Biochimica et Biophysica Acta, 659 (1981) 169—178
Elsevier/North-Holland Biomedical Press

BBA 69264

PURIFICATION AND PARTIAL CHARACTERIZATION OF PROSTAGLANDIN D_2 11-KETO REDUCTASE IN RABBIT LIVER

PATRICK Y -K WONG

Department of Pharmacology, New York Medical College, Valhalla, NY 10595 (U S A)

(Received November 6th, 1980)

Key words Prostaglandin 11-keto reductase, Prostaglandin D_2, NADPH dependence, (Rabbit liver)

Summary

The cytoplasmic NADPH-dependent prostaglandin D_2 11-keto reductase from rabbit liver was purified by a series of chromatographic procedures including isoelectric focusing The enzyme catalyzed the conversion of prostaglandin D_2 to prostaglandin $F_{2\alpha}$ and had a pH optimum of 7 0—7 5, and an isoelectric point of 5.8. The molecular weight was estimated to be 66 000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Enzymic activity was time and concentration dependent and required NADPH as cofactor.

Introduction

Prostaglandin D_2 is a significant product of arachidonic acid cascade in many tissues [1] Recently, prostaglandin D_2 has been identified as the major prostaglandin formed in rat brain and neuroblastoma cells [2] The enzyme, prostaglandin D_2 synthetase, has been purified to homogeneity and clearly distinguished from that of glutathione *S*-transferase [3] It has been demonstrated that prostaglandin D_2 is released by platelets during aggregation [4], and has also been found to be one of the potent inhibitors of platelet aggregation, with a potency only less than that of prostacyclin (prostaglandin I_2) and its metabolite, 6-keto-prostaglandin E_1 [5,6]

Recently, Ellis and coworkers [7] have found that over two-thirds of the prostaglandin D_2 metabolite of the monkey have the cyclopentane 1,3-diol ring structure (prostaglandin $F_{2\alpha}$), and they suggested that the infused prostaglandin D_2 may have been converted to prostaglandin $F_{2\alpha}$ by an 11-keto reductase before it was further metabolized by 15-hydroxyprostaglandin dehydrogenase and β-oxidation.

In the present investigation, we have demonstrated prostaglandin D_2 11-keto

0005-2744/81/0000—0000/$02 50 © Elsevier/North-Holland Biomedical Press

reductase activity in the rabbit liver and have purified this enzyme Thus, prostaglandin D_2 11-keto reductase may be a major enzymic pathway for the transformation of endogenous prostaglandin D_2 to prostaglandin $F_{2\alpha}$ Detailed studies on the occurrence and the distribution of the enzyme in various tissues of the rabbit by Reingold and coworkers [8] are presented in the accompanying paper

Materials and Methods

A Preparation of crude extract New Zealand white male rabbits (2—3 kg) were anesthetized with sodium pentobarbital (25 mg/kg, intravenous) After midline laparotomy, the liver was exposed The portal vein was cannulated and the liver was flushed with 200 ml of 50 mM Tris-HCl (buffer 1, pH 7.4), 0 1 mM dithiothreitol, removed and cut into small pieces 50 g of thin slices were homogenized in 5 vol ice-cold buffer 1 with a Polytron homogenization process which was repeated twice. The final homogenate was centrifuged at 8000 × *g* for 20 min The supernate was centrifuged further at 105 000 × *g* for 60 min in a Beckman model L-75 ultracentrifuge using a type W-28 rotor The supernatant is referred to as fraction I

B Ammonium sulfate fractionation All purification steps were carried out at 4°C The precipitate of 30—60% saturation of ammonium sulfate was prepared and resuspended in 20 ml of buffer 1 and dialyzed three times with 100 vol of buffer 1 (fraction II)

C DEAE-Sephadex chromatography The dialyzed fraction was loaded onto a DEAE-Sephadex (A50-120) column (4 × 30 cm) previously equilibrated with buffer 1. The column was first washed with 500 ml of the same buffer, then the enzyme was eluted with a step-wise gradient of NaCl (0.2 and 0 5 M) in buffer 1. Fractions containing 11-keto reductase activity were pooled and concentrated by an Amicon ultrafiltration cell using a 10 000 dalton cut-off membrane (PM-10) (fraction III)

