An Approach to Isolation of Thromboxane Synthase (TX-SYN) by Ligand Tethered Affinity Techniques

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Received November 14, 1990

The affinity chromatographic technique was applied to the isolation of Thromboxane Synthase, with a variety of imidazolyl alkanoic acids coupled Sepharose 2B including a gel (G in Table 4) which has one free COOH group in the bound affinity ligand. The effect of ligand structure on the "affinity" and "selectivity" for thromboxane synthase isolation is described.

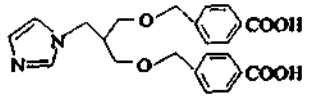
Introduction

Thromboxane-Synthase (TX-SYN) is one of the enzymes involved in the cardiovascular metabolism of arachidonic acid[1] and is known to by Cytochrome P450 enzyme. It catalyzes the transformation of prostaglandin endoperoxide (PGH_2)[2] to thromboxane A_2 (TXA_2)[3]. The latter induces platelet aggregation which is known to be a factor in thrombotic disorders.[4] Purification of TX-SYN has been attempted by several groups of investigators since 1976 when Moncad *et al.*[5] first localized the enzyme in platelet microsomes. The enzyme has been solubilized from both platelet and porcine lung and separated from cyclooxgenase by DEAE-cellulose column chromatography.[6] It has been reported to be purified to apparent homogeneity by conventional chromatographic techniques.[7] But this involves many purification steps and gives a very low yield. As a part of a program of study on interactions between Thromboxane Synthase and small molecules (including inhibitors and PGE_2[8]) at the molecular level, we needed to develop an easy and reproducible purification procedure.

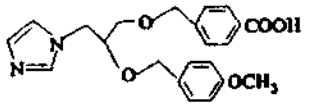
Conventional procedures of protein purification generally rely on small differences in the physiochemical properties of the proteins in the mixtures: solubility, charge, molecular size and shape. However one of the most characteristic properties of biological macromolecule is their ability to bind other molecule in a reversible and highly specific manner. The popularity of affinity chromatography is largely due to the following features: (1) the technique is rapid-complete separation and isolation is often achieved in a single purification step, and (2) high enrichment or concentration of the selected material can be achieved. Very subtle distinctions can be utilized as the separation basis. (3) The recovery (yield) of non-denatured, fully active material is usually high.

Prior to the start to this work, there had been a limited number of studies of the application of Inhibitor-tethered affinity techniques to the isolation of TX-SYN. Ullrich[9] had applied this technique to purify TX-SYN by using UK-37248[10] as a tethered ligand and 1-benzylimidazole as an eluant. Unfortunately a detailed description of this work has never appeared in the literature. But the use of a monocarboxylic acid as the affinity ligand presumably would fail to take advantage of the "selectivity"[11] of this class of thromboxane synthase inhibitors: the carboxylic group would be consumed in forming the linkage with the primary amine of the activated gel. The "selectivity" (TX-SYN as Cyclooxgenase) of this class of inhibitors depends on whether or not there is free carboxlyic acid terminal at the end of hydrocarbon chain while inhibitory power depends on the length (8.5-10 Å) between COOH terminal and sp2-N of the heterocyclic ring. Based on this consideration, the retention of a free carboxylic group on the tethered ligand should yield affinity media of superior selectivity. In order to retain one free carboxylic group for imparting TX-SYN selectivity to the affinity media, one must make heterocyclic derivatives which contain a COOH group at the ends of each of two hydrocarbon chains. For this purpose, we synthesized **A-1**, 2,2′[[(p-carboxyphenyl)methoxylmethyl]-N-ethyl imidazole[18] which has a carboxylic group at two chain termini.

As stated earlier, the choice of ligand (for example, variation in their chain length and structure) for successful affinity chromtography is very important. In this report, a variety of the gels (A-G, Table 4)[12] are examined for their capability to purifty TX-SYN by affinity chromotography. Gel G, which contained 2,2′-[[(p-carboxyphenyl]methoxy]methyl]-N-ethyl imidazole was of particular interest. Prior to appication of gel G to the isolation for TX-SYN, we needed to ascertain whether the designed unit of Gel G (with one of two COOH groups remaining free for presumably selective binding of TX-SYN) has measurable affinity of TX-SYN. Manley *et al.*[13] prepared compound **A-2**, [3′-(p-carboxyphenyl) methoxy-2′-(p-methoxyphenyl) methoxy]-N-propylimidazole which contains a COOH group at one of the two hydrocarbon branches. **A-2** was found to be an inhibitor which displayed an IC_{50} to 1.1×10^{-6} M.



