PURIFICATION AND PROPERTIES OF CHICKEN HEART PROSTAGLANDIN Δ^{13}-REDUCTASE

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SUMMARY: Prostaglandin Δ^{13}-reductase was purified extensively by ammonium sulfate fractionation, and DEAE-Sephadex-, hydroxylapatite-, and phosphocellulose chromatography. Enzyme activity was followed by radioimmunoassay with the use of an antiserum directed toward 15-keto-prostaglandin $F_{2\alpha}$ and antibodies directed toward 13, 14 dihydro-15-keto-prostaglandin $F_{2\alpha}$. The purified enzyme used NADPH as a cofactor much more effectively than NADH. It specifically reduced 15-keto-prostaglandins but not 15-hydroxy-prostaglandins. The enzyme was inhibited by p-chloromercuribenzoate. It had a relatively broad pH optimum (pH 7.4 to pH 8.5), and has a molecular weight estimated to be 70,000 to 80,000.

INTRODUCTION

Metabolism of prostaglandin proceeds through oxidation of the 15-hydroxyl group, reduction of the Δ^{13}-double bond and ω and β-oxidation of the side chains (1,2). There have been a number of studies concerned with the purification and properties of 15-hydroxyprostaglandin dehydrogenase (3,4). Purification and properties of prostaglandin Δ^{13}-reductase have not been reported. From *in vivo* and *in vitro* studies of the reduction of the Δ^{13}-double bond, several properties of prostaglandin Δ^{13}-reductase have been established (2).

With the use of serological methods to study prostaglandin metabolism, we have been able to purify and characterize several of these enzymes. In this communication we report a 7,000 fold purification of prostaglandin Δ^{13}-reductase from chicken heart and some of the properties of the purified enzyme.

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MATERIALS AND METHODS

Biologicals and Chemicals. [^{3}H] PGE_2 and [^{3}H] $PGF_{2\alpha}$ were purchased from New England Nuclear (Boston, Mass.). Unlabeled prostaglandins and prostaglandin metabolites were gifts from Dr. Udo Axen of the Upjohn Co. (Kalamazoo, Mich.). Pyridine nucleotides were purchased from Sigma Co. Chicken hearts were obtained from a slaughter house; the fat was removed and the defatted chicken hearts were stored at -70° until use. 15-keto-[^{3}H] $PGF_{2\alpha}$ and 15-keto-[^{3}H] PGE_2 were prepared by treating [^{3}H] $PGF_{2\alpha}$ and [^{3}H] PGE_2 with partially purified dog lung 15-hydroxyprostaglandin dehydrogenase.

Radioimmunoassays: Antibodies to 15-keto-$PGF_{2\alpha}$ were prepared in monkeys as described by Levine et al. (5). Rabbit anti-13, 14 dihydro-15-keto-$PGF_{2\alpha}$ was obtained from Clinical Assays, Inc., (Cambridge, Mass.). The procedures for radioimmunoassay have been described (5).

Measurement of Enzymatic Activity: Enzymatic activity was measured in a reaction mixture of 0.1 ml containing 1 mM NADPH 0.1 ng 15-keto-$PGF_{2\alpha}$ and enzyme. The reaction mixtures were incubated at 37° for 10 min and the enzymatic reaction was stopped by addition of 1 ml of cold Tris-HCl buffer (0.01 M, pH 7.4, containing 0.1% gelatin and 0.14 M NaCl) and incubation of this diluted solution in a boiling waterbath for 2 min. After centrifugation to remove denatured protein, the clear solution was analyzed for 15-keto-$PGF_{2\alpha}$ or 13, 14 dihydro-15-keto-$PGF_{2\alpha}$ by radioimmunoassay. The amount of 15-keto-$PGF_{2\alpha}$ that disappeared or the amount of 13, 14 dihydro-15-keto-$PGF_{2\alpha}$ that appeared was calculated from the competition between the product and 15-keto-[^{3}H] $PGF_{2\alpha}$ or 13, 14 dihydro-15-keto-[^{3}H] $PGF_{2\alpha}$ and their specific antibodies and comparison with the appropriate inhibition curve obtained with standard prostaglandin metabolites. The enzymatic activity is expressed as nanomoles of substrate lost per 10 min at 37°. Protein was determined by Lowry's method (6).