D DEAE-cellulose chromatography A DEAE-cellulose column (4 × 30 cm) was equilibrated with buffer 1 Fraction III was applied onto the column and the column was first washed with 500 ml of the same buffer The enzyme was then eluted by a step gradient of NaCl (0.1—0 2 M) in buffer 1 The enzyme activity was eluted as a single peak by the same buffer (0.1 M NaCl). Active fractions were pooled and concentrated immediately to 10 ml by using an ultrafiltration cell as described before

E Analytical isoelectric focusing and gel electrophoresis Enzyme fractions (10—20 mg of protein) obtained from the DEAE-cellulose column were applied to the top of a sucrose gradient focusing column (LKB) containing 1% Ampholine (pH range 3.5—10) Focusing was started at 250 V and the voltage was gradually raised to 400 V after 2 h. Total focusing time was 16 h. Fractions were collected and the prostaglandin D_2 11-keto reductase activity was monitored by the radiometric assay method described below

Electrophoresis, using discontinuous sodium dodecyl sulfate (SDS) polyacrylamide gels, was performed at 22°C in a slab gel which contained a linear 5—15% acrylamide gradient [9] *N,N*,-Methylene bisacrylamide was kept constant at 2 5%, the buffer system was that described by Laemmli [10] Electrophoresis was performed at 2 mA/gel until the marker dye (Bromophenol blue)

approached the end of the gels Protein was stained with Coomassie brilliant blue.

F Radiometric assay of prostaglandin D_2 11-keto reductase [5,6,8,9,12,14,-15(n)-^{3}H]Prostaglandin D_2 (100 Ci/mmol) was a gift from Dr David Ahern of New England Nuclear The purity of the [^{3}H]prostaglandin D_2 was established by thin-layer chromatography (TLC) (TLC plate, 0 25 mm thick, 5 × 20 cm silica gel precoated plastic sheets, Brinkmann) with two solvent systems (A) ethyl acetate/acetic acid (99 1, v/v), (B) organic phase of iso-octane/ethyl acetate/acetic acid/water (25 55 10 50, v/v) Radiochromatogram scans showed a single peak of [^{3}H]prostaglandin D_2 on TLC plates with R_F values of 0.65 for system A and 0.75 for system B.

The assay mixture contained NADPH (4 mM), [^{3}H]prostaglandin D_2 (500 00 dpm/5 nmol), 0.5—0.9 ml of the enzyme fractions collected from the columns or 10—25 μg of protein of the purified enzyme from the isoelectric focusing column, and with Tris-HCl buffer (pH 7 4, 50 mM) with 0 1 mM dithiothreitol to the final volume of 1 ml After incubation at 37°C for 60 min, the reaction was stopped by acidification (pH 3.0) and extracted with ice-cold ethyl acetate (3 ml) and dried under a stream of N_2. The residue was redissolved in 100 μl of dry acetone, applied to a TLC plate and separated in solvent system B with comigration of authentic prostaglandin D_2, prostaglandin E_2 and prostaglandin $F_{2\alpha}$. Radioactive zones on the TLC plates were located by radiochromatogram scanning (Packard radiochromatogram scanner, Model 7320) and identified by comparison with the mobilities of authentic standards. Zones corresponding to prostaglandin $F_{2\alpha}$ and prostaglandin D_2 were cut out and suspended in 10 ml of 0.4% Omnifluor and 20% Triton X-100 toluene liquid scintillation fluid and counted in a Beckman L-75 liquid scintillation counter The observed cpm in both prostaglandin $F_{2\alpha}$ and prostaglandin D_2 zones was converted to pmol of prostaglandin $F_{2\alpha}$ formed after 60 min of incubation The specific activity of the enzyme was expressed as pmol of prostaglandin $F_{2\alpha}$ formed/h per mg protein. Protein concentration was determined by using the method of Lowry et al. [11] using bovine serum albumin as a standard.

