PURIFICATION OF THE HUMAN PLACENTAL 15 HYDROXY PROSTAGLANDIN DEHYDROGENASE: PROPERTIES OF THE PURIFIED ENZYME

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1. Introduction

The 15 hydroxy prostaglandin dehydrogenase (PGDH) catalyses the oxidation of the 15 (S) OH of prostaglandins to a 15 keto group [1,2]. This is known to be of considerable importance for the inactivation of these compounds in vivo [2–5]. PGDH activity was first demonstrated in lung [2,6,7], then in other tissues of many animals [8,9].

First attempts at purifying the PGDH were carried out on lungs [1,10]. More recently partial purification of the placental PGDH [11–13] and of the kidney PGDH [14] were reported. We describe here a method which enables us to obtain milligram amounts of this enzyme from human placenta with a high specific activity. This relative large scale purification will afford us to perform structural studies of this protein.

2. Materials and methods

2.1. *Buffers*

Buffer I: 0.03 M potassium phosphate pH 7.2, 20% glycerol, 0.001 M EDTA. Buffer II: same buffer but 0.15 M phosphate. Buffer III: 0.01 M potassium phosphate, pH 7.2, 20% glycerol, 0.001 M EDTA, 0.01 M β-mercaptoethanol. Buffers IV, V, VI are the same as buffer III but with respectivly 0.03 M, 0.01 M, 0.15 M phosphate. Buffer VII: 0.1 M sodium pyrophosphate, pH 9.2.

2.2. *Preparation of placental supernatant fraction*

The fresh placentas (less than one hour after delivery) were homogenized (Ultra-Turrax) in 2 vol of buffer I. The homogenate was centrifuged (20 000 *g* for 30 min) and the supernatant was used for purification.

2.3. *Purification method*

Unless otherwise stated, all operations were carried out at 4°C. The three first steps of the purification procedure were the same for PGDH and 17β-oestradiol dehydrogenase (17β-E_2DH).

Automatic DEAE cellulose chromatography (Whatman DE 23): the apparatus was assembled and perfected in the laboratory [15]. Elution was carried out with a linear gradient obtained with buffer I and buffer II. Hydrophobic affinity chromatography: the preparation of the oestrone amino caproate Sepharose column and elution (buffer containing ammonium sulphate) have been already described [16] for the purification of the 17β-E_2DH. Second DEAE-cellulose chromatography (Whatman DE 32): elution was performed with a linear gradient of buffer IV and V. Hydroxy-apatite chromatography: (Biorad hydroxy-apatite). Columns (5 × 15 cm) were equilibrated in buffer III and elution was performed with two successive linear gradients: respectivly buffer III and buffer IV, buffer V and buffer VI. Third DEAE-cellulose chromatography (Whatman DE 32). Same conditions that described above.

2.4. *Analytical methods*

Spectrometric enzyme assay: the reaction cuvettes (1 cm light path), kept at 4°C, contained 0.6 μmole of NAD, 55 nmoles of substrate, 10 to 100 μlitre of enzyme solution, and buffer VII to a final volume of 1 ml. All the substrates used for kinetic studies were

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racemic synthetic prostaglandins: the substrate used for 'routine work' 10,11-dihydroprostaglandin A_2 (dihydro PGA_2) and PGA_2 were graciously given by Roussel-Uclaf. All other prostaglandins were generously supplied by Dr John E. Pike, of Upjohn Co.

Concentration of proteins by ammonium sulphate precipitation or by ultrafiltration (Diaflo membrane) were already described for the liver 17β-hydroxy-steroid dehydrogenase [17]. The method of Ornstein and Davis [18] was used for analytical polyacrylamide gel electrophoresis, that of Weber and Osborn [19] for polyacrylamide gel electrophoresis in sodium dodecyl-sulphate (SDS). The enzymatic activity staining was performed according to Latner [20]. The molecular weight was determined by polyacrylamide gel electrophoresis with different acrylamide concentration (method of Hedrick and Smith [21]) and by SDS polyacrylamide gel electrophoresis.

2.5. *Amino acid analysis*

The protein was hydrolysed under vacuum by 6 N HCl during 24 hr at 110°C and analysed with a Beckman Multichrom auto analyser.

