PROPERTIES OF A PARTIALLY-PURIFIED PREPARATION OF THE PROSTAGLANDIN-FORMING OXYGENASE FROM SHEEP VESICULAR GLAND

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ABSTRACT

The fatty acid oxygenase of sheep vesicular glands was solubilized with Tween-40 and purified 60-fold using ammonium sulfate precipitation and DEAE-cellulose chromatography. Glycerol (50%) stabilized the activity at all stages of purification and allowed long-term storage at -60°. The partially purified enzyme contained less than 0.7 nmoles of iron per mg of protein and less than 0.1 nmole of copper per mg of protein. Although the K_I values for aspirin, BL-2338, flurbiprofen and ibuprofen remained relatively unchanged during purification, the apparent K_I value for inhibition by indomethacin decreased from 120 to 2.7 μM.

ACKNOWLEDGEMENTS

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INTRODUCTION

Prostaglandins are formed from certain polyunsaturated fatty acids by oxidative cyclization and subsequent rearrangements (see reviews by Samuelsson (1,2)). Studies by Hamberg and Samuelsson (3) and Hamberg et al. (4) indicate that PGG_2 is the first stable compound formed from arachidonic acid in the presence of the microsomal fraction of sheep vesicular gland. The enzyme system responsible for formation of prostaglandins in bovine vesicular gland has apparently been separated into 2 components, a fatty acid oxygenase and an endoperoxide isomerase (5). The number of separate enzymes making up the synthetase system has not been determined; however, the oxygenase fraction appears to catalyze the formation of PGG_2, whereas the isomerase fraction appears to catalyze subsequent formation of PGE_2.

MATERIALS AND EXPERIMENTAL METHODS

Materials

All fatty acids were high-purity grade, obtained from the Hormel Institute (Austin, Minn.) and Nuchek Preps (Elysian, Minn.). Flubiprofen (U-27182) and Motrin (U-18573) were donated by the Upjohn Company (Kalamazoo, Mich.) as well as frozen sheep seminal vesicles and some samples of acetone powders of sheep vesicular glands. Indomethacin was a gift of the Merck Company (West Point, Pa.), BL-2338 was donated by Dr. P. Hebborn of Westwood Pharmaceuticals (Buffalo, N.Y.). Diethyldithiocarbamic acid was purchased from Sigma (St. Louis, Mo.). All other chemicals, reagent grade, were obtained from common commercial sources. Deionized, distilled water was used for the preparation of all solutions.

Preparation and Quantitation of Fatty Acids

Fatty acids were dissolved at 50 to 100 mM concentration in benzene containing 5 mM butylated hydroxy toluene (Calbiochem, Los Angeles, Cal.) and stored at 0°. Aqueous solutions of 5 to 10 mM concentration were prepared on the day of use by evaporating aliquots of stock benzene solutions under a stream of nitrogen and suspending the acid, by vigorous shaking, in 0.1 M Tris HCl, pH 8.5.

Determination of Fatty Acid-Dependent Oxygenase Activity

Oxygen absorption measurements were done on a Yellow Springs Instrument Company (Yellow Springs, Ohio) Model 53 Oxygen Monitor equipped with a Model 5301 Bath Assembly. A constant temperature of 30 ± 0.5°C was maintained with a Haake (Berlin, West Germany) Type F Thermo-regulated incubator bath. Oxygen absorption measurements were made using the "Air" position of the monitor and a 50 mV recorder setting, in addition to a voltage offset attached to the recorder. Continuous recordings were made with a Leeds and Northrup (Milano, Italy) Speedomax XL dual channel recorder. One channel recorded the oxygen electrode output, the other channel received the same signal after it had passed through a digital differentiator, which transformed the signal to the first

derivative (dO_2/dt) of the oxygen consumption curve. In most experiments, the total final volume of the reaction mixture in the sample chamber was 3.0 ml containing 0.5 μM hemoglobin and 0.67 mM phenol.