G Platelet preparations and aggregation studies Blood was drawn from volunteers who had not taken acetylsalicylic acid or other drugs for the preceding 10 days 9 parts of whole blood were mixed with 1 part of 3.8% sodium citrate to a total volume of 5 ml in a plastic tube The platelet-rich plasma was removed with a siliconized pipette after centrifuging blood at 150 × *g* for 10 min. Platelet-depleted plasma was prepared by centrifuging the remaining blood at 12 000 × *g* for 10 min. The final platelet count in platelet-rich plasma was adjusted to $2 \cdot 10^8$/ml with platelet-depleted plasma. The platelet aggregation studies were perfomed with 0.5-ml aliquots of platelet-rich plasma stirred at 1200 rev /min at 37°C in a dual-channel Payton aggregation module, and transcribed on a linear recorder (Payton Associates, Buffalo, NY)

Results

The purification steps of prostaglandin D_2 11-keto reductase, specific activities and percentage of yield during purification are summarized in Table I In

TABLE I

PURIFICATION OF PROSTAGLANDIN D_2 11-KETO REDUCTASE FROM RABBIT LIVER

Fraction	Total protein (mg)	Total activity (pmol)	Specific activity (pmol/mg protein)	Purification (-fold)	Percentage recovery
I Crude extract	2502	648	0 25	1	100
II Ammonium sulfate precipitate (30—60%)	1987	630	0 32	1 26	97 2
III DEAE-Sephadex	192	554	2 89	11 5	85 5
IV DEAE-cellulose	53	315	5 90	23 5	48 6
V Isoelectric focusing	6	153	25 52	101 7	23 6

this procedure, the prostaglandin D_2 11-keto reductase was separated by a DEAE-Sephadex column from other proteins (Fig. 1) by a step gradient of NaCl (0 2 and 0.5 M) The enzyme was eluted with 0.2 M of NaCl When the enzyme fractions were applied to a DEAE-cellulose column, the enzyme activity was eluted as one peak. When the material from the DEAE-cellulose column was focused in an isoelectric focusing column containing a pH gradient of 3 5—10, the enzyme activity was found to be located in the fractions where the pH was 5.8, indicating the isoelectric point (p*I*) of the enzyme (Fig. 2).

The enzyme activity was found to be time and protein concentration dependent and to have a pH optimum of 7 5. The effect of reduced pyridine nucleotides was measured at constant concentration or by an NADPH-generating sys-

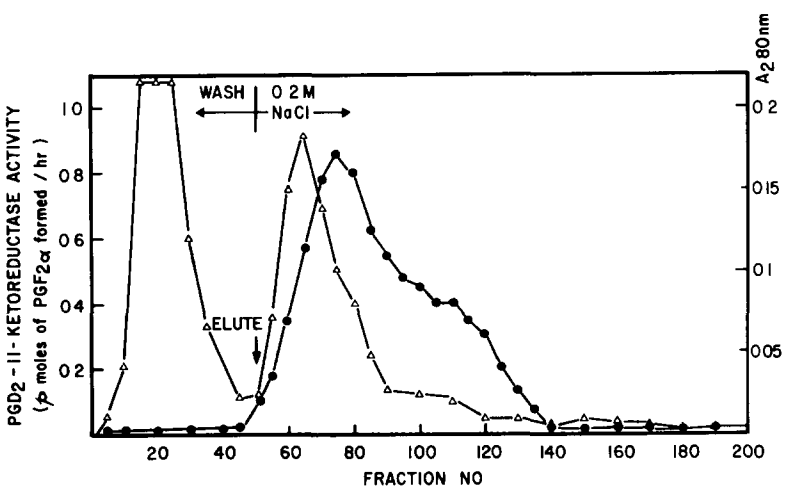


Fig 1 DEAE-Sephadex column chromatograph The desalted fraction of ammonium sulfate (30—60%) precipitation was applied to a DEAE-Sephadex column (4 X 30 cm) previously equilibrated with buffer 1 The enzyme was eluted with 0 1 M NaCl in buffer 1 10-ml fractions were collected, and the prostaglandin D_2 11-keto reductase activity assayed by the radiometric assay (●———●) Protein concentration was monitored by the absorbance at 280 nM (△———△)