A-1



A-2

One of the most critical steps in the chromatographic process is the elution of adsorbed material from the affinity matrix. The success of the purification may depend on the choice of methods used to recover the macromolecules. Specific elution with benzylimidazole[19] was examined. We also attempted to optimize other purification steps.

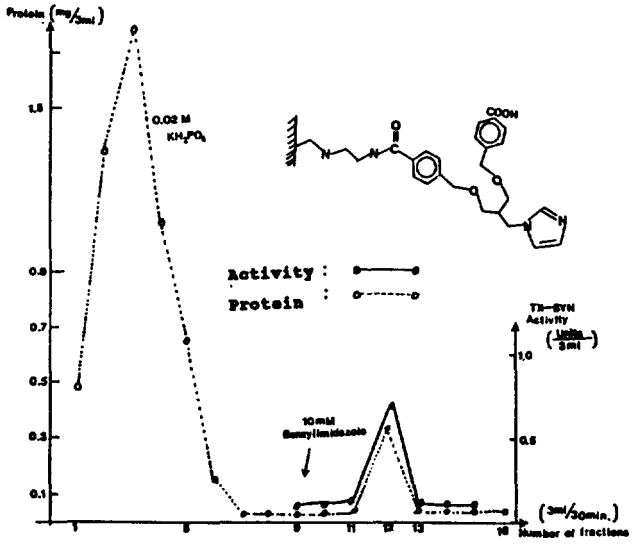


Figure 1. Elution profile with benzylimidazole

Results and Discussion

Specific Elution. Initial experiments was undertaken to determine if 10 mM benzylimidazole buffer (in 20 mM phosphate buffer) could elute TX-SYN from imdazole-loaded columns (see Figure 1). An imidazolyl substituted acid bearing gel (for example, Gel G) was packed in the column (0.8× 6 cm, Bio-Rad Lab.). The column was equilibrated with 20 mM phosphate buffer. After 0.6 m*l* (total activity 9.7 units, 6.9 mg of protein) of Tritox X-100 solubilized enzyme (specific activity 1.4 unit/mg) was loaded on to the column, the column was washed with 20 mM phosphate buffer until no futher protein was eluted (fraction 1-8 in Figure 1). The enzyme was then eluted in 3 m*l* fractions with 10 mM benzylimidazole buffer using gravity flow at 4℃. Each fraction was dialyzed at 4℃ against 3×15 m*l* of 0.1 M phosphate buffer, concentrated to 0.5 m*l* and assayed for TX-SYN activity. Total activity found was 0.72 units and recovery yield was 7.6%. Clearly further study was required.

Equilibration Time. The interaction of macromolecules with an immobilized affinant is a time dependent process.[16] In many instances, adsorption equilibration is attained very slowly. The kinetics were examined. The solubilized enzyme (0.6 m*l*, total activity 9.7 units) was loaded on to the column (0.8×6 cm) and after a specified time (10 min, 1 hour, 4 hour), the column was washed with 20 mM phosphate buffer. The fractions which showed the presence of protein were pooled, concentrated and assayed for TX-SYN acitivty. Finally the column was eluted with 30 m*l* of 10 mM benzylimidazole buffer. The eluant was also dialyzed, concentrated to 0.5 m*l* and assayed as before (Table 1). A 1 hour incubation seems to be sufficient to achieve the most effective adsorption.

Optimization of the Protein Loading Factor. The protein loading was optimized using 1 hour as the contact time. The results (see Table 2) showed that a 0.08 m*l* aliquot (0.92 mg of protein) was the proper amount for the column (0.8×6 cm) packed with Gel G. This procedure and recovery provided a high specific activity (48.8 units/mg, 38 fold increase).