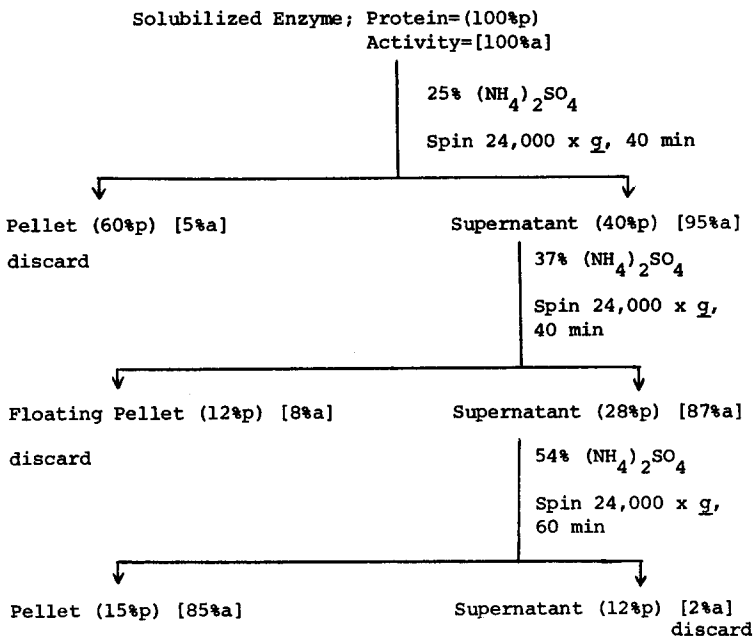
Solubilization of Acetone Powders

Solubilization of acetone powders was accomplished by adding 15 g of acetone powder (prepared as described by Wallach and Daniels (6)) to a mortar containing 15 ml of Tween 40. The powder was ground to a paste by slowly adding a solution of 0.1 M Tris HCl and 0.1 M DDC. The paste was worked with the pestle and more of the Tris-DDC solution was added until it had a smooth soupy consistency. The ground powder was then transferred to a blender, and more Tris-DDC was used to wash any remaining enzyme from the mortar into the blender. In all, 525 ml of the Tris-DDC was added. The enzyme solution was blended at room temperature for 10 minutes, and sonicated 3 times during the blending for 20 seconds each. The enzyme solution was then transferred to a chilled beaker and placed on ice.

Ammonium Sulfate Precipitation

The solubilized enzyme solution was stirred vigorously and 180 ml of saturated $(NH_4)_2SO_4$ in 0.1 M Tris HCl containing 30 mM DDC (pH 8.5) was added very slowly over approximately one hour. The solution was centrifuged for 40 minutes at 24,000 x g (Sorval GSA rotor). The super-

SCHEME I Ammonium Sulfate Precipitation



natant was carefully decanted, and 90 ml of the ammonium sulfate buffer solution was added slowly. Another centrifugation for 40 minutes at 24,000 x g resulted in a floating pellet that was carefully removed from the supernatant by pouring through cheesecloth. The supernatant was again placed on ice and an additional 180 ml of ammonium sulfate buffer was added (∿ 45 minutes). This final solution was spun for 60 minutes at 24,000 x g and the supernatant carefully decanted leaving a pinkish-brown pellet containing 85% of the solubilized enzyme activity (see Scheme I).

Desalting

The pellet from above was scraped into a small Dounce ball-type homogenizing tube and homogenized in 25 ml of buffer (0.01 M Tris HCl, pH 8.0 containing 1% Tween 40 and 10 mM DDC). The solubilized pellet was applied to a 2.5 x 100 cm column of Biogel P-2 and eluted with the same buffer. Fractions of 5 ml were collected and checked for salt with a conductivity bridge. Those samples with a conductivity >2 times the buffer conductivity were not used.

DEAE-Cellulose Chromatography

The desalted enzyme fractions were pooled (giving approximately 50 ml of 10 mg protein/ml) and applied to a column (2.0 x 60 cm) of DEAE-cellulose (Sigma) that had been equilibrated against a buffer containing 0.02 M Tris HCl, pH 8.0, containing 10 mM DDC and 1% Tween 40. The same solution was used for preparation of the elution gradients. The sample was washed onto the column with 50 ml of the equilibration buffer and eluted with a linear Tris HCl gradient from 0.02 M Tris HCl to 0.05 M Tris HCl. The elution pattern of this column is shown in Figure 1.

