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Purification and characterization of an NAD^+-dependent dehydrogenase that catalyzes the oxidation of thromboxane B_2 at C-11 from porcine liver. Development and application of 11-dehydro-thromboxane B_2 radioimmunoassay to enzyme assay

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11-Dehydro-thromboxane B_2 has been identified as a major metabolite of infused as well as endogenous thromboxane B_2 in mammalian plasma and urine. This metabolite is derived from thromboxane B_2 by enzymatic oxidation at C-11 catalyzed by 11-hydroxythromboxane B_2 dehydrogenase. A radioimmunoassay for 11-dehydro-thromboxane B_2 has been developed and used for enzyme assay, purification and characterization. Antibodies were generated against 11-dehydro-thromboxane B_2 conjugated to bovine thyroglobulin. Labeled marker was prepared by radioiodinating 11-dehydro-thromboxane B_2-tyrosine methyl ester conjugate. A sensitive radioimmunoassay capable of detecting 10 pg of 11-dehydro-thromboxane B_2 per assay tube was developed. The antibodies showed minimal crossreaction with thromboxane B_2 (0.03%), prostaglandin D_2 (2.76%) and other eicosanoids (< 0.03%). The enzyme activity was determined by assaying NAD^+-dependent formation of immunoreactive 11-dehydro-thromboxane B_2 from thromboxane B_2. The enzyme was found to be enriched in liver although significant activity was also detected in gastrointestinal tract and kidney in pig. The enzyme was purified from porcine liver cytosol to apparent homogeneity using conventional and affinity chromatography. The purified enzyme exhibited coenzyme specificity for NAD^+ and used thromboxane B_2 as a substrate. The enzyme also catalyzes NADH-dependent reduction of 11-dehydro-thromboxane B_2 to thromboxane B_2 indicating the reversibility of the enzyme catalyzed reaction. The apparent K_m values for thromboxane B_2, 11-dehydro-thromboxane B_2 and NAD^+ are 8.1, 8.0 and 23 μM, respectively. Subunit M_r was shown to be 55000, whereas the native enzyme M_r was found to be 110000 indicating that the enzyme is a dimer. The enzyme is sensitive to sulfhydryl inhibitions suggesting cysteine residues are essential to enzyme activity. The availability of a homogeneous enzyme preparation should allow further studies on the substrate specificity and the structure and function of the enzyme.

Introduction

Thromboxane (TX) A_2 is a labile yet potent vasoconstrictor and platelet activator [1]. It is derived from arachidonic acid via cyclooxygenase and thromboxane synthase catalyzed reactions [2]. This eicosanoid is readily hydrolyzed nonenzymatically to TXB_2 following its synthesis [1]. Further metabolism of TXB_2 appears to occur through two different pathways. One involves β-oxidation of TXB_2, resulting in the formation of 2,3-dinor-TXB_2 [3], whereas the other involves dehydrogenation of the hemiacetal alcohol group at C-11 leading to the production of 11-dehydro-TXB_2 [4]. Studies on major plasma and urinary metabolites of infused or endogenous TXB_2 have indicated that 11-dehydro-TXB_2 is a more predominant product than 2,3-dinor-TXB_2 [5,6]. In view of the fact that there is a readily ex vivo synthesis of TXB_2 but not of 11-dehydro-TXB_2 by blood cells, quantitation of 11-dehydro-TXB_2 has been suggested to provide a better index for assessing in vivo formation of thromboxane in circulation [6–8].

The enzyme that catalyzes the conversion of TXB_2 into 11-dehydro-TXB_2 requires NAD^+ as a coenzyme and is thus named NAD^+-dependent 11-hydroxythromboxane B_2 dehydrogenase [3]. It has been found to be

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Abbreviations: TX, thromboxane; TME, tyrosine methyl ester; PG, prostaglandin; HETE, hydroxyeicosatetraenoic acid.

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present in various mammalian tissues [7]. We have found that this enzyme is localized to the cytosolic fraction of various porcine tissues of which liver is particularly active. In order to gain further insight into the molecular properties of the enzyme and mechanisms of enzyme action and regulation, it is necessary to purify this enzyme to homogeneity. This paper describes a method for obtaining a homogeneous preparation from porcine liver cytosol, and some of its molecular and catalytic properties. A preliminary account of this study has been represented in an abstract form [9].