Thin Layer Chromatography. Thin layer chromatography was carried out with Eastman Chromagram Sheets (silica gel absorbent with fluorescent indicator) and the following solvent system: ethylacetate-2,2,4-trimethylpentane-acetic acid-water (90:50:20:100 V/V/V/V, upper layer). Pieces of thin layer plate, 2 x 1 cm were cut and counted for radioactivity in toluene scintillation fluid with a Packard liquid scintillation spectrometer. [^{3}H] PGE_2 (0.2 nmoles) and [^{3}H] $PGF_{2\alpha}$, 0.1 nmoles of 15-keto-[^{3}H] PGE_2 and 15-keto-[^{3}H] $PGF_{2\alpha}$ were incubated with purified prostaglandin Δ^{13}-reductase (2 units) and 1 mM NADPH in a reaction mixture of 0.2 ml. After incubation 2 hr. at 37°, the reaction mixtures were adjusted with 0.1 N HCl to pH 4.5 and extracted with equal volume of petroleum ether. This ether extraction was repeated two times and the combined ether phases were dried under nitrogen. The dried prostaglandin was then dissolved in 20 µl of ethanol. Five µl of the ethanol solutions were analyzed by thin layer chromatography.

Enzyme Purification. Chicken hearts (2 kg) were cut into small pieces and suspended in three times their weight of ice-chilled phosphate buffer (0.092 M, pH 7.3, containing 0.1 mM dithiothreitol). They were homogenized with a Waring blender that was operated at top speed for 2 min, in ice water; after stopping the blender for 2 min, they were homogenized for an additional 2 min. The resulting suspensions were centrifuged at 10,000 xg for 20 min. The supernatant fluids were then centrifuged at 78,000 xg for 3 hours in a Spinco Model L2-65B ultracentrifuge with the use of a rotor, type 30. Solid ammonium sulfate was added to the clear supernatant fluids with stirring to 30% saturation. The precipitate was removed by centrifugation at 10,000 xg for 20 minutes after stirring for 30 minutes at 0°. The supernatant solution was then brought to 60% saturation of ammonium sulfate and equilibrated again for 30 minutes. The precipitate, insoluble in 60% ammonium sulfate, was collected by centrifugation (10,000 xg for 20 min) and was dissolved in phosphate buffer (0.092 M, pH 7.3, 0.1 mM dithiothreitol) and dialyzed against 100 volumes of phosphate buffer. The dialysate was applied

to a DEAE-Sephadex column (4.0 x 60 cm) that had previously been equilibrated with phosphate buffer until the absorbence at 280 nm of the eluate was less than 0.03. Those fractions containing prostaglandin Δ^{13}-reductase activities were pooled, concentrated by precipitation with ammonium sulfate (65% saturation) and the precipitates resuspended into phosphate buffer and dialyzed against phosphate buffer. The dialysate was applied to a hydroxylapatite column (2.5 x 60 cm) that had previously been equilibrated with phosphate buffer. The column was washed with phosphate buffer until the absorbence at 280 nm was less than 0.03. The column was then eluted with four column volumes of phosphate buffer containing 0 to 1 M KCl linear gradient. Prostaglandin Δ^{13}-reductase was eluted at 0.15 M KCl and the active fractions were pooled. The pooled enzyme was dialyzed against 0.015 M phosphate buffer containing 0.1 mM dithiothreitol. The dialysate was applied to a phosphocellulose column (1.5 x 30 cm) and then washed with one column volume of 0.015 M phosphate buffer (0.1 mM dithiothreitol). For this phosphocellulose chromatography, the phosphocellulose was precycled by treatment with five volumes 0.5 N NaOH for 30 min, filtered and rinsed with distilled H_2O until the rinse was about pH 8. The cake was stirred with five volumes of 0.5 N HCl for 30 minutes and rinsed with distilled H_2O until the pH was about 4. The resin was then suspended in two volumes of 0.092 M phosphate buffer, pH 7.3 and titrated with 6 N KOH until the pH was 7.3. The resin was filtered and suspended in 0.015 M phosphate buffer, pH 7.3. The column was eluted with four column volumes of 0.015 to 0.092 M phosphate buffer linear gradient. The prostaglandin Δ^{13}-reductase was eluted at 0.05 M phosphate buffer. The pooled prostaglandin Δ^{13}-reductase was stored at -20°.