3. Results

3.1. *Purification*

Table 1 summarises the purification procedures and the results. A 3000- to 5000-fold purification was achieved with a 5% yield. The highest specific activity obtained was 2500 mUI/mg. Each purification was carried out with 4 to 8 placentas and gave 2 to 4 mg of protein. Separation of 17β-E_2DH and PGDH was achieved only after hydroxy-apatite chromatography. The 17β-E_2DH was eluted with the first gradient and the PGDH with the second.

After analytical polyacrylamide gel electrophoresis, specific enzymatic staining showed one or two PGDH bands (depending of the batch of enzyme) which were both revealed by all the substrates used (either dihydro PGA_2, $PGF_{2\alpha}$ or PGE_1). Relative migration of these two isozymic bands in gel of different acrylamide concentration showed that they have the same molecular weight. Graphic determination of their mol. wt. gave a value of 50 to 55 000. Protein staining showed a slower band, very thin which has been identified to the 17β-E_2DH and which represents less than 10% of the total protein.

Table 1
Purification of the placental PGDH

	Activity, mIU	Protein, mg	Specific activity, mIU/mg	Yield, %	Purification, X-fold
Homogenate	9000	1500	0.6		
Automatic DEAE cellulose (Whatman 23)	6300	525	12	70	20
Hydrophobic affinity chromatography	4797	41	117	54	195
DEAE-cellulose (Whatman 32)	2350	12	195	26	325
Hydroxy-apatite	1175	1.2	979	13	1631
DEAE-cellulose (Whatman 32)	576	0.3	1866	6	3110

1 mIU = 1 nmole oxidized substrate per minute. Purification was carried out with 3 to 6 placenta. Protein values were calculated for one placenta.

3.2. *Stability*

Enzymatic solutions in buffer containing 20% glycerol and 10^{-2} M β-mercaptoethanol lost only 10% of their activity after two days at 4°C. They could be stored at −24°C for one month with less than 20% loss of activity.

3.3. *Kinetic constants*

Kinetic constants obtained with different substrates and inhibitors are given in tables 2 and 3. The best substrates were PGE_1, PGE_2 and dihydro PGA_2. The 15-methyl PGE_2 was not substrate and had a K_i of $6.4 \cdot 10^{-4}$ M. The inhibition was of the competitive type. Methylation of the carboxylic function of PGA_2 prevented the oxidation of the substrate by the PGDH and decreases highly its affinity. Inhibition by this methyl ester is of mixed type ($K_i = 16 \cdot 10^{-4}$ M).

Oestrone and progesterone had an inhibitory effect on the PGDH at a concentration of $5 \cdot 10^{-5}$ M. Inhibition is competitive for the progesterone ($K_i = 5.10^{-5}$ M) and seems to be of mixed type for the oestrone ($K_i = 10^{-4}$ M). The enzyme was strictly NAD-dependent.

Table 2
Substrate* specificity of the placental PGDH

Substrate	K_m μM.	Relative V_{max} (p. 100 of PGE_1)
Prostaglandin E_1	2.9	100
Prostaglandin E_2	4.0	97
Prostaglandin $F_{1\alpha}$	57.0	91
Prostaglandin $F_{2\alpha}$	26.3	97
Prostaglandin A_1	8.7	109
Prostaglandin A_2	50.0	24
10,11-dihydro PGA_2	4.5	111

* All substrates used are racemic compounds.

Table 3
Inhibition of placental PGDH by different prostaglandins derivatives and by steroids

Substrate	K_i M	Type of inhibition
15 methyl $PGF_{2\alpha}$	$6.4 \cdot 10^{-4}$	competitive
PGA_2 methyl ester	$1.6 \cdot 10^{-4}$	mixed
Progesterone	$7 \cdot 10^{-5}$	competitive
Oestrone	10^{-4}	mixed (?)

The K_m for NAD was $5 \cdot 10^{-5}$. The K_m for dihydro PGA_2 was independent from NAD concentration in the range of 4 to 12 K_m.