Experimental procedures

Materials

NAD^+, $NADP^+$, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, *N*-ethyl maleimide, *p*-chloromercuribenzoate, sodium mersalyl, dithio-bis(nitrobenzoate), dithiothreitol, Reactive blue agarose, DEAE-cellulose, charcoal, triethylamine, ethyl chloroformate, sodium dodecyl sulfate (SDS), 2-mercaptoethanol, tyrosine methyl ester (TME), arachidonic acid, bovine thyroglobulin, bovine serum albumin, carbonic anhydrase, ovalbumin, trypsinogen, yeast alcohol dehydrogenase, lysozyme, cytochrome *c*, and Coomassie brilliant blue R250 were all obtained from Sigma. Hydroxyapatite, Bio-Sil TSK-250 column and protein assay reagents was purchased from Bio-Rad. Cellulose was purchased from Whatman. 11-Dehydro-thromboxane B_2 (11-dehydro-TXB_2), thromboxane B_2 (TXB_2), prostaglandin D_2 (PGD_2), prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), 15-keto-prostaglandin E_2 (15-keto-PgE_2), 15-keto-prostaglandin $F_{2\alpha}$ (15-keto-$PGF_{2\alpha}$), 5-hydroxyeicosatetraenoic acid (5-HETE), 12-hydroxyeicosatetraenoic acid (12-HETE), 15-hydroxyeicosatetraenoic acid (15-HETE) and leukotriene B_4 were supplied by the Upjohn Company. TXB_2 antisera and TXB_2 radioimmunoassay were produced and carried out as described previously [10]. $Na^{125}I$ was purchased from Amersham. Porcine liver and other tissues were kindly given by Weber Farm at Cynthiana, KY.

Purification of 11-hydroxythromboxane B_2 dehydrogenase

Purification was carried out in a 4° C cold room.

Step 1: DEAE-cellulose chromatography. Porcine liver cytosol (1.2 g protein) was applied to a DEAE-cellulose column (2.5 × 17 cm) which was previously equilibrated with 0.01 M potassium phosphate buffer (pH 7.5) containing 1 mM EDTA and 0.5 mM dithiothreitol. The column was eluted with 400 ml of the equilibrating buffer. The enzyme activity appeared in the unbound fractions (No. 10–20) as shown in Fig. 2.

Step 2: First hydroxyapatite chromatography. The above pooled active fractions were applied to a hydroxyapatite/cellulose (1 : 2) column (2.5 × 37 cm) equilibrated with 0.01 M potassium phosphate buffer (pH 7.5) containing 0.5 mM dithiothreitol. The column was washed with 600 ml of the equilibrating buffer and then eluted with 0.1 M potassium phosphate buffer (pH 7.5) containing 0.5 mM dithiothreitol. The active fractions (Nos. 106–125) (Fig. 3) were pooled and diluted with 10 vol. of cold water.

Step 3: Reactive blue agarose affinity chromatography. The above fractions was applied to a blue agarose column (2.5 × 16 cm) equilibrated with 0.01 M potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 0.5 mM dithiothreitol and 20% ethylene glycol. The column was washed with 200 ml of the equilibrating buffer followed by 200 ml of the equilibrating buffer containing 0.2 M KCl. The column was finally eluted with the last buffer containing 1 mM NAD^+. The active fractions (Nos. 49–56) (Fig. 4) were pooled and concentrated to 10 ml with an Amicon ultrafiltration system using a PM-10 membrane.

Step 4: Second hydroxyapatite chromatography. The above concentrated fraction was diluted with 10 vol. of cold water and applied to a hydroxyapatite/cellulose (1 : 2) column (1.5 × 16 cm) equilibrated with 0.01 M potassium phosphate buffer (pH 7.5) containing 0.5 mM dithiothreitol. The column was washed with 50 ml of the equilibrating buffer followed by 50 ml of 0.05 M potassium phosphate buffer (pH 7.5) containing 0.5 mM dithiothreitol. The column was eluted with 250 ml of 0.1 M potassium phosphate buffer (pH 7.5) containing 0.5 mM dithiothreitol. The active fractions (Nos. 22–32) (Fig. 5) were pooled and concentrated to 7 ml with an Amicon ultrafiltration system and stored in aliquots at −80° C.

Preparation of 11-dehydro-TXB_2-thyroglobulin conjugate

11-Dehydro-thromboxane B_2 was conjugated to bovine thyroglobulin according to the procedure previously described for TXB_2 [10]. Briefly, 11-dehydro-thromboxane B_2 (4 mg) was first dissolved in 4 ml of 10% ethanol in 0.02% aqueous Na_2CO_3. Bovine thyroglobulin (12 mg) was added followed by 20 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride. The reaction mixture was adjusted to pH 5.5 and stirred for 20 h at 4° C. The product was dialyzed against water, lyophilized and stored at −20° C.

Production of 11-dehydro-TXB_2 antiplasma

The production of antibodies was carried out in a similar manner as that described previously for TXB_2 [10]. The above conjugate (1 mg) was dissolved in 1 ml of saline and emulsified in an equal volume of complete Freund's adjuvant. The emulsion was injected into a rabbit's back at multiple sites. A total of two rabbits were injected. Booster injections at the same dose were carried out at monthly intervals. One week to 10 days after each booster injection the rabbit was bled by

venous puncture of the ears. Heparinized blood was collected into a tube and centrifuged at 2000 × *g* for 20 min. The plasma was collected and stored at −20 °C.