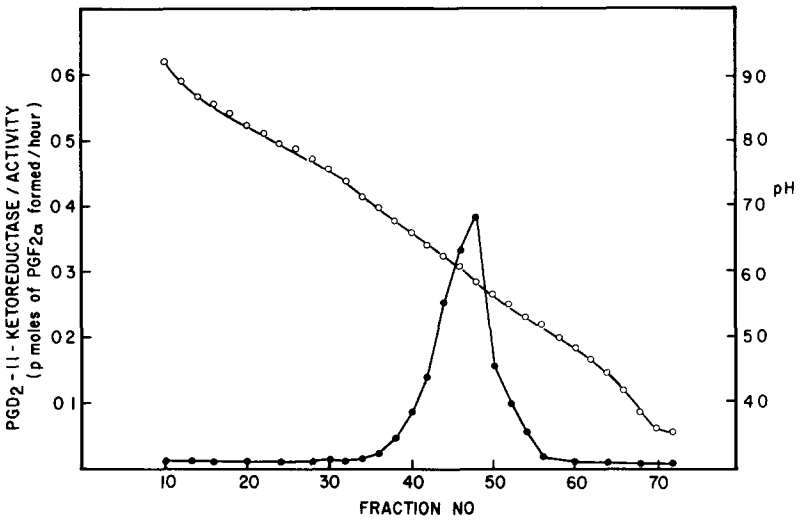


Fig 2 Purification of prostaglandin D_2 11-keto reductase by isoelectric focusing The enzyme fractions obtained from DEAE-cellulose column chromatography were concentrated by ultrafiltration Approx 20 mg of protein were electrofocused Prostaglandin D_2 11-keto reductase activity (•———•) and the pH value (○———○) were determined at every second fraction Prostaglandin D_2 11-keto reductase activity was determined by radiometric assay

tem which contained the following mixture $NADP^+$, 2 0 mM, glucose 6-phosphate, 3.5 mM, and 2 units of glucose-6-phosphate dehydrogenase. The conversion of [^{3}H]prostaglandin D_2 to [^{3}H]prostaglandin $F_{2\alpha}$ only occurred in the presence of NADPH (4 mM) and the NADPH-generating system, with a specific activity of 5.1 and 4.8 pmol/mg protein, respectively. NADH (4 mM) had lower activity (1.6 pmol/mg protein) and, with NAD^+ or $NADP^+$ as cofactor, activity was not detectable. The result suggested that 11-keto reductase is an NADPH-dependent enzyme. The K_m value for [^{3}H]prostaglandin D_2 was estimated to be 200 μM. After electrophoresis on an SDS-polyacrylamide gel, the enzyme was found to be one major band with a molecular weight estimated to be 66 000, as compared to protein with known molecular weight on the same gel (Fig. 3)

The enzyme was clearly distinguishable from 9-hydroxyprostaglandin dehydrogenase based on the following observations (1) no 9-hydroxyprostaglandin dehydrogenase activity was detected with the purified fraction of 11-keto reductase when either prostaglandin $F_{2\alpha}$ or 6-keto prostaglandin $F_{1\alpha}$ was used as substrate; (2) NAD^+ was not a required cofactor for the conversion of prostaglandin D_2 to prostaglandin $F_{2\alpha}$, (3) the p*I* value of 11-Keto reductase was 5.8, whereas that of 9-hydroxyprostaglandin dehydrogenase was found to be 5.0. [12].

Radiochromatogram scans of the extracts of the incubation of [^{3}H]prostaglandin D_2, with purified 11-keto reductase and NADPH (4 mM) as cofactor, showed one major radioactive peak corresponding to the mobilities of prostaglandin $F_{2\alpha}$ (Fig. 4A and B) Controls assayed at zero time and boiled enzyme did not show any conversion of [^{3}H]prostaglandin D_2 to [^{3}H]prostaglandin $F_{2\alpha}$ (Table I and Fig 4) Since prostaglandin D_2 is a potent inhibitor of platelet

