Volume 320, number 2, 107–110 FEBS 12275 April 1993

Kinetic mechanism of ketoreductase activity of prostaglandin F synthase from bovine lung

Oleg A. Barski and Kikuko Watanabe

Department of Enzyme and Metabolism, Osaka Bioscience Institute, 6-2-4 Furuedai, Suita, Osaka 565, Japan

Received 4 January 1993; revised version received 17 February 1993

The kinetic mechanism of ketoreductase activity of bovine lung prostaglandin F synthase, expressed in *E. coli*, was investigated. Data on initial velocity and radioisotope exchange between [^{3}H]prostaglandin D_2 and 9α,11β-prostaglandin F_2 suggest that the enzyme obeys the ping-pong mechanism. Using a fluorescence technique we obtained a binding constant of 3 μM for NADPH. This is in close correlation with the kinetically determined intrinsic Michaelis constant for NADPH. Activation energy of the redox process was determined from the temperature dependence of maximal velocities for nitrobenzaldehyde and menadione and was found to be 119 and 96 kJ/mol, respectively.

Ping-pong mechanism; Kinetics; Prostaglandin F synthase; Binding constant; Aldo-keto reductase; NADPH

1. INTRODUCTION

Prostaglandin (PG) $F_{2\alpha}$ is widely distributed in various mammalian organs and shows a variety of biological activities. PGF synthase from bovine lung is a cytosolic monomeric protein with an M_r of about 37,000. It catalyzes the reduction of PGD_2 to 9α,11β-PGF_2 and that of PGH_2 to $PGF_{2\alpha}$ and prefers NADPH to NADH as a reducing agent [1]. It was shown that the enzyme possesses two different activities. PGH_2 possessing the 9,11-endoperoxide group is reduced at one active site. The reduction of the keto group of PGD_2 as well as various carbonyl compounds such as phenanthrenequinone, nitroacetophenone, nitrobenzaldehyde, and menadione to hydroxyl groups takes place at another active site [1]. At present, the amino acid sequence of the enzyme is known [2], and the recombinant protein has been expressed in *E. coli* [3]. However, little is known about the mechanism of action of this enzyme. In the present work the kinetic properties of recombinant PGF synthase are investigated with particular emphasis on the ketoreductase activity of the enzyme.

2. MATERIALS AND METHODS

2.1. *Materials*

[5,6,8,9,12,14,15-^{3}H(N)]PGD_2 was purchased from DuPont-New England Nuclear. 4-Nitroacetophenone was from Nacalai Tesque, and 4-nitrobenzaldehyde and potassium phosphates were from Wako Pure Chemical Industries. NADPH was obtained from Sigma, and PGs were kindly donated by Ono Pharmaceutical Co.

Correspondence address: K. Watanabe, Department of Enzyme and Metabolism, Osaka Bioscience Institute, 6-2-4 Furuedai, Suita, Osaka 565, Japan. Fax: (81) (6) 872 4818.

Abbreviation: PG, prostaglandin.

2.2. *Enzyme purification*

Recombinant PGF synthase was expressed in *E. coli* and purified as described previously [3].

2.3. *Radioisotope exchange*

The exchange reaction between [^{3}H]PGD_2 and 9α,11β-PGF_2 was assayed in a mixture containing 100 mM potassium phosphate buffer (pH 6.5), 0.2 μCi [^{3}H]PGD_2, various concentrations of cold PGD_2, 1 mM 9α,11β-PGF_2, and enzyme in a total volume of 50 μl. The reaction was initiated by addition of the enzyme, and the mixture was incubated at 37°C for the desired time. Blanks contained the same mixture without enzyme. The reaction was stopped by the addition of 250 μl of cold diethyl ether/methanol/0.2 M citric acid mixture (30:4:1), and PGs were extracted into the organic phase. A 50-μl aliquot of the organic phase was then applied onto silica gel TLC plates (F254, Merck), and 9α,11β-PGF_2 was separated from PGD_2 in diethyl ether/ methanol/ acetic acid (90:2:0.1). Radioactivity was determined by liquid scintillation counting, and the rate of radioisotope exchange was calculated from the percentage of radioactivity found in 9α,11β-PGF_2.

2.4. *Enzyme assay*

Enzyme activity was measured at 37°C (except for temperature dependence studies) by monitoring with a double beam spectrophotometer Hitachi U-3200, the decrease in the absorbance at 340 nm due to the consumption of NADPH. The reaction was initiated by addition of the enzyme to the mixture containing 100 mM potassium phosphate buffer (pH 6.5), NADPH, and second substrate in a 0.5 ml volume. The reference cuvette contained the full mixture except enzyme, and enzyme activity was the difference between the rate observed for the full reaction mixture minus that of the blank. Buffers were degassed and equilibrated with argon prior to use to decrease the spontaneous oxidation of NADPH.