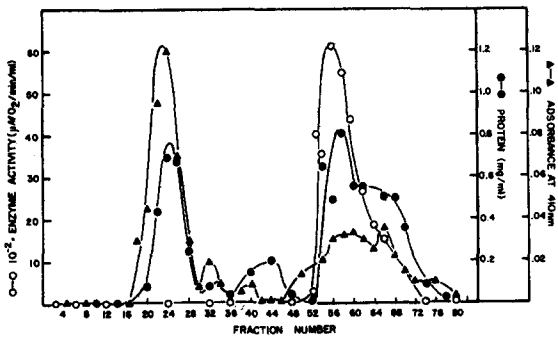


Figure 1. DEAE-Cellulose Chromatography Elution Profile

Approximately 50 ml of 10 mg/ml protein from P-2 column (see Methods) was applied to the DEAE-cellulose column (described in Methods). The flow rate was about 0.5 ml per min and approximately 12 ml fractions were taken. After 100 ml of buffer was eluted a 600 ml linear gradient from 0.02 to 0.05 M Tris was started.

RESULTS

Enzyme Purification

Solubilization and Stabilization. The acetone powder preparation of the fatty acid oxygenase from sheep vesicular gland was solubilized with non-ionic detergents. The results are summarized in Table I. Tween 40 was selected for use in further purification studies since it was found

TABLE I Solubilization of the Fatty Acid Oxygenase from Sheep Vesicular Gland with Non-Ionic Detergents

The acetone powder preparation (50 mg) was ground in a mortar for 2 minutes with 0.1 ml of detergent while 10 additions (0.5 ml each) of buffer (0.1 M Tris HCl containing 0.67 mM phenol) were added. Solutions (2% detergent) were poured into 1/2" x 2 1/2" cellulose nitrate tubes and spun at 200,000 x g for 30 min in a L3-50 ultracentrifuge (Beckman, Palo Alto, Calif., SW50.1 rotor). Supernatant was removed with a Pasteur pipette, diluted, and assayed for oxygenase activity. All assay mixtures contained less than 0.1% detergent. Protein was measured by the method of Lowry et al. (7) with bovine serum albumin as the standard. One unit of enzyme activity causes a μM decrease in oxygen per minute under the conditions described in Methods.

Detergent	Protein (mg/ml)	Total Activity (μM O_2/min)	Specific Activity (units/mg)	% of Activity Soluble
Tween 40	7.8	400	10.3	88
Tween 20	9.2	300	6.5	66
Triton X-100	10.0	300	6.0	66
Cutscum	10.2	275	5.1	60

to solubilize the greatest percent of oxygenase activity. The solubilized enzyme was quite unstable, losing greater than 90% of the oxygenase activity in 120 hours at 4°. Attempts to stabilize the enzyme activity with 1% albumin, 10% glycerol, 10% acetone, 3 mM ammonium sulfate, or 3 mM ferrous sulfate were unsuccessful. However, as shown in Table II, the presence of 5 mM DDC allowed greater than 50% recovery of the oxy-

TABLE II Stabilization of Solubilized Fatty Acid Oxygenase Activity with Diethyldithiocarbamate

Two solutions of Tween 40-solubilized acetone powder were prepared as described in Table I. One solution contained 5 mM DDC in the buffer. The solutions were placed on ice and aliquots were withdrawn for assay of oxygenase activity after listed times.

Time (hours)	Oxygenase Activity (total units)	
	+DDC	-DDC
0.2	343	254
60.0	274	48
120.0	196	24
160.0	186	5

genase activity after 120 hours at 4°C. Following solubilization with Tween 40 the oxygenase was further purified by ammonium sulfate precipitation (see Scheme I in Methods).

Stimulation by Hemoglobin. Complete recovery of enzyme activity during the ammonium sulfate treatment was possible if hemoglobin (0.2 μM was included when the final ammonium sulfate pellet was assayed. Oxygenase activity of acetone powder or solubilized acetone powder preparations was not stimulated by hemoglobin. However, following ammonium sulfate and subsequent purification steps, hemoglobin consistently stimulated oxygenase activity (3 to 5-fold) and was therefore included during assay of the partially purified oxygenase.

DEAE Chromatography, Atomic Absorption Spectra, and Absorption Spectra.