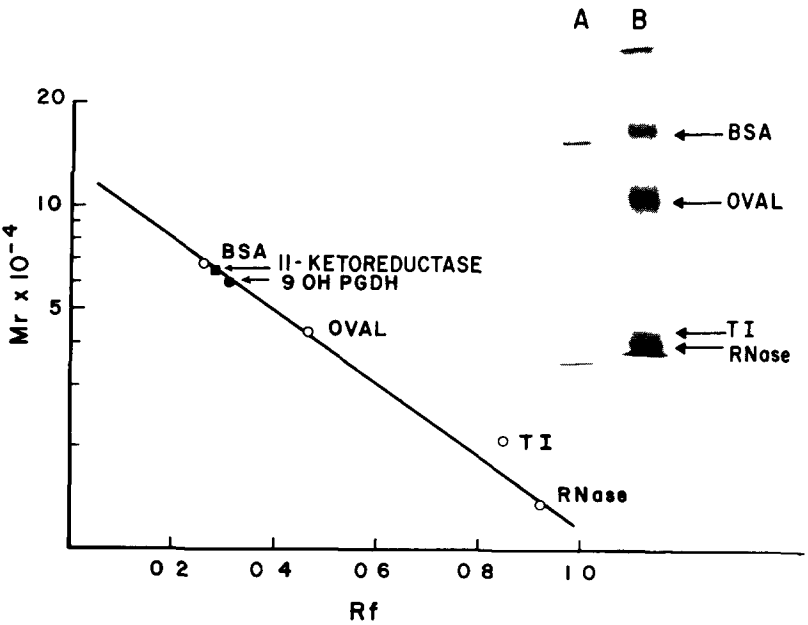


Fig 3 SDS-polyacrylamide gel electrophoresis of the purified prostaglandin D_2 11-keto reductase The purified enzyme obtained from isoelectric focusing (20 μg) was applied to an SDS-polyacrylamide gel (A) A slab gel with a 5—15% linear polyacrylamide gradient was prepared according to the method of Laemmli [10] Protein was stained with Coomassie Brilliant blue The molecular weight was calibrated with bovine serum albumin (BSA, M_r = 67 000), ovalbumin (OVAL, M_r = 42 000), soybean trypsin inhibitor (TI, M_r = 21 000) and ribonuclease A (RNAase, M_r = 13 700) (B) A indicated that the molecular weight of the prostaglandin D_2 11-keto reductase in rabbit liver was estimated to be 66 000

aggregation, the loss of platelet anti-aggregatory activity of the prostaglandin D_2 after incubation with the purified 11-keto reductase was also examined. Fig 4C and D shows the disappearance of anti-aggregatory activity of the extracts of the assay mixtures after they were incubated for 60 min with the purified enzyme in the presence of NADPH (4 mM) as cofactor The loss of anti-aggregatory activity coincides with the conversion of [^{3}H]prostaglandin D_2 to [^{3}H]prostaglandin $F_{2\alpha}$, the latter being inactive on platelet aggregation [13].

Discussion

Many tissues, including platelets, generate prostaglandin D_2 from arachidonic acid or endoperoxides (prostaglandin G_2 and prostaglandin H_2) [1] Although the biological actions of various prostaglandins have attracted much attention, the effects of prostaglandin D_2 have received little interest. The interest in prostaglandin D_2 has further declined since the discovery of prostacyclin [14] Recently, two groups of investigators reported the transformation of prostaglandin H_2 to prostaglandin D_2 with rat brain homogenates [15,16] These investigators clearly demonstrated the enzymatic synthesis of prostaglandin D_2, and suggested a physiologically significant role for this prostaglandin which has occasionally been regarded as a degradation product of prostaglandin endoperoxides Futhermore, Shimizu et al. [3] and Christ-Hazelhof and Nugteren