2.5. *Binding studies*

Titration of intrinsic fluorescence of the enzyme with NADPH was carried out on a Shimadzu RF-5000 spectrofluorophotometer. Various concentrations of NADPH were added to a 1.35 μM enzyme solution in 100 mM potassium phosphate buffer (pH 6.5) at 21°C.

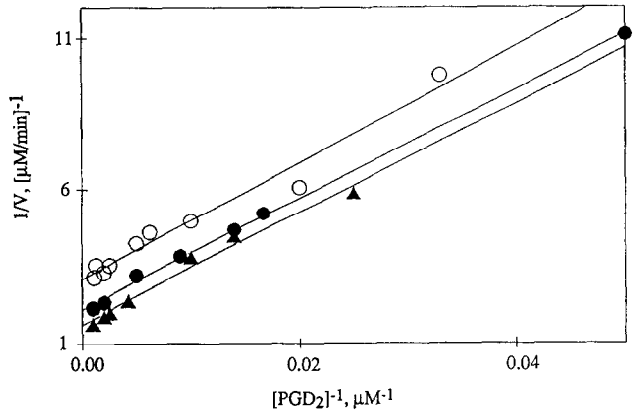

Fig. 1. $1/V$ versus $1/[PGD_2]$ under various NADPH concentrations. NADPH concentrations were: 3 μM (○), 10 μM (●), and 80 μM (▲).

Fluorescence was measured with excitation at 281 nm and emission at 330 nm. Each measurement was corrected for dilution effect. The correction factor of the inner filter effect was determined by titration of a tryptophan solution of approximately the same fluorescence as the enzyme with NADPH [4]. The difference between initial enzyme fluorescence intensity and corrected values in each point, ΔF, reflected the concentration of bound cofactor. The data were plotted according to the equation

$$\frac{1}{1-R} = \frac{L_0}{K_s R} - n\frac{E_0}{K_s},$$

where $R = \Delta F/\Delta F_{max}$, L_0 = total NADPH concentration, E_0 = total enzyme concentration, K_s = dissociation constant of PGF synthase – NADPH complex, and n = number of NADPH binding sites per one molecule of enzyme.

3. RESULTS

3.1. *Radioisotope exchange*

In order to establish the kinetic mechanism of ketoreductase activity of PGF synthase, the radioisotope exchange between [^{3}H]PGD_2 and 9α,11β-PGF_2 in the absence of $NADP^+$/NADPH was followed. Appearance of radio-label in PGF_2 under the action of enzyme would imply a ping-pong mechanism of catalysis. The PGF synthase spectrum taken at an enzyme concentration of 10 μM had a single maximum at 280 nm without shoulder at 260 nm or absorbance at 340 nm, thus indicating that the enzyme preparation was free of $NADP^+$/NADPH. As shown in Table I, radio-label transfer to 9α,11β-PGF_2 was proportional to the enzyme concentration, but the exchange did not proceed in the absence of cold 9α,11β-PGF_2. Accumulation of radio-label in 9α,11β-PGF_2 was time-dependent. Radioisotope exchange initial velocity exhibited Michaelis dependence with respect to PGD_2 concentration giving a K_m of 174 ± 33 μM when the 9α,11β-PGF_2 concentration was fixed at 1 mM.

3.2. *Initial velocity studies*

The double reciprocal plots of the initial velocity versus PGD_2 concentration at various fixed levels of NADPH showed a parallel pattern consistent with a ping-pong mechanism (Fig. 1). Secondary plots of V_m, K_m, and V_m/K_m versus NADPH concentration are shown in Fig. 2. From these plots the intrinsic K_m values of 3.4 ± 0.6 μM for NADPH and 141 ± 6 μM for PGD_2 were calculated; and the V_m/K_m for PGD_2 was independent on NADPH concentration.

