 10.** Catalytic inactivation of thromboxane synthase by PGH\(_2\). In one set of test tubes, thromboxane synthase (1 \( \mu \)g) was assayed in the presence of 23 \( \mu \)M PGH\(_2\) (4.6 nmol) for 30, 60, or 90 s. In another set of test tubes, the enzyme reaction was carried out identically but with the addition of fresh PGH\(_2\) (4.6 nmol) at 30, 60, or 90 s (arrows). The reaction was continued for an addition 30 s. TXB\(_2\) and HHT formed were quantitated as described under "Experimental Procedures." Point at the end of each dashed line is the amount of TXB\(_2\) or HHT formed in the tube that has received fresh PGH\(_2\) 30 s before. Each point is the mean of duplicate assays. HHT, ●; TXB\(_2\), ○; HHT/TXB\(_2\), □.

**FIG. 11.** Inactivation of thromboxane synthase by PGH\(_2\) and the protection by OKY-046 and U-46,619.

Thromboxane synthase was immobilized on \( S. \) aureus cells by 1 \( \mu \)g of TS\(_2\) antibody. The enzyme activity was assayed in the presence of various amounts of inhibitors or was pretreated with PGH\(_2\) in the absence or presence of the inhibitors. The activity was assayed after the removal of PGH\(_2\) and inhibitors. Details of the experiment are described under "Experimental Procedures."
that the inactivation also occurred at the active site of the enzyme. Again, almost the same degree of inhibition and protection was observed for the formation of HHT and TXB₂.

**DISCUSSION**

Previous attempts from our laboratory and others to purify thromboxane synthase by conventional column chromatography have met with difficulties and resulted in limited success. As a consequence, more specific means are needed for the purification of this important enzyme. This has led us to produce monoclonal antibodies against thromboxane synthase for immunoaffinity purification of the enzyme. The TS₂ antibody used in this report belongs to the IgG₁ subclass and has a high affinity toward porcine lung thromboxane synthase (Kₐ = 3.1 × 10⁶ M⁻¹). The tight binding of this antibody to the enzyme has allowed us to wash the antibody column with a very stringent condition (1% Lubrol-PX and 1% SDS-PAGE) in the crude porcine lung sample. The purification of this important enzyme has allowed us to wash the antibody column with a very stringent condition (1% Lubrol-PX and 1% SDS-PAGE). The enzyme can either rearrange to form TXA₂ or break down to HHT. The three-step purification procedure described herein is simple and very reproducible. Porcine lung thromboxane synthase has been purified 625-fold with 5% yield in activity. The low yield of activity was poorer. Other conditions such as 4 M MgCl₂, 1 M NaSCN, or 0.1% SDS failed to elute the enzyme.

The purified enzyme has a subunit molecular weight of 53,000 and was electrophoretically homogeneous as judged by SDS-PAGE. That the 53,000-dalton polypeptide is thromboxane synthase is supported by the fact that rabbit antiserum to thromboxane synthase was further demonstrated by the immunoblotting experiment (Fig. 4). A single polypeptide was recognized by the antiserum in crude human platelet enzyme preparation (Mₐ = 50,000) and purified porcine lung enzyme preparation (Mₐ = 53,000). The presence of an additional polypeptide (Mₐ = 14,000) recognized by the antiserum in the crude porcine lung sample has posed the question that this polypeptide might be a subunit of thromboxane synthase or a fragment of the enzyme. However, the variations of the content of this polypeptide in different preparations and the lack of this peptide in purified samples (Fig. 2 and Fig. 4, lanes 1 and 3) obviate these possibilities. Our data (not shown) indicated that this polypeptide was a proteolytic fragment of the 53,000-dalton protein which retained the antigenic determinant recognized by the antiserum. The difference in polypeptide(s) recognized by the antiserum in the mononucleuses of human platelets and human lung fibroblast cells suggests that thromboxane synthase exists in different molecular forms in human tissues.

Purified thromboxane synthase appeared to be a hemoprotein since it exhibits an absorption maximum at 418 nm. Ullrich and Haurand (24) have postulated that platelet thromboxane synthase is a cytochrome P-450 type enzyme. They found that enrichment of thromboxane synthase activity (37-fold) was parallel with the increase of cytochrome P-450 concentration (40-fold) during chromatography and the partially purified enzyme exhibited Soret bands of absorbance at 418 nm, where we were unable to detect the typical α and β band absorption bands generally observed in the oxidized form of cytochrome P-450 at the enzyme concentration used (198 μg/ml). More studies such as the identification of the heme moiety and the determination of CO difference spectrum need to be done before a conclusion can be drawn.