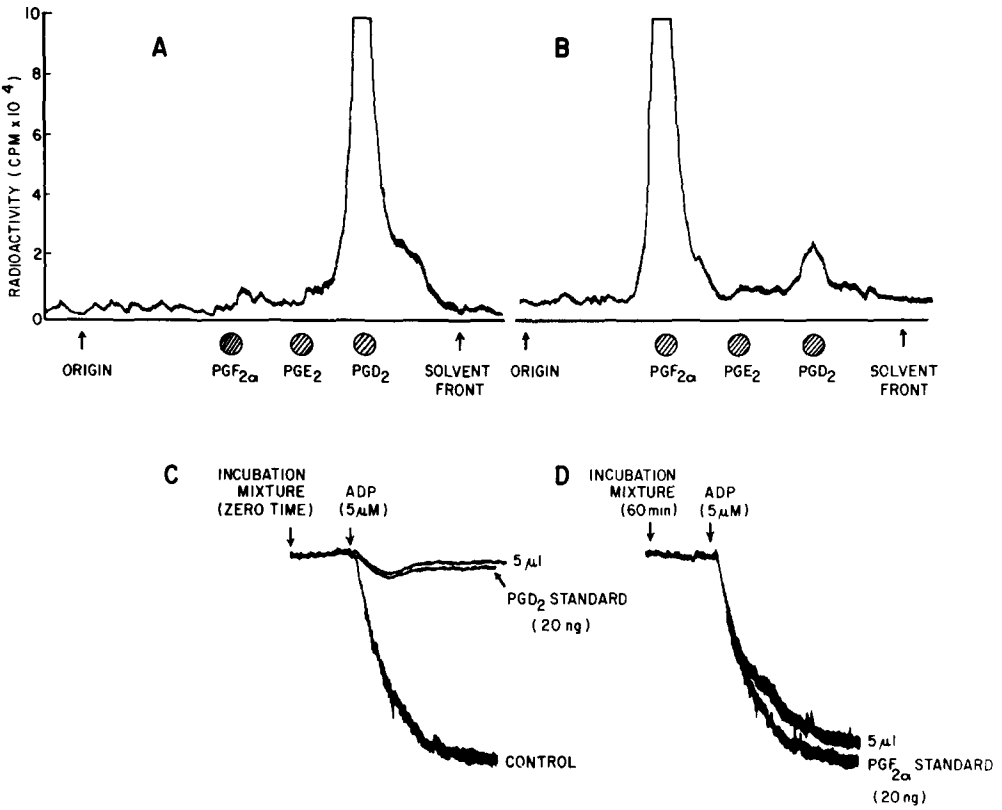


Fig 4 (A, B) Radiochromatograph scan of the radiometric assay of prostaglandin D_2 11-keto reductase and (C, D) bioassay of the prostaglandin D_2 11-keto reductase on platelet aggregation (A, B) Purified enzyme was incubated with [^{3}H]prostaglandin D_2 (500 000 dpm, 5 nmol), NADPH (4 mM) and buffer 1 to a volume of 1 ml After incubation at 37°C for 0 (A) and 60 min (B), the reactions were terminated by acidification and extraction The radioactive products extracted were separated by TLC (C, D) The enzymes were assayed as described above, except that unlabeled prostaglandin D_2 (14 nmol) was added to the incubation mixture before the addition of the enzymes After incubation at 37°C for 0 (C) and 60 min (D), the reactions were terminated and extracted as described The extracted products were resuspended in Tris-HCl buffer (pH 7 5, 50 mM) and a 5 μl aliquot of each suspension, equivalent to 20 ng of prostaglandin D_2, and were tested for their biological activity on the inhibition of ADP-induced platelet aggregation as compared to authentic prostaglandin D_2 and prostaglandin $F_{2\alpha}$ standards [5] $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$, PGE_2, prostaglandin E_2, PGD_2, prostaglandin D_2