Table 1. Equilibration time

Equilibration time	Percent recovery	Total activity
	Buffer Wash	Eluant
10 min	54.4%	7.6%
1 hour	33.4%	24.3%
4 hours	29.9%	5.9%

Table 2. Optimization Protein Loading

Amount of Protein	Percent recovery of total activity		Specific Activity of eluant (Units/mg)
	Buffer Wash	Eluant	
0.05 m*l* (0.57 mg)	10.4%	34.7%	29.3
0.08 m*l* (0.92 mg)	10.8%	48.6%	48.8
0.12 m*l* (1.3 mg)	30.0%	27.0%	38
0.6 m*l* (6.9 mg)	33.3%	24.3%	18.7

Table 3. Purification of TX-SYN with a Variety of Imidazolyl Acid Tethered Gels

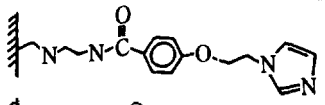
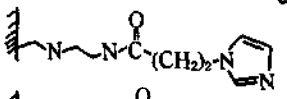
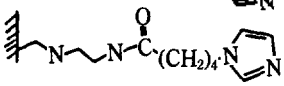
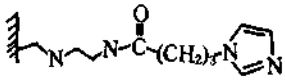
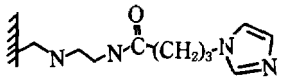
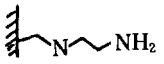
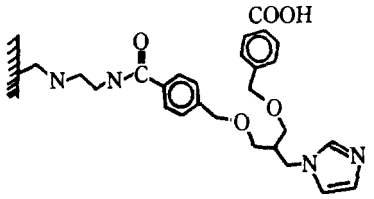
Gel	Percent of recovery of total acitivity in the		Specific activity (Eluant)	Degree of purification
	Buffer Wash	Eluant		
A	17.5	32.1	27.3	20
B	13.5	42	60.1	44.5
C	15.4	51.1	78.7	57.9
D	11.2	50.5	80.0	58.8
E	21.4	24.2	20.0	14.8
F	59.6	10.1	13.2	9.7
G	10.8	48.6	48.8	35.8

The Effect of Affinity Ligand Structure. A variety of imidazolyl acids tethered Sepharose 2B gel were employed, using the optimized amount (0.08 m*l*, 0.92 mg of protein) of enzyme and 1 hour as the contact time. The results appeared in Table 3 and 4. It is interesting to note that Gel F, which is 2-amino ethyl Sepharose, can immobilize TX-SYN (13.2%) and gives a 9.7 fold increase of specific activity. But it was found, based on percent recovery in the eluant and specific activity, that imidazole containing gels consistently retained TX-SYN more strongly and provided because its presumed selectivity toward TX-SYN might have been partly reduced by a high chance of nonspecific binding of other protein around the long hydrocarbon chain, or by steric hindrance of the desired specific binding process.

Experimental

Enzyme Isolation, Solubilization. Six units of outdated human platelet concentrates from the Puget Sound Blood Bank (Seattle) were pooled in an EDTA washed container and microsomal enzyme was prepared[15] using a Beckman

Table 4. Imidazolyl Acid Coupled Gel Used in This Study

Gel	Ligand	Amount of ligand bound (mmol/g dry gel)
A		0.17
B		0.24
C		0.20
D		0.19
E		0.15
F		0.9
G		0.35

Ultracentrafuge. For the solubilized enzyme, the resulting microsomal pellet was resuspended in 2 m*l* of 0.8% Triton X-100 in 100 mM phosphate buffer (pH 7.5).

Biosynthesis of [1-^{14}C] PGH_2. Previously frozen seminal vesicles (20 g)[20] were homogenized with a blender in 200 m*l* of ice cooled 100 mM phosphate buffer (pH 7.4, 1 mM EDTA). The homogenate was filtered through cheese cloth and centrifuged at 8500×g for 15 min and the supernatant was again filtered through four layers of gauze and spun at 100,000×g for 1 hour at 4℃. The supernatant and soft top layer was discarded and the microsomal pellet was resuspended in about 150 m*l* of 100 mM phosphate buffer containing 10 mg/m*l* of bovine albumin (BSA, Sigma) with the aid of a Teflon blade homogenizier. The added BSA binds any free arachidonic acid avidly and thus helps to keep the blank low. The material was then spun again at 100,000 ×g for 1 hour and resuspended in the same buffer without BSA. A further spin at 100,000×g for 1 hour resulted in the final pellet which was resuspended in 15 m*l* of 100 mM phosphate buffer (pH 7.5).