The ammonium sulfate precipitate containing the oxygenase activity was dissolved in buffer, desalted, and chromatographed on DEAE-cellulose (see Methods). The purification results are shown in Table III. The specific activity of the fraction containing the highest activity from the DEAE-cellulose column was increased 62.5-fold over the acetone powder. The pooled enzyme peak contained 26% of the original activity and 1.1% of the protein.

In addition to activity and protein measurements, the DEAE-cellulose fractions were examined by atomic absorption spectroscopy for the presence of copper and iron. Results in Figure 2 indicate that the fractions containing enzyme activity contained less than 0.7 nmole of Fe per mg of protein and less than 0.1 nmole of Cu per mg of protein. Fractions that

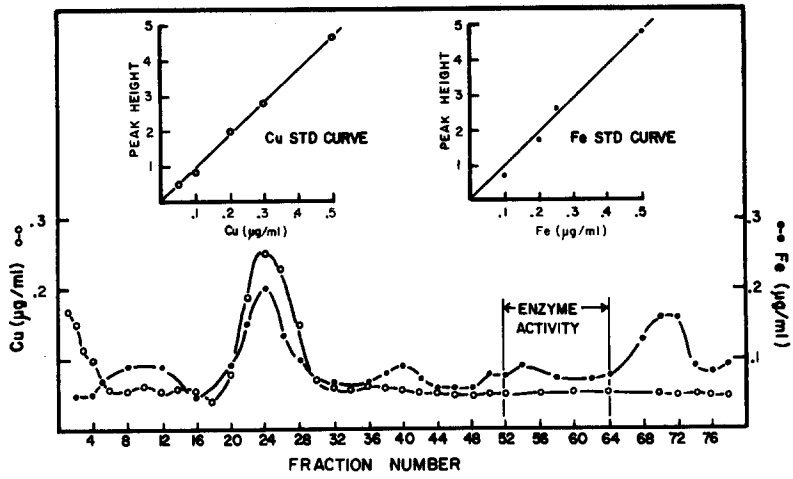


Figure 2. Iron and Copper Content of DEAE-Cellulose Samples

Metals were determined using a Model 306 (Perkin-Elmer, Norwalk, Conn.) Atomic Absorption Sepctrophotometer. Iron measurement was carried out with the Fe-lamp under the following conditions: Source 30 mAmp, wavelength 248.3 nm, slit = 3. Copper measurement was carried out with the Cu-lamp under the following conditions: Source 15 mAmp, wavelength 325.7 nm, slit = 4.

TABLE III Purification of the Fatty Acid Oxygenase from Sheep Vesicular Gland

Procedural details are described in Methods. Values in parentheses represent the average of 3 separate experiments. One unit of enzyme activity causes a μM decrease in oxygen per minute under the conditions described in Methods. Protein was measured by the method of Lowry et al. (1951).

Purification Step	Volume (ml)	Units/ml	Total Units	Protein mg/ml	Units/mg Protein	Yield (%)	Purification
Acetone Powder			2,100,000		200	100	
Solubilizate	560	3,393	1,900,000	13.0	261	90.5 (82)	1.3 (1.3)
$(NH_4)_2SO_4$ (54%)	16	112,500	1,800,000	60.0	1,875	85.7 (66)	9.4 (10)
P-2 Eluate	50	19,000	950,000	10.0	1,900	45.2 (40)	9.5 (10)
DEAE-Cellulose (tube 56)*	12	5,000	50,000	0.4	12,500	2.4	62.5
DEAE-Cellulose (tubes 52 through 64)*	200	2,800	550,000	0.6	4,667	26.2 (16)	23.3 (19)

*See Figure 2.

were high in iron and copper preceded the fractions containing enzyme activity and a small amount of iron was eluted after the enzyme activity. Absorption spectra of the partially purified oxygenase are shown in Figure 3. A peak at 412 nm was observed in the spectrum of the enzyme under aerobic conditions. The peak was eliminated by reducing the sample with sodium dithionite. The pyridine hemochrome had 2 maxima, one at 412 and one at 396 nm.