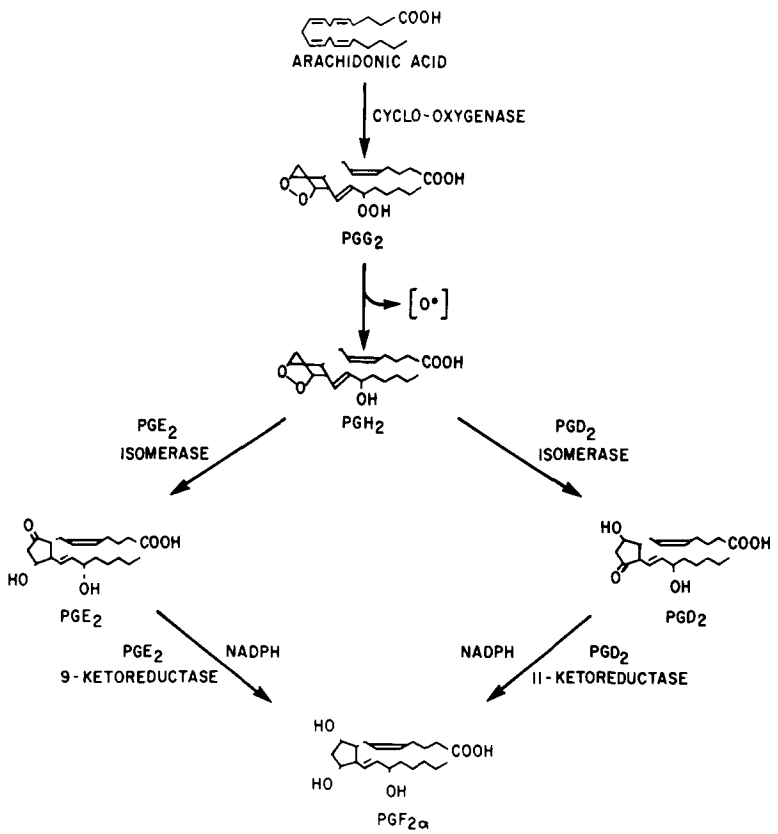
[20] have demonstrated that, in the rat brain and spleen, prostaglandin D_2 is enzymically transformed from prostaglandin H_2 by the enzyme prostaglandin D_2 synthetase Although the physiological role of prostaglandin D_2 has begun to unfold, very little is known about the enzyme system(s) which is responsible for the metabolism of this prostaglandin.

In the present studies we have purified the enzyme, prostaglandin D_2 11-keto reductase, from rabbit liver as indicated by a single band on gel electrophoresis and isoelectric focusing Hensby [17] first reported the conversion of prostaglandin D_2 to prostaglandin $F_{2\alpha}$ in sheep blood in vitro. Ellis et al. [7] reported that the conversion of prostaglandin D_2 to prostaglandin $F_{2\alpha}$ metabolites strongly suggested the presence of a unique enzymic system in the metabolism of prostaglandin D_2 in vivo. The purified prostaglandin D_2 11-keto reductase

from rabbit liver was able to convert prostaglandin D_2 to the corresponding prostaglandin $F_{2\alpha}$, but not $F_{2\beta}$ (unpublished data), suggesting that the reduction of prostaglandin D_2 to prostaglandin $F_{2\alpha}$, catalyzed by 11-keto reductase, was stereospecific.

The apparent action of 11-keto reductase in the metabolism of prostaglandin D_2 is analogous to the demonstrated role of a 9-keto reductase in the metabolism of prostaglandin E_2 [18]. Since prostaglandin D_2 is a poor substrate for 15-hydroxyprostaglandin dehydrogenase [19], prostaglandin D_2 may be converted to prostaglandin $F_{2\alpha}$ before it is able to be metabolized by the 15-hydroxyprostaglandin dehydrogenase (Fig. 5)

In their study on the distribution of 11-keto reductase in various tissues of the rabbit, Reingold et al [8] found this enzyme to be present in highest concentration in the liver This fact, together with our results regarding prostaglandin D_2 11-keto reductase, suggests the rapid interconversion of prostaglandin D_2 to prostaglandin $F_{2\alpha}$ in the liver The loss of biological activity of prostaglandin



CONVERSION OF PGD_2 TO $PGF_{2\alpha}$ BY PGD_2 11-KETOREDUCTASE IN RABBIT LIVER

Fig 5 Proposed metabolic pathways of prostaglandin D_2 by prostaglandin D_2 11-keto reductase in rabbit liver PG, prostaglandin

D_2 in passage through the rabbit liver may result from its conversion to prostaglandin $F_{2\alpha}$ by the enzyme 11-keto reductase Studies on incubation of prostaglandin D_2 with the purified enzyme resulted in the loss of its anti-platelet aggregatory activity. This finding is consistent with the conversion of [^{3}H]prostaglandin D_2 to [^{3}H]prostaglandin $F_{2\alpha}$, and suggests that 11-keto reductase may be an important metabolic pathway for the metabolism of prostaglandin D_2 in situ Thus, reports on the estimation of prostaglandin $F_{2\alpha}$ generation by tissues, as measured by its metabolites, must be re-examined to account for the possible contribution by the conversion of prostaglandin D_2 to prostaglandin $F_{2\alpha}$ Furthermore, measurements of the biological effect of prostaglandin D_2 in vivo will depend upon whether prostaglandin $F_{2\alpha}$ has a similar or opposite biological effect to that of prostaglandin D_2. Since prostaglandin $F_{2\alpha}$ is a potent vasoconstrictor [8,16] and has no effect on platelet aggregation [13], the conversion of prostaglandin D_2 to prostaglandin $F_{2\alpha}$, catalyzed by the enzyme prostaglandin D_2 11-keto reductase, may result in the potentiation of the musculotropic activity and elimination of the anti-aggregatory activity of prostaglandin D_2