Preparation of 11-dehydro-TXB_2-TME conjugate

The 11-dehydro-TXB_2-TME conjugate was synthesized according to a procedure previously described for the synthesis of TXB_2-TME conjugate [10]. Briefly, 11-dehydro-TXB_3 (1 mg) was dissolved in 0.2 ml of dimethyl formamide containing 2 μl of triethylamine. Ethyl chloroformate (0.5 μl) in 0.1 ml of dimethylformamide was added and incubated at 0 °C for 15 min. TME (1.3 mg) and triethylamine (0.6 μl) in 0.2 ml of dimethylformamide were then added and the reaction mixture was stirred at room temperature for 2 h. The solvent was evaporated to dryness and the residue was dissolved in methanol and spotted on a silica gel G plate (2 × 20 cm) developed in a solvent system of ethyl acetate/ acetic acid/ isoctane/ water (11 : 2 : 5 : 10, organic phase). The 11-dehydro-TXB_2-TME conjugate ($R_F = 0.33$) migrated behind 11-dehydro-TXB_2 ($R_F = 0.53$) and ahead of TME ($R_F = 0$). The conjugate was scraped off the plate and extracted twice with 0.6 ml of ethanol. Silica gel was removed by centrifugation at 2000 × *g* for 5 min after each extraction. The conjugate was stored at −20 °C. The efficiency of conjugation was greater than 80%.

Radioiodination of 11-dehydro-TXB_2-TME conjugate

Radioiodination was carried out as described previously [10]. Briefly, the conjugate (0.5 μg) was dissolved in 0.5 M sodium phosphate buffer (pH 7.5) and 0.3 mCi of $Na^{125}I$ was added. Iodination was initiated by the addition of 5 μl of chloramine T (5 mg/ml in H_2O) and terminated 30 s later by the addition of 50 μl of sodium metabisulfite (5 mg/ml in H_2O). The reaction mixture was extracted twice with 0.6 ml of ethylacetate. The organic phase was evaporated under a stream of nitrogen and chromatographed on a silica gel G plate (2 × 20 cm) developed in a solvent system of 1% acetic acid in ethylacetate. The plate was subjected to autoradiography and the major radioactive peak which was [^{125}I]-11-dehydro-TXB_2-TME was eluted twice with 0.6 ml of ethanol. The ethanol extract was kept at −20 °C and used for radioimmunoassay. The labeled conjugate could be used for 2 months without substantial loss in sensitivity.

Radioimmunoassay procedure

Radioimmunoassay was run in duplicate in 10 × 75 mm glass test tubes at room temperature. The standard assay buffer was 0.05 M Tris-HCl (pH 7.5) containing 0.1% gelatin. The incubation mixture (0.4 ml) contained 0.2 ml of 11-dehydro-TXB_2 standards, 0.1 ml of appropriately diluted antiplasma and 0.1 ml of labeled conjugate (10000 cpm). The incubation was carired out for 1 h. Separation of bound from free labeled conjugate was made by adding a charcoal suspension followed by centrifugation as described rpeviously [10]. All tubes were allowed to have the same contact time with charcoal by using Luckham LP/35 stopper which withhold 0.2 ml of the charcoal suspension. The bound form (the supernatant) was counted and the concentration of each sample was calculated by a computer built in the LKB minigamma counter.

Preparation of porcine tissue cytosolic fraction

Porcine liver or other tissue was suspended in 3 vol. of cold 0.05 M Tris-HCl buffer (pH 7.5) and homogenized in a Waring Blendor for 2 min. The homogenate was centrifuged at 8000 × *g* for 10 min and the supernatant was further centrifuged at 100000 × *g* for 60 min. The supernatant designated as the cytosolic fraction was used for purification.

Enzyme assay

11-Hydroxythromboxane B_2 dehydrogenase was assayed by determining the NAD^+-dependent formation of 11-dehydro-TXB_2 from TXB_2. The assay mixture contained 5.4 nmol TXB_2, 1 μmol NAD^+ and enzyme preparation in a final volume of 1 ml of 0.05 M Tris-HCl buffer (pH 7.5). The reaction was initiated by the addition of enzyme and the incubation was carried out at 37 °C for 15 min. The reaction was terminated by the addition of 50 μl of 1 M HCl. An unreacted sample was also made by adding 50 μl of 1 M HCl prior to the addition of enzyme into the assay mixture. The acidified sample was alkalinized by adding 70 μl of 1 M Tris base to ensure dicarboxylate formation before further dilution with radioimmunoassay buffer for 11-dehydro-TXB_2 immunoreactivity measurement. The difference in 11-dehydro-TXB_2 immunoreactivity between the zero time and the 15 min was taken as the amount of 11-dehydro-TXB_2 synthesized during 15 min of incubation. One unit of the enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 nmol of 11-dehydro-TXB_2 per min under the above standard assay conditions.

Protein determination

Protein concentration was determined by the dye binding method of Bradford [11] using bovine serum albumin as a standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subunit molecular weight determination. Proteins were fractionated by SDS-PAGE according to the method fo Laemmli [12]. The protein samples were denatured and reduced in 1% SDS containing 1% mercaptoethanol at 100 °C for 3 min and then allowed to cool to room temperature prior to electrophoresis. The gel was stained with Coomassie blue R in $CH_3OH/H_2O/CH_3COOH$ (45 : 45 : 10) and

destained in the same solvent. The molecular weight of the subunit of 11-hydroxythromboxane B2 dehydrogenase was estimated from its mobility in SDS-PAGE relative to those of the following proteins of known subunit molecular weights: bovine serum albumin (68 000), ovalbumin (45 000), carbonic anhydrase (30 000), trypsinogen (24 000) and lysozyme (14 000). The logarithm of the molecular weight of different proteins was plotted against their relative mobilities. The molecular weight of the subunit of the enzyme was estimated from the linear plot.