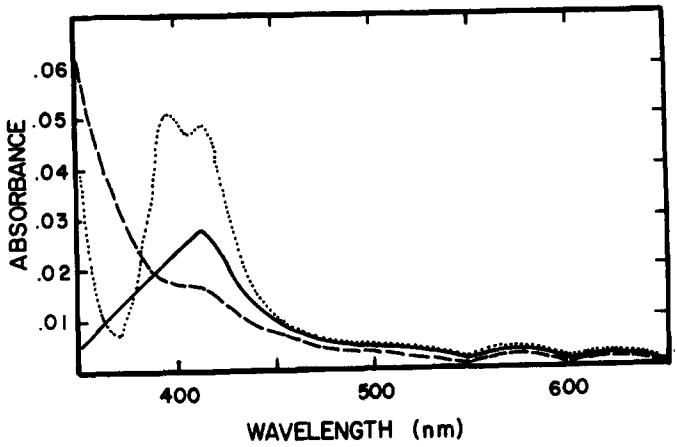


Figure 3. Oxidized, Reduced, and Pyridine Hemochrome Spectra of the Partially Purified Fatty Acid Oxygenase

Absorption spectra were obtained with a Cary Model 14 spectrophotometer. Protein concentrations of approximately 0.6 mg/ml were employed. An oxidized sample (represented by the solid line) was reduced by adding a few crystals of sodium dithionite (represented by dashed line). Pyridine hemochrome (dotted line) was obtained by adding a few crystals of sodium dithionite to a solution of the protein in 0.05 M NaOH-pyridine (5:1, v/v).

The stability of the partially purified enzyme was examined. A 0.6 mg/ml solution of the enzyme lost approximately 30% of its activity upon freezing. Concentrating the enzyme 6-fold to 3.6 mg/ml protein gave increased stability (Table IV). The enzyme was more stable when stored in 30% glycerol which prevented the solutions from freezing when kept at -20°C. 50% glycerol and storage at -60°C appeared to completely prevent decay of the partially purified enzyme.

Inhibition of Oxygenase Activity by Antiinflammatory Agents.

The effects of a number of antiinflammatory agent, known to inhibit acetone powder and microsomal preparations of the oxygenase, were examined at various stages during the enzyme purification. K_I values (Table V) for aspirin, flurbiprofen, ibuprofen and BL2338 were relatively unchanged during the purification whereas the K_I of indomethacin showed a marked decrease during the purification procedure. Plots of reciprocal velocity versus reciprocal of substrate concentration (20:3) (n-6) for the par-

TABLE IV Stability of the Partially Purified Oxygenase from Sheep Vesicular Gland

Days Stored	% Remaining Activity			
	(0°C) (0.6 mg/ml)	(0°C) (3.5 mg/ml)	(-20°C) (2.5 mg/ml) 30% Glycerol	(-60°C) (3.5 mg/ml) 50% glycerol
1	100	100	100	100
3	100	60	88	96
5	66	56	91	95
11	28	24	47	96
15	11	20	52	95
39	--	--	36	95

One unit of enzyme activity causes a μM decrease in oxygen per minute the conditions described in methods.

TABLE V Estimated K_I Values[a] for Inhibitors

Enzyme Preparation[b]	Indomethacin (μM)	Aspirin (mM)	Flurbiprofen (μM)	Ibuprofen (μM)	BL-2338 (μM)
Microsomes (300)	120.5 (3)	∿ 5.4 (4)	4.2 (4)	7 (4)	6.6 (2)
Acetone Powder (300)	94.2 (3)	>17 (3)	1.4 (4)	3.3(4)	1.1 (2)
Ammonium Sulfate (4,000)	15 (2)	19 (3)	1.0 (2)	7 (2)	1.8 (2)
DEAE partially purified (8,000)	2.7 (4)	21 (2)	2.8 (2)	8.8(2)	0.6 (4)

[a]The number of assays used to estimate the apparent K_I values are given in parentheses

[b]The specific activity is noted in parentheses for each preparation

tially purified enzyme in the presence of indomethacin (Fig. 4) showed curvature, however, a K_I value of approximately 2μM was estimated by replotting the data by the method of Dixon (8). This K_I value for indomethacin inhibition of the partially purified enzyme is almost 2 orders of magnitude smaller than the K_I value (150 μM) observed with acetone powder and microsomal preparations.

