PURIFICATION AND PARTIAL CHARACTERIZATION OF AN NADH-LINKED Δ^{13}-15-KETOPROSTAGLANDIN REDUCTASE FROM HUMAN PLACENTA

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SUMMARY: A Δ^{13}-15-ketoprostaglandin reductase has been isolated from human placenta and purified 800-fold. The enzyme utilizes NADH as a cofactor but not NADPH. It reduces the 13,14 double bond in 15-ketoprostaglandin E_1, E_2 and $F_{2\alpha}$. The K_M apparent for NADH is 54.8 μM and the K_M apparent for 15-ketoprostaglandin E_2 is 7.0 μM. The partially purified enzyme contains no 15-hydroxyprostaglandin dehydrogenase activity.

INTRODUCTION

The first two steps in the metabolic breakdown of prostaglandins in many mammalian tissues are oxidation of the secondary alcohol group at C-15 to a ketone, followed by reduction of the Δ^{13} double bond (1,2):

(eq.1) 15-hydroxyprostaglandin + NAD^+ ⇄ 15-ketoprostaglandin + NADH + H^+

(eq.2) 15-ketoprostaglandin + NADH + H^+ ⇄

13,14-dihydro-15-ketoprostaglandin + NAD^+

Although the enzyme which catalyzes the first step in this pathway, 15-hydroxyprostaglandin dehydrogenase (EC 1.1.1.141), has been studied extensively and recently purified to homogeneity from human placenta (3), the enzyme catalyzing the second step, Δ^{13}-15-ketoprostaglandin reductase, has received little attention. This paper describes the partial purification and characterization of a human placental Δ^{13}-15-ketoprostaglandin reductase.

MATERIALS AND METHODS

Materials. NAD, NADH, and NADPH were obtained from P-L Biochemicals; F-254 silica gel thin layer chromatography plates from Brinkman. Prostaglandins and prostaglandin metabolites were generously provided by Drs. J. Pike and U. Axen of the Upjohn Company.

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Enzyme Assays. Δ^{13}-15-ketoprostaglandin reductase activity was measured with a Gilford recording spectrophotometer. A cuvet with a 1-cm path contained 3 ml of aqueous solution at 25 ± 0.5° consisting of 30 μmol of potassium phosphate (pH 7.0), 680 nmol of NADH and 114 nmol of 15-keto-PGE_2 added in .02 ml 95% ethanol. The reaction was initiated by addition of enzyme. The blank cuvet contained no 15-keto-PGE_2 or ethanol; enzyme was included in the blank only when NADH oxidase was present--this background activity was completely removed from the enzyme solutions by the TEAE-cellulose chromatography step. Oxidation of NADH was followed by measuring the change in absorbance at 340 nm. One unit of enzyme activity is defined as the amount of enzyme which catalyzes the oxidation of 1 μmol of NADH per minute under the conditions of assay. The enzyme was not measured in the presence of 15-hydroxyprostaglandin dehydrogenase, i.e., prior to DEAE-cellulose chromatography.

15-hydroxyprostaglandin dehydrogenase was measured spectrophotometrically by the method of Jarabak (4), except that the reaction mixture contained 30 μmol rather than 100 μmol of potassium phosphate, and PGE_2 was used as substrate.

Protein Determinations. Estimates of protein concentration were made by absorption measurements at 280 nm (5), assuming that a solution containing 1 mg/ml protein has an absorbance of 1.0 in a cuvet of 1-cm light path.

Mass Spectrometry of the Reaction Product. The product was prepared by incubating 1.4 μmol 15-keto-PGE_2, 6.8 μmol NADH, and 7.7 mU of the partially purified enzyme in 5 ml of .01 M potassium phosphate (pH 7.0) for 1 hour at 37°. The solution was acidified with citric acid, extracted with 3 volumes of ethyl acetate, and evaporated to dryness under nitrogen. The residue was dissolved in chloroform, applied to a 5 x 20 cm silica gel plate, and developed in chloroform: methanol: acetic acid (90:5:5) (6). The product was located by reference to a 13,14-dihydro-15-keto-PGE_2 standard after a brief exposure to iodine vapors. The spot was scraped from the plate, and the metabolite was eluted from the silica gel by refluxing overnight in a Soxhlet extractor with 125 ml ethyl acetate. The O-methyloxime-trimethylsilyl (MO-TMS) derivatives of the product and of standard 13,14-dihydro-15-keto-PGE_2 were prepared by the procedure of Green (7) and analyzed using a Finnigan 1015 mass spectrometer equipped with a vapor phase chromatography inlet.