Molecular weight determination of the native enzyme by gel filtration on a HPLC column. A Bio-Sil TSK-250 column (7.5 × 300 mm) was equilibrated with 0.05 M potassium phosphate buffer (pH 7.5). The purified enzyme was applied into the column alone and along with five standard proteins, namely, thyroglobulin (670 000), immunoglobulin G (158 000), ovalbumin (45 000), myoglobin (17 000) and vitamin B_{12} (1350). The column was eluted with the equilibrating buffer at a flow rate of 1 ml/min. The molecular weight of the unknown protein was estimated from the V_e/V_0 vs. log[M.W.] plot, where V_e is the elution volume and V_0 is the void volume.

Results

11-Dehydro-TXB_2 antibody production and specificity

Both rabbits immunized with 11-dehydro-TXB_2-thyroglobulin conjugate produced detectable antibodies to the hapten 2 months afer the initial injection. The antibody titers increased with each booster injection for about 6 months. Although each rabbit gave a high titer antiplasma, one gave a more specific and sensitive antiplasma than the other. The standard displacement curve and crossreactivity studies of the best antiplasma are shown in Fig. 1 and Table I. As little as 10 pg per assay tube can be detected. The antibodies showed minimal crossreaction with PGD_2 (2.76%) and 5-HETE (0.29%) and virtually no crossreaction with any other prostaglandins, hydroxy fatty acids and leukotriene B_4. The absence of crossreaction with TXB_2 is particularly valuable for studying enzymatic conversion of TXB_2 to 11-dehydro-TXB_2.

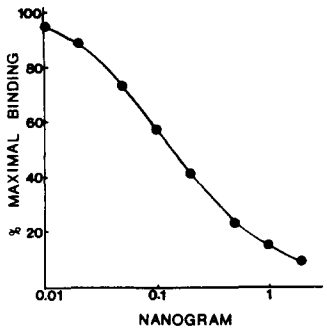

Fig. 1. Standard displacement curve for 11-dehydro-TXB_2.

TABLE I

Crossreaction of eicosanoids with 11-dehydro-TXB_2 antiplasma

Compounds	Amount required to inhibit 50% of maximal binding (ng)	% Crossreaction
11-Dehydro-TXB_2	0.13	100
PGD_2	4.70	2.76
5-HETE	45.0	0.29
TXB_2	520	0.03
PGE_2		< 0.03
$PGF_{2\alpha}$		< 0.03
6-Keto-$PGF_{1\alpha}$		< 0.03
15-Keto-PGE_2		< 0.03
15-Keto-$PGF_{2\alpha}$		< 0.03
12-HETE		< 0.03
15-HETE		< 0.03
LTB_4		< 0.03
Arachidonic acid	< 0.03	

Tissue distribution of 11-hydroxythromboxane B_2 dehydrogenase activity

The NAD^+-dependent formation of 11-dehydro-TXB_2 from TXB_2 catalyzed by crude porcine tissue cytosol was found to be both time and protein dependent and was inhibitable by prior boiling of the cytosol suggesting an enzymatic catalyzed reaction. A screen of various porcine tissues indicated that liver cytosol exhibited the highest specific activity in catalyzing the formation of 11-dehydro-TXB_2 from TXB_2 as shown in Table II. Gastrointestinal tract as well as kidney also possessed some enzyme activity. Other tissues appeared to show little activity.

Purification of 11-hydroxythromboxane B_2 dehydrogenase

In order to characterize this 11-dehydrogenase activity, the enzyme was purified from porcine liver cytosol. The enzyme was not retained by DEAE-cellulose at low ionic strength as shown in Fig. 2. However, it was adsorbed by hydroxyapatite at the same ionic strength.

TABLE II

Distribution of 11-hydroxythromboxane B_2 dehydrogenase in porcine tissues

Organs	Activities (mU/mg protein)
Liver	347.96
Small intestine	14.82
Kidney	11.27
Stomach	8.49
Colon	3.84
Brain	2.22
Heart	0.46
Spleen	0.44

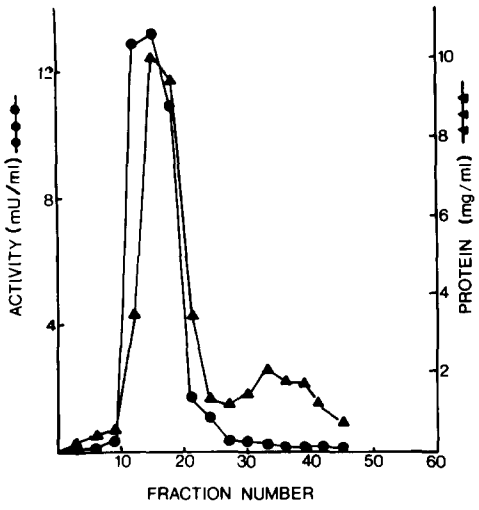

Fig. 2. DEAE-cellulose chromatography of 11-hydroxythromboxane B_2 dehydrogenase. Porcine liver cytosol (1.2 g protein) was applied to a DEAE-cellulose column (2.5×17 cm) and the chromatography was carried out as described in the Experimental procedures. Fractions of 8 ml were collected.