3.4. *Inactivation by thiol reagent*

The enzyme was completly inactivated by $5 \cdot 10^{-5}$ M Ellman's reagent and by $5 \cdot 10^{-4}$ M phenyl mercury acetate. Inhibition by $5 \cdot 10^{-5}$ M Ellman's reagent was prevented by addition of $3 \cdot 10^{-6}$ M NAD or $2.7 \cdot 10^{-6}$ M dihydro-PGA_2. The inactivation of the PGDH by $5 \cdot 10^{-4}$ M phenyl mercury acetate was partly reversed by β-mercaptoethanol (50% of the initial activity were recovered whatever could be the duration of incubation with the inhibitor).

4. Discussion

In the present study a 3000- to 5000-fold purification of the human placental PGDH was achieved. In the best conditions, when the enzyme had a specific activity of 2500 mUI/mg, it still contained traces of 17β-E_2DH. These two enzymes were very difficult to separate with preservation of the PGDH activity:

Both PGDH and 17β-E_2DH were retained on oestrone Sepharose columns. This unexpected result can be explained, either by the affinity of the PGDH for oestrone (which is an inhibitor of the enzyme: $K_i = 10^{-4}$ M) or by eventual hydrophobic interactions occurring in the presence of ammonium sulphate between the 17β-E_2 DH retained on the phase and the PGDH [22].

Separation by gel filtration on Sephadex columns was only partially effective due the small difference between the molecular weight of these two enzymes, and had a poor yield in PGDH due to its lability. The greater lability of the PGDH when compared to the 17β-E_2DH facilitated on the contrary the 17β-E_2DH purification: 17β-E_2DH solutions were easily rid of the PGDH by handling the material at room temperature and by using buffers without β-mercaptoethanol. Nevertheless a good separation of PGDH from 17β-E_2DH was achieved by hydroxy-apatite chromatography. Evidence of this separation was given by the amino acid analysis of the purified PGDH which showed an amino acid composition different from that of the 17β-E_2DH, especially in methionine percentage.

From the yield of the purification procedure describ-

ed above it can be calculated that each placenta (300 g) contains about 5 to 10 mg of PGDH. It is at the present time the richest source of PGDH known: purification from lungs gave very poor yields; the best results are a 15-fold purification with a 45% yield and specific activity 4 nmoles/min/mg [10,22].

Two bands of PGDH activity were often seen on polyacrylamide gel; this finding, which is not constant, was also reported by Schlegel [12]. The behaviour of these bands in gels of increasing acrylamide concentration gives evidence that they have the same mol. wt. (50 – 55 000). Mol. wt. values determined by SDS polyacrylamide gel electrophoresis were a little lower 42 000. This result is in good agreement with the mol. wt. of 44 000 given recently for placental PGDH by Braithwaite and Jarabak [13].

Our kinetic data showed that the K_m for PGE_1, PGE_2, PGA_1 and dihydro PGA_2 are in the 10^{-6} M range. These values and the corresponding V_{max} are in good agreement with those of Jarabak [11] but quite different from those recently reported by Schlegel [12]. This discrepancy cannot be related to a difference in pH conditions of kinetic measurements, for pH conditions were also different in Jarabak's experiments, who yet reported similar K_m values. However, such a difference could be explained by the low NAD concentration ($1 \cdot 10^{-5}$M, lower than K_m) used by Schlegel, or by differences in molecular forms induced by different methods of purification as suggested by this autor. These molecular forms, or the presence of contaminating inhibitor(s) in the substrate, might also explain the different V_{max} values given for $PGF_{2\alpha}$ by Schlegel.

The kinetic behaviour of the dihydro PGA_2 is quite interesting. PGA_2 is not a good substrate but suppression of the 10,11 double bond highly increases the affinity of this substrate for the enzyme. The relativly low K_m of PGF may have physiological and pharmacological incidences.

When comparing the affinity of the placental PGDH for its substrates to PGDH of other organs, affinity of the same order of magnitude (10^{-6}M) is found for the placental and the kidney PGDH and a little lower (10^{-5}M) for the lung PGDH.

Inhibition of PGDH by steroids was investigated by Schlegel [12] on very crude enzymatic preparations. The inhibitory effect of progesterone and oestrone reported in this present work on purified PGDH was effective at only very high concentrations of steroid (10^{-5}M) and the type of inhibition is not clearly definite in the case of oestrone.

Whether regulation of PGDH by these hormones is of physiological importance awaits further experiments with very low concentrations of both prostaglandins and steroids.

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