RESULTS AND DISCUSSION

Detection of Prostaglandin Δ^{13}-Reductase Activity. Reduction of the Δ^{13}-double bond of 15-keto-$PGF_{2\alpha}$ was measured by disappearance of serologic activity when assayed with monkey antibodies directed toward 15-keto-$PGF_{2\alpha}$ (7) and appearance of serologic activity when measured with antibodies directed toward 13, 14 dihydro-15-keto-$PGF_{2\alpha}$. The serologic specificity of the monkey antiserum has already been described (5). The 15-keto group of the prostaglandin is immunodominant ($PGF_{2\alpha}$ reacts poorly with these antibodies). But the Δ^{13}-double bond is also recognized since its reduction results in a loss of serologic activity. Loss of serologic activity could also have resulted from degradation of 15-keto-$PGF_{2\alpha}$ other than by reduction of the Δ^{13}-double bond. Therefore antibodies which recognize the reduced Δ^{13}-double bond, anti-13, 14 dihydro-15-keto-$PGF_{2\alpha}$, were used to identify the product of this reaction. The serologic specificity of the anti-13, 14 dihydro-15-keto-$PGF_{2\alpha}$ is shown in Fig. 1. With 15-keto-$PGF_{2\alpha}$ as substrate, reduction of the Δ^{13}-double bond by the prostaglandin Δ^{13}-reductase led to a loss of serologic activity when measured with anti-15-keto-$PGF_{2\alpha}$, while the serologic activity

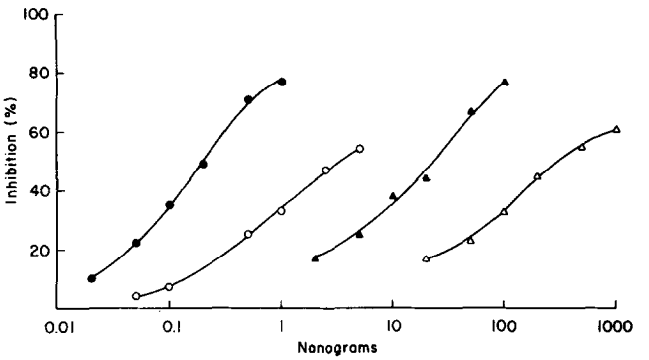

Figure 1. Serologic specificity of rabbit anti 13,14-dihydro-15-keto-$PGF_{2\alpha}$. Inhibition of 13,14-dihydro-15-keto-[^{3}H]$PGF_{2\alpha}$ anti-13,14-dihydro-15-keto-$PGF_{2\alpha}$ binding; 13, 14-dihydro-15-keto-$PGF_{2\alpha}$ (●), 15-keto-$PGF_{2\alpha}$ (o), 13,14-dihydro-15-keto-PGE_2(▲) and $PGF_{2\alpha}$ (Δ). Each reaction mixture contained 0.6 pico moles of 13,14-dihydro-15-keto[^{3}H]$PGF_{2\alpha}$, 0.1 ml of a 1:10,000 dilution of rabbit anti 13,14-dihydro-15-keto-$PGF_{2\alpha}$ and 0.1 ml of inhibitor in a final volume of 0.3 ml. After 1 hour incubation at 37°, 0.1 ml of a 1:100 dilution normal rabbit serum and 0.1 ml of goat anti-rabbit-γ-globulin were added and the reaction mixtures were incubated overnight at 2-4. The precipitate obtained from centrifugation (1000 xg for 30 min) was dissolved in 0.2 ml of 0.1 N NaOH and counted.

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of the same reaction mixtures increased when measured with these antibodies to 13, 14 dihydro-15-keto-$PGF_{2\alpha}$.

Purification of Prostaglandin Δ^{13}-Reductase. Purification of the Δ^{13}-reductase is summarized in Table I. The enzymatic activity shown in Table I must be regarded as qualitative since less than optimal conditions of substrate concentration may have been used (1 μg 15-keto-$PGF_{2\alpha}$/ml). Prostaglandin E 9-ketoreductase and prostaglandin Δ^{13}-reductase did not bind to the DEAE-Sephadex column while the 15-hydroxyprostaglandin dehydrogenase did. The prostaglandin Δ^{13}-reductase from chicken heart binds to hydroxylapatite and was separated from prostaglandin E 9-ketoreductase at this step. In the last step of purification, prostaglandin Δ^{13}-reductase did bind to phosphocellulose after reducing the concentration of phosphate buffer as detailed in Materials and Methods. The purified enzyme contained no 15-hydroxyl-prostaglandin dehydrogenase or prostaglandin E 9-ketoreductase activity.