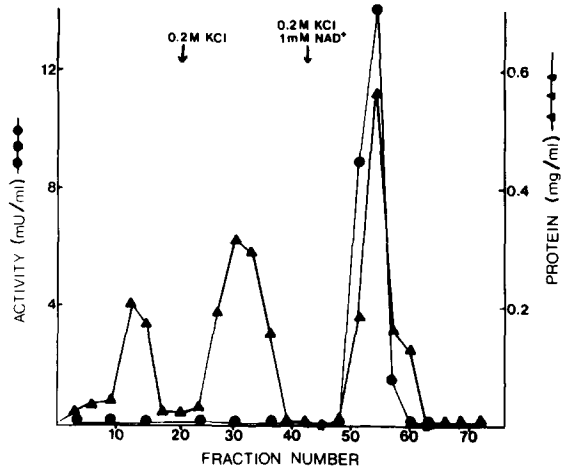

Fig. 4. Reactive blue agarose affinity chromatography of 11-hydroxythromboxane B_2 dehydrogenase. The first hydroxyapatite active fractions were applied to a reactive blue agarose column (2.5×16 cm) and the chromatography was carried out as described in the Experimental procedures. Fractions of 8 ml were collected.

The enzyme was eluted by 0.1 M phospahte buffer as shown in Fig. 3. Following the concentration of the eluate and decrease in the ionic strength, the enzyme was bound to the reactive blue agarose. The enzyme could be specifically eluted by 1 mM NAD^+ as shown in Fig. 4. Slight contamination by impurity in this fraction was completely removed by second hydroxyapatite chromatography as shown in Fig. 5. The purities of the enzyme preparations at each step of the purification process are shown in Fig. 6. The final enzyme preparation was judged to be homogeneous by SDS-PAGE analysis. The overall purification resulted in a 216-fold purified enzyme with a 22% recovery of enzyme activity (Table III).

Molecular and biochemical characterization

Molecular weight of the native enzyme and the subunit. HPLC analysis of the purified enzyme along with other standard proteins in a gel filtration column indicated that the native enzyme has a molecular weight of 110000 (Fig. 7). SDS-PAGE analysis revealed that the subunit molecular weight is 55000, indicating that the native enzyme is a dimer (Fig. 8).

Coenzyme specificity. Both NAD^+ and $NADP^+$ were tested for their ability to serve as coenzymes for the

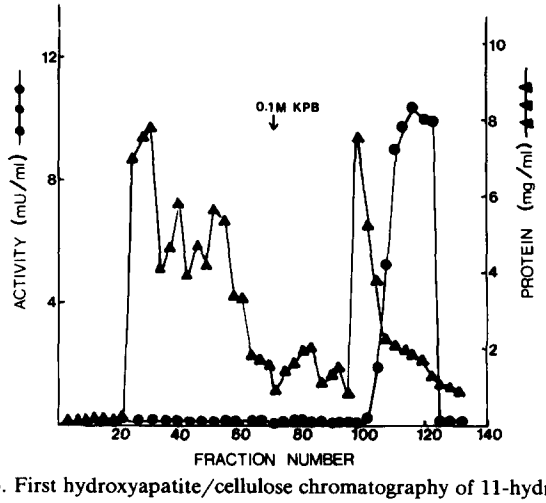

Fig. 3. First hydroxyapatite/cellulose chromatography of 11-hydroxythromboxane B_2 dehydrogenase. The DEAE-cellulose flow through fraction was applied to a hydroxyapatitecellulose column (2.5×37 cm) and the chromatography was carried out as described in the Experimental procedures. Fractions of 8 ml were collected.

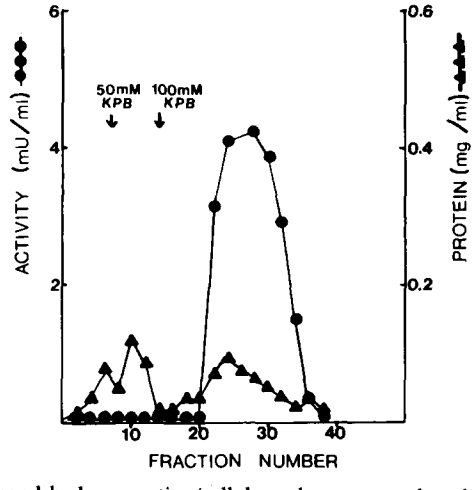

Fig. 5. Second hydroxyapatite/cellulose chromatography of 11-dehydrothromboxane B_2 dehydrogenase. The reactive blue agarose active fractions were concentrated, diluted with water and applied to a second hdyroxyapatite/cellulose column (1.5×16 cm). Chromatography was carried out as described in the Experimental procedures. Fractions of 8 ml were collected.