To prepare PGH2, the sodium salt of arachidonic acid (Sigma, 5 mg) and 0.2 m*l* (101 μCi) of an ethanol solution of [1-^{14}C] labelled arachidonic acid (Amersham, 195 μCi/μg) was used. The solvent from arachidonic acid solution was removed in a stream of argon and 0.4 m*l* of 100 mM sodium carbonate was added to form the salt of the fatty acid. This required extensive vortexing until the entire precipitate was dissloved. The incubation buffer was made by adding 0.2 m*l* of 100 mM KOH aqueous solution to the 100 m*l* of the solution (pH 7.4, 100 mM phosphate, 1 mM EDTA, 2 mM Phenol, 1 mM L-trpytophan, 0.1 mg/m*l* of hemoglobin power, 8.8 mg/m*l* of sodium p-hydroxymecuribenzoate salt). 80 m*l* of the above incubation buffer was warmed to 37℃ in an erlenmeyer flask under vigorous stirring to assure oxgen equilibration with room air. Next, the resuspended microsomal pellet obtained from 20 g of vesicles was added and preincubated for 5 min. The substrate was rapidly added and incubated for 45 seconds. The enzyme reactions were quenched by adding a mixture of 300 m*l* of ether and 10 m*l* of 100 mM citric acid precooled to −20℃. After shaking for 1 min the top layer was transferred to a 1 *l* separatory funnel. The ether layer was dried over $MgSO_4$ and filtered. The ether phase was roto-evaporated taking care that the round bottom flask always remained at a temperature well below 0℃. The residue was applied to a silicic acid column (500 mg SiO_2 packed in hexane) in a 5×200 mm column with a glass wool plug. Elution with 15 m*l* of 1 : 9 ether : petroleum ether afforded recovered arachidonic acid, and then 5 m*l* of 2 : 8 mixture is followed by 45 m*l* of a 4 : 6 ratio (which eluted PGG_2), 30 m*l* of 6 : 4 (which eluted PGE_2, together with PGD_2). Five milliliter fractions were collected throught and identified by TLC. The homogeneous fractions of PGH_2 were pooled and solvent was removed by evaporation in an inert gas (N_2) stream. PGH_2 (1.69 mg) thus obtained was dissolved in 5 m*l* of dimethoxyethane and had a specific activity of *ca.* 2400 dpm/nmole. The solution was kept at −70℃ untiluse.

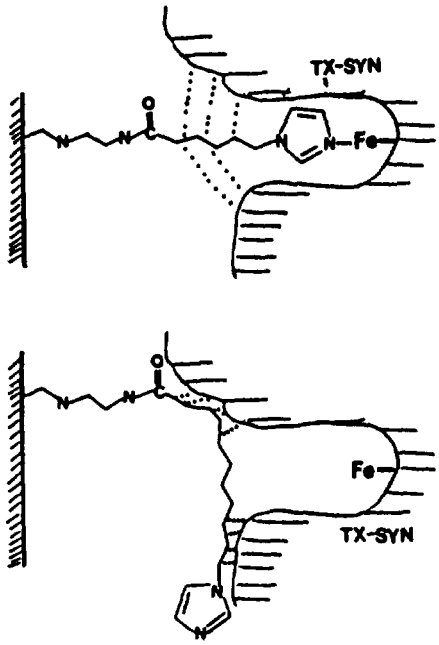


Figure 2. Models for Interaction between TX-SYN and imidazole containing matrix (Dependence on Spacer Arms).

Enzyme Assays. The activity of TX-SYN preparation was assayed by following Yoshimoto's procedure using [1-^{14}C] PGH_2 in 5 μ*l* DME (4.5 nmole, typically 10100 dpm). One unit of activity is defined as that amount to afford 1 nmole of TXB_2 per minute at room temperature.

Protein Determination and Radiotracer Quantitation. The amount of protein was determined by the Bradford method.[17] For tracer studies using ^{14}C-labelled prostaglandin, a Beckman LS-7500 Scintillation Counter was used.