Cofactor Requirements. The purified prostaglandin Δ^{13}-reductase from chicken

TABLE I

PURIFICATION OF PROSTAGLANDIN Δ^{13}-REDUCTASE FROM CHICKEN HEART

Fraction	Activity (units)	Total Protein (mg)	Specific Activity (units/mg protein)X10^3	Yield %
10,000 x g supernatant fluid	43.8	60,480	0.7	100
78,000 x g supernatant fluid	41.6	47,160	0.9	95
Ammonium Sulfate Precipitate (30-60%)	39.7	18,540	2.1	91
DEAE-Sephadex	34.9	9,000	3.9	80
Hydroxylapatite	20.9	1,250	16.7	48
Phosphocellulose	12.3	1.8	6833.3	28

heart used NADPH as a cofactor much more effectively than NADH (Fig. 2). Prostaglandin Δ^{13}-reductases of human placenta; dog lung; swine lung, kidney and liver; monkey heart, lung, liver, spleen, kidney and uterus also used NADPH more effectively than NADH. NADH also serves as a cofactor for these Δ^{13}-reductases, but it is less than 10% as effective. Dog lung prostaglandin Δ^{13}-reductase has also been partially purified by the same procedures as were used to purify the Δ^{13}-reductase from chicken hearts.

Unlike 15-hydroxyprostaglandin dehydrogenase, which is inhibited by reduced pyridine nucleotides (7) or prostaglandin E 9-ketoreductase, which is inhibited by oxidized pyridine nucleotides (8), prostaglandin Δ^{13}-reductase is not affected by oxidized pyridine nucleotides.

Substrate Specificities. Serologic assays for measuring reduction of the Δ^{13}-double bond, are limited at this time to 15-keto-$PGF_{2\alpha}$ or 15-keto-PGE_2. It was necessary to employ other methods, such as thin layer chromatography to study the enzymatic activity of the Δ^{13}-reductase on other substrates. The

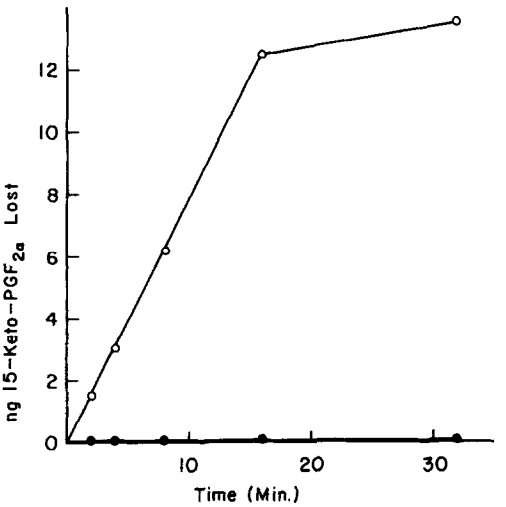
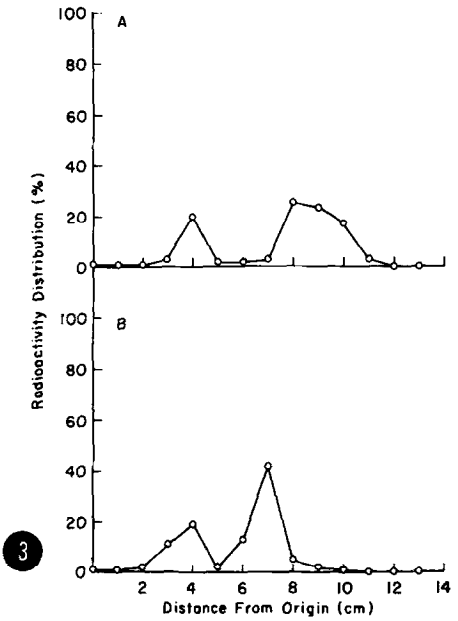


Figure 2. Cofactor-dependence of prostaglandin Δ^{13}-reductase. Purified prostaglandin Δ^{13}-reductase (1.8 μg) was incubated with 0.1μg 15-keto-$PGF_{2\alpha}$ and 1 mM NADPH (o) or 1 mM NADH (●) in a reaction mixture of 0.1 ml. At various times 10 μl were diluted with 0.1 ml of tris-HCl buffer (0.01M, pH 7.4, containing 0.1% gelatin and 0.15M NaCl) and boiled 2 min. and then analyzed for disappearance of 15-keto-$PGF_{2\alpha}$ serologic activity with monkey antiserum to 15-keto-$PGF_{2\alpha}$.