Enzyme Purification. Two normal human term placentas were chilled immediately after delivery, and all subsequent steps were carried out at 4°. The villous tissue was dissected from the membranes. It was homogenized in 75-100 g portions at top speed for 2 min in a Waring Blender with two volumes of 5 mM potassium phosphate (pH 7.0), 20% glycerol, 1 mM EDTA (Buffer A) and then centrifuged at 10,000 x g for 45 min. Solid ammonium sulfate was added to the supernatant to achieve 50% saturation (.298 g/ml) while the pH was maintained at 7.0 by the addition of 3 N NH_4OH. After 1½ hrs the solution was centrifuged at 10,000 x g for 45 min. Solid ammonium sulfate was added to the supernatant to achieve 80% saturation (.197 g/ml), while maintaining the pH at 7.0. After 1½ hrs this solution was centrifuged at 10,000 x g for 45 min and the precipitate was dialyzed against two changes of 40 volumes each of Buffer A. The dialyzed enzyme solution was centrifuged for 15 min at 39,000 x g. The supernatant solution was applied to a 4 x 35 cm DEAE-cellulose column which had been equilibrated with Buffer A. The protein that was not bound to the DEAE-cellulose was collected and the pH adjusted to 8.0 by the addition of 1 N NaOH. This solution, about 400 ml, was concentrated 10-fold by ultrafiltration in an Amicon Diaflo apparatus, using a UM-10 membrane. The concentrated solution was applied to a 4 x 45 cm TEAE-cellulose column equilibrated with 5 mM Tris-HCl (pH 8.0), 20% glycerol, 1 mM EDTA. Then the column was washed with the same buffer. The enzyme was not bound to the TEAE-cellulose. Fractions of highest specific activity were combined, concentrated by ultrafiltration to about 20 ml, and then applied to a 5 x 95 cm Sephadex G-100 column that had been equilibrated with 20 mM potassium

phosphate (pH 7.0), 20% glycerol, 1 mM EDTA. The Sephadex column was developed with the same buffer. Fractions of highest specific activity were combined and the pH was adjusted to 6.0 by the addition of 1 N HCl. This solution was partially desalted by ultrafiltration in a Diaflo apparatus with repeated addition of cold 20% glycerol, until the conductivity was about .2 mMHO when measured at 4°. Then it was applied to a 2 x 12 cm CM-cellulose column that had been equilibrated with 5 mM potassium phosphate (pH 6.0), 20% glycerol, 1 mM EDTA. The column was washed with 200 ml of buffer. A linear gradient was used for elution: chamber 1 contained 500 ml of 5 mM potassium phosphate (pH 6.0), 20% glycerol, 1 mM EDTA, and chamber 2 contained 500 ml of 100 mM potassium phosphate (pH 6.0), 20% glycerol, 1 mM EDTA.

RESULTS

Enzyme Purification. The purification is summarized in Table I. The partially purified enzyme did not contain NADH oxidase or 15-hydroxyprostaglandin dehydrogenase activity. Although the reported enrichment is 820-fold, the purification is actually greater because the reductase could not be assayed spectrophotometrically before Step 3, due to the presence of the 15-

TABLE I

PURIFICATION OF Δ^{13}-15-KETOPROSTAGLANDIN REDUCTASE FROM HUMAN PLACENTA

Step	Activity (units)	% Recovery	Total Protein (mg)	Specific Activity X 10^3 (units/mg protein)
1. Centrifuged homogenate[a]			52,580	
2. 50-80% ammonium sulfate precipitate			21,988	
3. DEAE-cellulose	.748	100	15,000	0.05
4. TEAE-cellulose	.497	66	184	2.7
5. Sephadex G-100	.407	54	26.3	15.0
6. CM-cellulose	.181	24	4.46	40.5

[a] A total of 604 g of villous tissue was used for this step

hydroxyprostaglandin dehydrogenase. More than 99% of the dehydrogenase is bound to DEAE-cellulose and is removed by this step.