TABLE III

Purification of 11-hydroxythromboxane B_2 dehydrogenase from porcine liver

Procedure	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Recovery (%)	Purification (fold)
Cytosol	1242	21.98	0.018	100	1
DEAE-cellulose	525	22.00	0.042	100	2.4
First hydroxyapatite	71.5	11.43	0.160	52.0	9.0
Blue agarose	6.8	6.13	0.902	28.0	51.0
Second hydroxyapatite	1.2	4.75	3.88	21.6	216

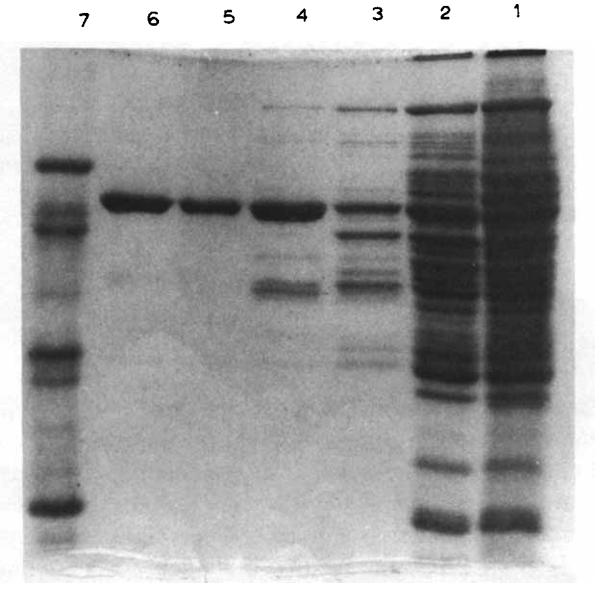

Fig. 6. SDS-PAGE analysis of the enzyme preparations at different stages of purification. From the right: (1) cytosol (120 μg); (2) DEAE-cellulose fraction (80 μg); (3) first hydroxyapatite fraction (30 μg); (4) reactive blue agarose fraction (30 μg); (5) second hydroxyapatite fraction (10 μg); (6) second hydroxyapatite fraction (20 μg); and (7) molecular weight standards (from top to bottom, bovine serum albumin (68000), ovalbumin (45000), trypsinogen (24000) and lysozyme (14000).

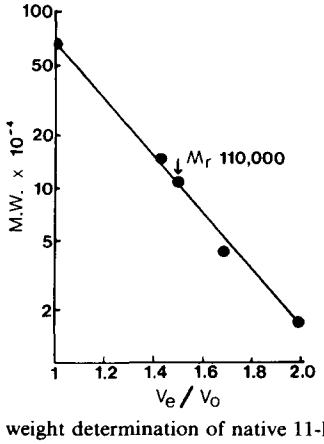

Fig. 7. Molecular weight determination of native 11-hydroxythromboxane B_2 dehydrogenase by gel filtration. The purified enzyme was chromatographed alone as well as co-chromatographed with thyroglobulin (670000), immunoglobulin G (158000), ovalbumin (45000), and myoglobin (17000) on a Bio-Sil TSK-250 HPLC column and eluted as described in the Experimental procedures.

oxidation of TXB_2. NAD^+ appeared to be much more active in being a coenzyme for enzyme catalyzed oxidation of TXB_2 (Fig. 9). The apparent K_m for NAD^+ was determined from a double-reciprocal plot and was found to be 23 μM.

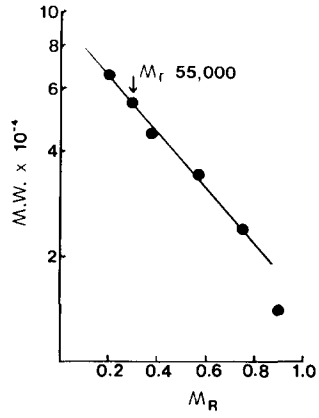

Fig. 8. Subunit molecular weight determination of 11-hydroxythromboxane B_2 dehydrogenase. The purified enzyme and the molecular weight standards, namely, bovine serum albumin (68000), ovalbumin (45000), carbonic anhydrase (30000), trypsinogen (24000) and lysozyme (14000) were subjected to SDS-PAGE as described in the Experimental procedures.

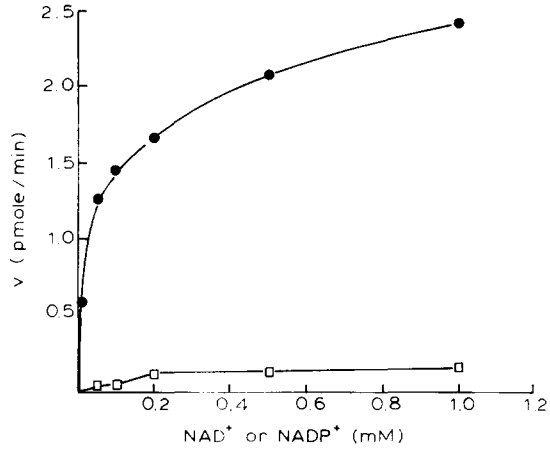

Fig. 9. Coenzyme specificity of 11-dehydro-thromboxane B_2 dehydrogenase. The purified enzyme was assayed in the presence of 5.4 μM of TXB_2 at different concentrations of NAD^+ (●) or $NADP^+$ (□) as described in the Experimental procedures.