Using the immunoaffinity-purified enzyme, we have unequivocally demonstrated that thromboxane synthase catalyzed the formation of both TXA₂ and HHT (Figs. 6 and 7). The formation of both products by the enzyme required no cofactors or metal ions and was not affected by exogenous phospholipids or heme-containing compounds, indicating that the enzyme is self-sufficient for catalysis. The fact that both HHT and TXA₂ are formed by the same enzyme has raised the question whether these two products are formed at the same active site. It has been demonstrated that the formation of HHT was dependent on the formation of TXA₂ because trapping of TXA₂ with methanol did not affect the amount of HHT formed (25). The two products can be formed in two separate active sites which independently transform PGH₂ into the corresponding products or they can be formed in the same active site which generates a common intermediate that can either rearrange to form TXA₂ or break down to HHT and malondialdehyde. The results we obtained could not differentiate the active site for TXA₂ formation from that for HHT formation. The similar kinetic parameters such as Kₐ, activation energy, and time course of formation and the identical responses to pH and to structurally unrelated inhibitors strongly suggest that both products are probably formed in the same active site through a common intermediate. Such a mechanism should call for the same Kₐ, activation energy, and the same degree of inhibition by inhibitors provided that the inhibitory effects are exerted at the step leading to the formation of the intermediate. These are all consistent with our results.

One dilemma in interpreting the mechanism of the simultaneous formation of HHT and TXA₂ by thromboxane synthase is the inconsistent ratios of HHT to TXB₂ observed. The ratio reported in the literature varied from 1 to 2.1 (1, 12, 20, 33-35). Using purified thromboxane synthase, we have found that the ratio of HHT to TXB₂ increased with increasing concentrations of PGH₂ reaching 2.1 at PGH₂ concentrations greater than 50 μM (Fig. 9). At a fixed concentration of PGH₂ (23 μM), the ratio also varied slightly depending on the reaction conditions, such as temperature, pH, and reaction time. In general, the ratio fell into the range of 1.6-2.0. Thus the discrepancy of the reported values about the ratio of HHT to TXB₂ might be largely due to the reaction conditions used.
Hall and Tai (23) have shown that a heat-stable, non-dialyzable factor in solubilized porcine lung microsomes can stimulate the formation of HHT from PGH₂. The presence of this or similar factor(s) in crude thromboxane synthase preparations may also account for the observed difference in HHT/TXB₂ ratio.

The physiological role of HHT remains enigmatic at present. The concomitant enzymic formation of this compound with TXA₂ and the existence of unidentified factor(s) catalyzing its formation from PGH₂ suggest that HHT itself or its formation might have a profound yet unrecognized role. We have recently found that HHT is an excellent substrate for NAD⁺-dependent 15-hydroxyprostaglandin dehydrogenase (27). Whatever role HHT may play it appears to be transient in vivo. It is conceivable that the greater rate of HHT formation in TXA₂-synthesizing tissues and the non-enzymic diversion of PGH₂ to HHT in some other tissues might serve as an important regulatory mechanism in controlling the biosynthesis of prostaglandins and thromboxanes.

The rapid and irreversible self-inactivation of thromboxane synthase by PGH₂ has been shown to occur in solubilized and antibody-immobilized enzyme (Fig. 10 and Table II). The protection from inactivation by reversible inhibitors suggests that the inactivation by PGH₂ or 15-HPETE occurs at the active site of the enzyme. We have found that the enzyme was also very sensitive to N-chlorosuccinimide (IC₅₀ = 200 μM, data not shown). Since PGH₂, 15-HPETE, and N-chlorosuccinimide are all oxidative reagents, the susceptibility of the enzyme to these reagents suggests that oxidative destruction of the enzyme might account for its rapid inactivation. It is possible that the heme group of the enzyme is the target for these reagents. Such a mechanism has been shown to occur in prostaglandin endoperoxide synthase and prostacyclin synthase (36, 37) and may represent a common regulatory mechanism in the production of the autacoids derived from arachidonic acid.

REFERENCES