Identification of Product. Incubation of 15-keto-PGE_2 with NADH and the Δ^{13}-15-ketoprostaglandin reductase gives a product which behaves identically to 13,14-dihydro-15-keto-PGE_2 on thin layer chromatography; it moves faster than 15-keto-PGE_2 and does not absorb ultraviolet light at 254 nm (signifying the loss of the α,β unsaturated ketone). The mass spectrum of the MO-TMS derivative of the product is identical to that of 13,14-dihydro-15-keto-PGE_2, showing major peaks at 465, 375, 340, 325, 265 and 232 mass units (8).

Cofactor Requirements. The purified enzyme utilizes NADH but not NADPH as a cofactor. Neither supernatants obtained by centrifugation of placental homogenates nor the ammonium sulfate precipitates of these supernatants contain NADPH-linked Δ^{13}-15-ketoprostaglandin reductase activity.

pH Dependence. The activity of the Δ^{13}-15-ketoprostaglandin reductase shows a wide pH range with a small peak at pH 5.5. There is little change in activity between pH 6.5 and 7.5. Below pH 6.0, spontaneous oxidation of NADH is significant.

K_M for NADH and 15-keto-PGE_2. Michealis-Menten kinetics were observed for both substrate and cofactor. The K_M apparent for NADH is 54.8 μM when the concentration of 15-keto-PGE_2 is 37 μM; the K_M apparent for 15-keto-PGE_2 is 7.0 μM when the NADH concentration is 180 μM.

Substrate Specificity. The Δ^{13}-15-ketoprostaglandin reductase reduces the 13,14 double bond in 15-keto-PGE_1, -PGE_2, and -$PGF_{2\alpha}$; 15-hydroxyprostaglandins are not substrates for the enzyme.

DISCUSSION

Both the 15-hydroxyprostaglandin dehydrogenase and the Δ^{13}-15-ketoprostaglandin reductase are present in placental tissue. Incubation of crude placental extracts with 15-keto-PGE_2 and NADH will give PGE_2 and NAD due to the reversibility of the dehydrogenase reaction (eq. 1) as well as 13,14-

dihydro-15-keto-PGE_2, the product of the reductase reaction (eq. 2). Since the rate of reduction of 15-keto-PGE_2 by the dehydrogenase is 0.6 times as rapid as the rate of oxidation of PGE_2 by this enzyme under the conditions of the dehydrogenase assay[1], and since the dehydrogenase activity is more than 10 times greater than the Δ^{13}-15-ketoprostaglandin reductase activity in placental tissue, most 15-keto-PGE_2 will be converted to PGE_2 rather than 13,14-dihydro-15-keto-PGE_2 by crude placental extracts. Thus, an estimate of reductase activity in placental extracts cannot by obtained by spectrophotometric methods which measure the disappearance of either 15-keto-PGE_2 or NADH until the 15-hydroxyprostaglandin dehydrogenase has been removed. Chromatography on DEAE-cellulose effects this separation, and the reductase may be assayed spectrophotometrically after this step. Although the dehydrogenase reaction has been shown to be completely or partially reversible in a number of tissues besides placenta (9,10) many investigators fail to take this into account when measuring reductase activity. It is likely that the estimates of Δ^{13}-15-ketoprostaglandin reductase in various swine tissues (11) are too high for this reason.

It is of interest to compare placental Δ^{13}-15-ketoprostaglandin reductase to chicken heart Δ^{13}-15-ketoprostaglandin reductase, the only other prostaglandin reductase which has been purified extensively (12). Placenta is a much richer source than chicken heart: while chicken heart contains .00219 mU/g of tissue when assayed at 37°, the human placental preparation described here contains 1.2 mU/g when assayed at 25°. This value must be considered a minimum estimate for placental tissue, since the enzyme cannot be assayed spectrophotometrically in crude homogenates. The specific activity of the partially purified placental enzyme is 60 times that of the partially purified chicken heart enzyme. We have not been able to confirm the report of Lee and Levine (12) that placental tissue contains an NADPH-dependent

[1]Westbrook and Jarabak, unpublished observations.

Δ^{13}-15-ketoprostaglandin reductase. The chicken heart reductase utilizes NADPH as cofactor much more effectively than NADH (12), but the placental enzyme is specific for NADH. Both enzymes, however, show the same substrate specificity for 15-ketoprostaglandins and will not reduce 15-hydroxyprostaglandins. This specificity appears to be general among prostaglandin reductases (11), and implies that the initial steps in prostaglandin metabolism are the same in the human placenta as they are in other tissues (2).

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