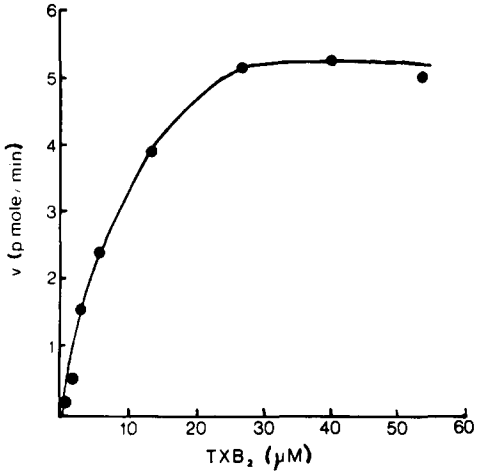

Fig. 10. The effect of increasing concentrations of TXB_2 on 11-hydroxythromboxane B_2 dehydrogenase activity. The purified enzyme was assayed in the presence of 1 mM of NaD^+ at different concentrations of TXB_2 as described in the Experimental procedures.

Substrate saturation studies. The effect of TXB_2 concentrations on 11-hydroxythromboxane B_2 dehydrogenase activity was examined in Fig. 10. A normal hyperbolic dependency was observed. The apparent K_m for TXB_2 was estimated from a double-reciprocal plot and was found to be 8.1 μM.

Reversibility of the reaction. The reduction of 11-dehydro-TXB_2 to TXB_2 was examined at different concentrations of 11-dehydro-TXB_2 in the presence of 1 mM NADH. Again the formation of TXB_2 was determined by a specific radioimmunoassay for TXB_2 (crossreaction of TXB_2 antibodies with 11-dehydro-TXB_2 was less than 0.3%). Fig. 11 shows a normal Michaelis-Menten kinetics for 11-dehydro-TXB_2. The apparent K_m for 11-dehydro-TXB_2 was estimated to be 8 μM.

Effect of sulfhydryl inhibitors. The effect of four different sulfhydryl inhibitors on the activity of the purified enzyme is shown in Table IV. The enzyme was

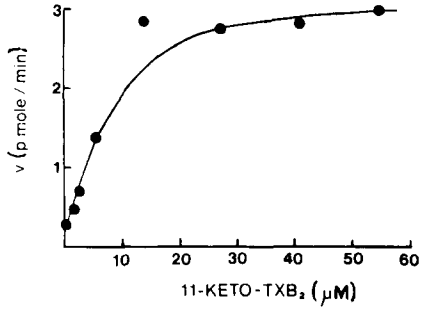

Fig. 11. The effect of increasing concentrations of 11-dehydro-thromboxane B_2 on 11-hydroxythromboxane B_2 dehydrogenase activity. The purified enzyme was assayed in the presence of 1 mM of NADH at different concentrations of 11-dehydro-TXB_2 as described in the Experimental procedures.

TABLE IV

The effect of sulfhydryl inhibitors on 11-hydroxythromboxane B_2 dehydrogenase

Inhibitors		Activities
None		100%
N-Ethylamleimide	0.1 mM	21%
	1 mM	4%
p-Chloromercuribenzoate	0.1 mM	19.8%
	1 mM	5.4%
Sodium mersalyl	0.1 mM	22.0%
	1 mM	14.4%
Dithio-bis(nitrobenzoate)	0.1 mM	50.2%
	1 mM	38.1%

significantly inhibited by all four inhibitors at 0.1 mM indicating that the sulfhydryl group of the enzyme is essential to the enzyme activity.

Discussion

Studies on an enzyme require a sensitive and specific enzyme assay. Although 11-hydroxythromboxane B_2 dehydrogenase is a NAD^+-dependent enzyme, it appears that the enzyme exhibits a relatively low K_m for TXB_2 and is not sufficiently active to be assayed by following the reduction of NAD^+ at 340 nm. We have developed a radioimmunoassay for 11-dehydro-TXB_2 sensitive enough to detect minute transformations of TXB_2 to 11-dehydro-TXB_2 catalyzed by the enzyme. This immunological approach of enzyme assay has several advantages. Firstly, the radioimmunoassay of 11-dehydro-TXB_2 is sensitive to the low pg level. The amount of enzyme needed for an assay can be relatively small. For example, a few micrograms of crude liver homogenate is sufficient for an enzyme assay. Secondly, the radioimmunoassay of 11-dehydro-TXB_2 is very specific. The crossreactivity of the antibodies with substrate TXB_2 is less than 0.03%. Therefore, assay of the enzyme can be carried out at relatively high concentrations of TXB_2 to achieve a greater reaction rate without any concern of high background level of 11-dehydro-TXB_2 which can be due to antibody crossreaction with the substrate TXB_2. Furthermore, the enzyme assay can be carried out at any stage of purification since any eicosanoids present in the crude enzyme extract will not significantly affect the quantitation of 11-dehydro-TXB_2 simply because the antibodies are highly specific. Thirdly, a great number of enzyme assays can be carried out concurrently in a single incubation and subsequent radioimmunoassay. This is particularly efficient for assaying large numbers of fractions during column chromatography.