enzymatic conversions of PGE_2, $PGF_{2\alpha}$, 15-keto-PGE_2 and 15-keto-$PGF_{2\alpha}$ to their 13, 14 dihydro metabolites can be detected by thin layer chromatographic techniques. The data, shown in Fig. 3, depict the thin layer chromatograms of 15-keto-[^{3}H]PGE_2, and 15-keto-[^{3}H]$PGF_{2\alpha}$ after treatment with prostaglandin Δ^{13}-reductase. The higher Rf value for the enzymatically treated sample is equivalent to the Rf of 13, 14 dihydro-15-keto-PGE_2 chromatographed under the same conditions. The conversion of 15-keto-[^{3}H]$PGF_{2\alpha}$ to 13, 14 dihydro-15-keto-$PGF_{2\alpha}$ by this enzyme is shown in Fig. 4. Again the Rf of the new peak of radioactivity after enzymatic treatment is equivalent to the Rf of 13, 14 dihydro-15-keto-$PGF_{2\alpha}$. Under identical conditions [^{3}H]PGE_2 and [^{3}H]$PGF_{2\alpha}$ were not converted to 13, 14 dihydro[^{3}H]PGE_2 and 13, 14 dihydro[^{3}H]$PGF_{2\alpha}$. The specificity of prostaglandin Δ^{13}-reductase is such that it reduces the $\Delta^{13,14}$-double bond only when the 15-keto group is there. This confirms other investigators' studies on the specificity of this enzyme (2).



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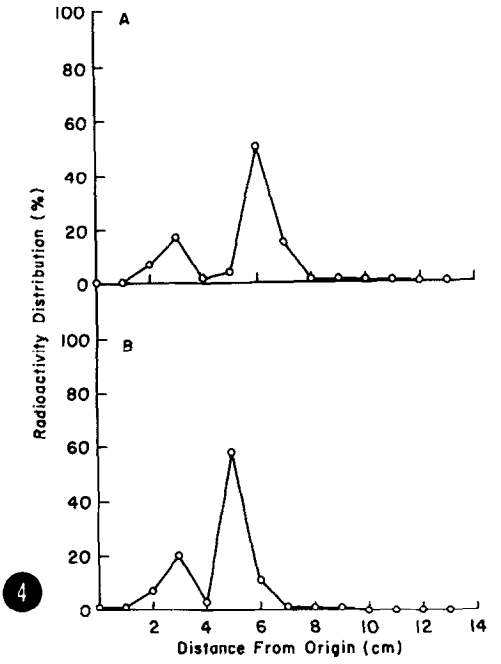

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Figure 3. Thin layer chromatography of prostaglandin Δ^{13}-reductase-treated 15-keto-[^{3}H]PGE_2 (A) and untreated 15-keto-[^{3}H]PGE_2 (B). The 15-keto-[^{3}H] PGE_2 preparation was contaminated with [^{3}H]PGE_2 (4 cm from origin). After prostaglandin Δ^{13}-reductase treatment, [^{3}H]PGE_2 was not changed while practically all 15-keto-[^{3}H] PGE_2 (7 cm from origin) was changed to 13, 14-dihydro-15-keto-[^{3}H]PGE_2 (9 cm from origin). Each reaction mixture of 0.3 ml contained 5.4 µg prostaglandin Δ^{13}-reductase, 1 mM NADPH and 0.1 nmole 15-keto-[^{3}H]PGE_2. After incubation at 37 for 2 hours, the reaction mixtures were extracted with petroleum ether and processed as described in Materials and Methods.

Figure 4. Thin Layer chromatography of prostaglandin Δ^{13}-reductase treated 15-keto-[^{3}H]$PGF_{2\alpha}$ (A) and untreated 15-keto-[^{3}H]$PGF_{2\alpha}$ (B). The 15-keto-[^{3}H]$PGF_{2\alpha}$ was contaminated with [^{3}H]$PGF_{2\alpha}$ (3 cm from origin). After prostaglandin Δ^{13}-reductase treatment, [^{3}H]$PGF_{2\alpha}$ was not changed while 15-keto-[^{3}H]$PGF_{2\alpha}$ (5cm from origin) was converted to 13,14-dihydro-15-keto-[^{3}H]$PGF_{2\alpha}$ (6cm from origin). Assay conditions were the same as those for Figure 3.

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pH Optimum. The activity of prostaglandin Δ^{13}-reductase has relatively broad pH spectra. No significant differences in the activity were observed on varying the pH from 7.4 to 8.5.

Estimation of Size. With the use of Sephadex G-200 chromatography and several proteins of known size, the molecular weight of prostaglandin Δ^{13}-reductase was estimated to be 70,000 to 80,000; larger than 15-hydroxyprosta-

glandin dehydrogenase (60,000 to 70,000) or prostaglandin E 9-ketoreductase (45,000 to 55,000).

Effect of Sulfhydryl Blocking Reagents. Prostaglandin Δ^{13}-reductase is not affected by iodoacetate, iodacetamide, Ellman's reagent and N-ethylmaleimide at a concentration of 1×10^{-3} M. It is inhibited up to 50% by 1×10^{-3} of p-chloromercuribenzoate.

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