Acknowledgements

The author is indebted to Dr. John Pike and Dr. Udo Axen of the Upjohn Company for the gift of prostaglandins, and to Dr. David Ahern of New England Nuclear for the [^{3}H]prostaglandin D_2. I thank Dr. J C McGiff and Dr. P Needleman for their helpful discussion of this manuscript, and Dr. R N. Wallace for his assistance on the electrophoresis experiments The technical assistance of Mr Warren H Lee and Mrs Patricia H.-W Chao on this study, and the assistance of Mrs Pamela Blank and Mrs. Sallie McGiff in the preparation of this manuscript, are gratefully acknowledged This study was supported in part by USPHS grant HL-25316, DE-04591 and the Westchester Heart Association. P.Y -K W. is a recipient of the NIH Heart, Lung and Blood Institute Research Career Development Award (HL-00811).

References

1 Samuelsson, B , Goldyne, M , Granstrom, E , Hamberg, M , Hammarstrom, S and Malmsten, C (1978) Annu Rev Biochem 47, 997—1029
2 Shimizu, T , Mizuro, N , Amano, T and Hayaishi, O (1979) Proc Natl Acad Sci U S A 76, 6231—6234
3 Shimizu, T , Yamamoto, S and Hayaishi, O (1979) J Biol Chem 254, 5222—5228
4 Oelz, O , Oelz, R , Knapp, H R , Sweetman, B J and Oates, J A (1977) Prostaglandins 13, 225—234
5 DiMinno, G , Silver, M J and DeGaetano, G (1979) Br J Haematol 43, 637—647
6 Wong, P Y -K , McGiff, J C , Sun, F F, and Lee, W H (1979) Eur J Pharmacol 60, 245—248
7 Ellis, C K , Smigel, M D , Oates, J A , Oelz, O and Sweetman, B J (1979) J Biol Chem 254, 4152—4163
8 Reingold, D F , Kawasaki, A and Needleman, P (1981) Biochim Biophys Acta 659, 179—188
9 Wallace, R W , Lynch, T J , Tallant, A A and Cheung, W Y (1978) J Biol Chem 254, 377—382
10 Laemmli, U K (1970) Nature 227, 680—685
11 Lowry, O H , Rosebrough, N J , Farr, A L and Randall, R J (1951) J Biol Chem 193, 265—275
12 Wong, P Y -K , Lee, W H , Reiss, R F and McGiff, J C (1980) Fed Proc 39, 392
13 Marcus, A J (1978) J Lipid Res 19, 793—826
14 Johnson, R A , Morton, D R , Kinner, J H , Gorman, R , McGuire, J C , Sun, F F , Whittaker, N , Bunting, S , Salmon, J , Moncada, S and Vane, J R (1976) Prostaglandins 12, 915—928

15 Abdel-Halim, M S , Hamberg, M , Sjoquist, B and Anggard, E (1977) Prostaglandins 14, 633—643
16 Sun, F F , Chapman, J P and McGuire, J C (1977) Prostaglandins 14, 1055—1074
17 Hensby, C N (1974) Prostaglandins 8, 369—375
18 Wong, P Y -K , Terragno, D A , Terragno, N A and McGiff, J C (1977) Prostaglandins 13, 1113—1125
19 Sun, F F , Armour, S B , Bockstanz, V R and McGuire, J C (1976) Adv Prostaglandin Thromboxane Res 2, 163—169
20 Christ-Hazelhof, E and Nugteren, D H (1979) Biochim Biophys Acta 572, 43—51