References

1. For a summary; Rhee, Jk. Ph. D Thesis, University of Washignton (1989).
2. PGH_2 is a known inducer of platelet aggregation. Its half life in aqueous solution is 5-30 min.
3. TXA_2 is the product formed from PGH_2 by the action of Thromboxane Synthase. It decomposes to inactive TXB_2 in queous solution.
4. "Modern Phamacology", second edition: Craig, Charles R., Stitzel, Robert E.
5. (a) P. Neediman, and S. Moncada, *Nature (London)* **261**, 558 (1976); (b) S. Moncada, P. Needlman, S. Bunting, and JR Vane, *Prostaglandins*, **12**, 323 (1076).
6. (a) T. Yoshimoto, S. Yamamoto, M. Okuma, and O. Hayaishi, *J. Biol. Chem.*, **252**, 5871 (1977); (b) S. Hammarstrom and U. Diczfalusy, *Adv. Prostaglandins and Thromboxane Research* **6**, 267 (1980).
7. M. Haurand and V. Ullrich, *J. Biol. Chem.* **260**, 15059 (1985).
8. PGE_2 shows two different functions depending on the concentration. At low concentration, it promotes aggregation induced by other agents (ADP, Arachidonic acid, etc) while, at high concentrations, it inhibits aggregation. It has now been shown to be an effector of TX-SYN (unpublished results, Andersen, NH, Rhee, Jk.) increasing the production of TXA_2 from PGH_2 at very low concentrations.
9. V. Ullrich, M. Haurand, *Adv. Prostaglandin Thromboxane Leukotrienes Research* **11**, 105 (1983).
10. p-Carboxyphenoxy-N-ethyl imidazole.
11. The basic structural requirements for selective inhibitor are a 1-imidazolyl or 3-pyridyl moiety at one end of the molecule and a carboxylic acid group at the other. Further the distance between the carboxylic group and the nitrogen atom at the 3 position or in the pyridine moiety should be between 8.5 and 10 Å. Imidazole derivatives not containing a COOH group show strong inhibitory power for fatty acid cyclooxgenase as well as TX-SYN. Cyclooxgenase is also known to be a metallo enzyme containing iron. The presence of the COOH enhances inhibitor selectivity for TX-SYN.
12. The procedures of preparation of Gel A-G were described in a separate paper submitted to *Bull. Korean. Chem. Soc.* Volume 13, p. 221(1992).
13. P. W. Manley, N. M. Allanson, R. G. Booth, and DP. Tuffin, *J. Med. Chem.*, **30**, 1588 (1987).
14. (a) CA. Mullin and B. D. Mammock, *Arch. Biochem. Biophys.* **216**, 423 (1982); (b) K. Pommerenig, M. Kuhn, W. Jung, and M. Benes, *Int. J. Biol. Macromol.*, **1**, 79 (1979).
15. (a) T. Yoshimoto and S. Yamamoto, Methods in Enzymology **86**, pp. 106 (1982); (b) F. F. Sun, *Biochem. Biophys. Res. Commun.*, **74**, 1432 (1977).
16. P. Mohr, and K. Pommerenig, Affinity chromatography, A series of Monographs Volume 33 (1985).
17. MM. Bradford, *Anal Biochem*, **72**, 248 (1976).
18. For the synthesis of A-1, See "Small Molecule Agents that Inhibit or Enhance Thromboxane Synthase Activity" Submitted to *Eur. J. Pharmacology* (Niels H. Andersen and Jaekeol Rhee), See ref. 1.
19. Benzylimidazole is also known to be a weak inhibitor of TX-SYN with a demonstrated moderate affinity for TX-SYN.
20. Sheep seminal vesicles were obtained from Dr. Robert Solomon. Department of Chemistry, Case Western University, Cleveland, U.S.A.

Structure and Bonding of Perovskites $A(Cu_{1/3}Nb_{2/3})O_3$ (A=Sr, Ba and Pb) and Their Series of Mixed Perovskites

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Some perovskites $A(Cu_{1/3}Nb_{2/3})O_3$ ($A=Sr^{2+}$, Ba^{2+} and Pb^{2+}) and their series of mixed perovskites have been prepared by solid state reaction. Single perovskite phase was obtained in Sr or Ba rich samples, but pyrochlore phase was found in Pb rich samples. The stability of perovskite phase is dependent on the ionicity of bonding as well as the tolerance factor. All the obtained perovskites have tetragonal symmetry distorted by Jahn-Teller effect of Cu^{2+}. In the case of $Sr(Cu_{1/3}Nb_{2/3})O_3$, some superlattice lines caused by threefold enlarging of fundamental unit cell were observed. And, the symmetry of B site octahedron and the bonding character of B-O bond have been studied by IR, ESR and diffuse reflection spectroscopy. It appeared that the symmetry and the bonding character are influenced by such factors as the size and the basicity of A cation.

Introduction

Many compounds with the general formula ABO_3 having perovskite structures have been prepared and studied by many investigators.[1-4] These compounds exhibit many interesting physical properties such as ferroelectricity, piezoelectricity, and larger variation in electric and magnetic behaviours.[5-8] These properties depend on not only the crystal