Development of radioimmunoassay for 11-dehydro-TXB_2 have been reported by three different groups [13–15]. Each group claimed to have generated highly specific antibodies similar to ours. This may be attributed to the fact that the antibodies generated may be directed against the dicarboxylate form of 11-dehydro-TXB_2 (open form) which is structurally very different from the hemiacetal form of TXB_2 as well as many other eicosanoids. The radioimmunoassay was carried out at neutral or slightly alkaline pH which favors 11-dehydro-TXB_2 to exist as a dicarboxylate (open form) resulting in high specificity of the assay. One unique feature of our assay is that the labeled hapten is an ^{125}I-labeled tyrosine methyl ester derivative. This allows direct counting of the bound and free forms by a gamma counter without the use of a scintillation fluid.

The four-step purification of the enzyme results in a 216-fold of purification with 22% activity recovery. The procedure included a step of affinity chromatography with blue agarose which had been shown to be an efficient step for purifying prostaglandin related dehydrogenase [16,17]. The enzyme could be eluted specifically with NAD^+. A further step of hydroxy-apatite chromatography resulted in a homogeneous preparation. The subunit molecular weight of the enzyme (55 000) as determined by SDS-PAGE is exactly half of the molecular weight of the native enzyme (110 000) estimated by gel filtration indicting the native enzyme consisted of two identical subunits. Among all the other prostaglandin related dehydrogenases/reductases, only NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase exhibits a similar dimeric structure [18]. Furthermore, the molecular weight of the enzyme appears to be higher than any of the known prostaglandin-related dehydrogenases/reductases. Similar to all these enzymes NAD^+-dependent 11-hydroxythromboxane B_2 shows no evidence of existing in multiple forms.

The purified enzyme preparation catalyzes not only 11-dehydrogenation of TXB_2 but also 11-keto reduction of 11-dehydro-TXB_2 indicating that the enzyme catalyzes reversible oxido-reduction of the substrates. The apparent V_{max} of the reduction reaction is approximately two-thirds that of the oxidation reaction indicating that the enzyme catalyzed reaction is freely reversible. This reversible reaction appears to be similar to those catalyzed by NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase [19] and $NADP^+$-linked 15-hydroxy/9-hydroxyprostaglandin dehydrogenase [20], but is in contrast to that catalyzed by NAD^+-dependent 9-hydroxyprostaglandin dehydrogenase which is of quasi-irreversible nature [17]. The biological significance of the reversibility of the NAD^+-dependent 11-hydroxy-thromboxane B_2 dehydrogenase catalyzed reaction is not apparent since the biological activity of TXB_2 remains unclear.

The specificity of NAD^+-dependent 11-hydroxy-thromboxane B_2 dehydrogenase remains to be determined. Whether the enzyme catalyzes the oxido-reduction of eicosanoids other than TXB_2 at C-11 is not clear. Two different oxido-reductases that catalyze the reduction of PGD_2 at C-11 have been described [21–24]. One is concerned with the reduction of PGD_2 to $9\alpha,11\beta$-PGF_2 which is NADPH specific [21]. The enzyme has been purified to homogeneity from bovine lung and exhibits an M_r of 30 500 [22]. The other is involved in the reduction of PGD_2 to $PGF_{2\alpha}$ which is also NADPH specific, although the stereochemistry of the product at C-11 was not unequivocally established [23,24]. This enzyme has also been purified from rabbit liver and found to have an M_r of 66 000 [23]. Both enzymes are clearly different from the enzyme described in this paper based on the difference in coenzyme specificity and molecular weight. The possibility of the purified enzyme catalyzing the oxido-reduction of TXB_2 at carbons other than C-11 also remains to be determined. Although most of the prostaglandin-related dehydrogenase reductases catalyze position specific oxidation/reduction, there is one exception, this being $NADP^+$-linked 15-hydroxy/9-hydroxyprostaglandin dehydrogenase catalyzing oxido-reduction at C-9 and C-15 [20]. This enzyme exhibits a M_r of 29 500 which is lower than that of the current enzyme. Another NAD^+-dependent dehydrogenase which catalyzes the oxidation of prostanoids at C-9 employs 15-keto-13,14-dihydro-$PGF_{2\alpha}$ as the best substrate [17]. This enzyme exhibits an M_r of 34 000 which is lower than that of the current enzyme. Whether the purified enzyme described herein catalyzes the oxido-reduction of a wide variety of hydroxyl/carbonyl compounds similar to $NADP^+$-linked 15-hydroxy/9-hydroxyprostaglandin dehydrogenase from porcine kidney [25] is not clear. However, it is unlikely that this enzyme catalyses the oxidation of TXB_2 at C-15 alone since 15-dehydro-TXB_2 exists in hemiacetal form at neutral pH much like TXB_2 which shows little crossreaction with the antibodies.

The availability of a homogeneous enzyme preparation should allow probing into these key questions concerning the enzyme. Furthermore, preparation of monospecific antiserum against the enzyme also becomes a feasible task. Applications of the antiserum to exploring the relatedness of various prostaglandin-related dehydrogenases and to probing the structure and function of the enzyme will be the subject of future investigations.

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