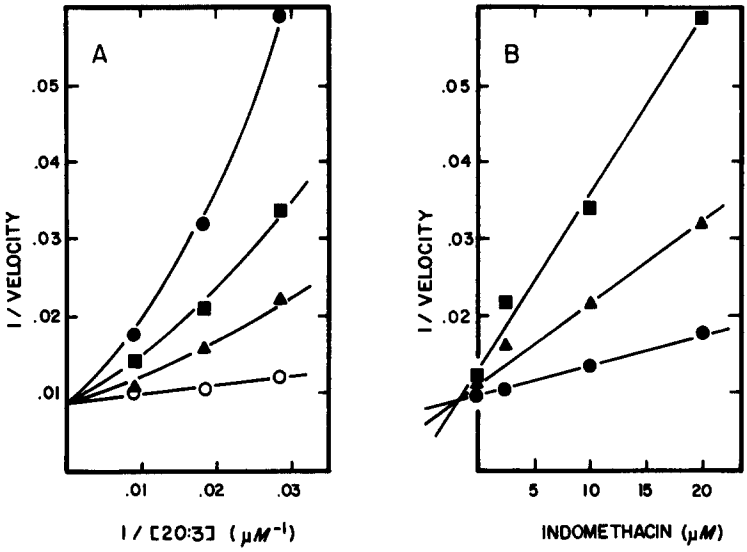


Figure 4. Indomethacin Inhibition of the Oxygenation of 20:3 by the Partially Purified Oxygenase

(A) Reciprocal of velocity versus reciprocal of the concentration of 20:3 (n-6) in the presence of various levels of indomethacin. The partially purified enzyme was added to reaction chambers containing mixtures of inhibitor and substrate and 0.2 μM hemoglobin. Units of velocity are μM O_2/min/mg protein. Levels of indomethacin: ●-●, 20 μM; ■-■, 10 μM; ▲-▲, 3.3 μM; o-o, no indomethacin.

(B) Data from (A) are replotted by the method of Dixon (1953). Levels of 20:3 (n-6): ■-■, 36 μM; ▲-▲, 53 μM; ●-●, 107 μM.

DISCUSSION

Enzyme Purification

Solubilization of the vesicular gland prostaglandin biosynthetic system was first reported by Samuelsson et al. in 1967 (9). The solubilization and purification described in a later report (1) involved treatment of microsomes with the non-ionic detergent cutscum which solubilized 62% of the enzyme activity, and ammonium sulfate precipitation (40-60% saturated) resulted in a 1.8-fold purification. Table III illustrates that treatment of an acetone powder preparation of vesicular gland with Tween 40 solubilized 80 to 90% of the oxygenase activity. The majority of the enzyme activity precipitated between 37 and 45% ammonium sulfate, and a 9 to 10-fold increase in specific activity was observed, in contrast to the earlier report (1). Perhaps the increased purification was aided by protection of the oxygenase with added DDC. The DDC may stabilize by preventing endogenous substrate fatty acids from destroying the oxygenase, since at the concentrations used (5-50 mM), DDC is an effective inhibitor of the fatty acid oxygenase (10).

The stimulation of the oxygenase activity by hemoglobin observed in these studies may be similar to that reported earlier (11) for microsomes of bovine seminal vesicles. Miyamoto et al. (5), using a partially purified oxygenase from bovine glands, also reported that hemoglobin was a necessary cofactor for the formation of PGH, from 20:3 (n-6). Hemoglobin, or heme compounds, may also be responsible for the reported 8-fold stimulation of prostaglandin synthesis by adding boiled supernatant to washed microsomes (1).

One question, yet unresolved is whether or not the oxygenase is a metallo-protein. The oxygenase from sheep vesicular gland is inhibited by a wide variety of iron and copper complexing agents, including DDC and cyanide (10), suggesting the requirement for iron and/or copper in the oxygenase. The DEAE-cellulose fractions containing the highest specific activity (Figure 1), had very low concentrations of copper (<0.1 nmole/mg protein) and also low concentrations of iron (<0.7 nmole/mg protein). Spectra of the partially purified oxygenase (i.e. maxima at 412 nm) indicated that the iron present may be in a heme form, however, the absorbance maxima did not correspond to any of the naturally-occurring heme proteins (12). The highly purified fatty acid oxygenase from soybeans contains one atom of non-heme iron per molecule (13,14), but it is not appreciably inhibited by metal complexing agents such as azide, cyanide, DDC, fluoride, or EDTA (15).

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