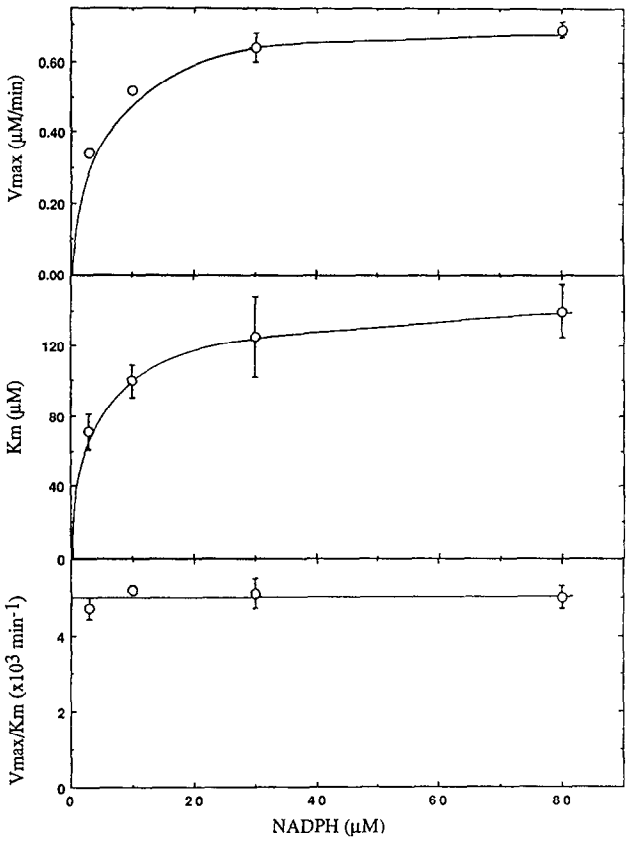

Fig. 2. Dependencies of maximal velocity (V_m) (upper panel), Michaelis constant (K_m) (middle panel), and V_m/K_m (lower panel) for PGD_2 on NADPH concentration. V_m, K_m, and V_m/K_m were calculated from the data of Fig. 1. The plot obtained at 30 μM NADPH was too close to that obtained at 80 μM, and therefore is not shown at Fig. 1.

4-Nitroacetophenone is another substrate reduced by the enzyme at the same active site as used for PGD_2. The initial velocity of nitroacetophenone reduction versus NADPH concentration plots in double reciprocal coor-

Table I

Velocity of radioisotope exchange between PGD_2 and 9α,11β-PGF_2 in the absence of $NADP^+$/NADPH. [^{3}H]PGD_2, 0.2 μCi; PGD_2, 30 μM; 9α,11β-PGF_2, 1 mM.

Condition	Velocity (nM/min)
Without enzyme	36
Enzyme 0.04 mg/ml	99
Enzyme 0.11 mg/ml	182
Without 9α,11β-PGF_2, enzyme 0.11 mg/ml	28

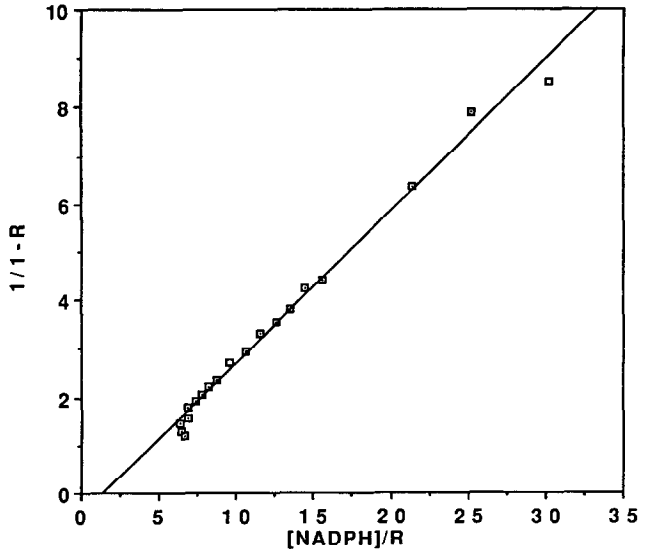

Fig. 3. Linear transformation of data on NADPH binding to PGF synthase. Experimental conditions and data processing are described in section 2.

dinates obtained at two concentrations of nitroacetophenone (2 and 60 μM) also exhibited a parallel pattern as in the case with PGD_2 (data not shown).

3.3. *NADPH binding studies*

In a separate experiment the affinity of enzyme for NADPH was measured by quenching of enzyme fluorescence. The result shown in Fig. 3 confirms the existence of only one NADPH binding site per enzyme molecule and indicates that K_s has a value of 3.0 ± 0.1 μM.

3.4. *Temperature dependence experiments*

4-Nitrobenzaldehyde and menadione are the best substrates for PGF synthase in terms of maximal velocity; activity of expressed enzyme towards menadione is twice that of nitrobenzaldehyde. The activation energy was determined from the temperature dependencies of catalytic constants for these two substrates. Concentrations of substrates and NADPH that greatly exceeded their Michaelis constants were used. Substrate concentration dependencies were checked at several temperatures to demonstrate that the chosen conditions represented maximal velocity. The temperature dependence of initial velocity of nitrobenzaldehyde reduction is presented in Fig. 4. Activation energy values were calculated to be 119 ± 1 kJ/mol for nitrobenzaldehyde and 96 ± 12 kJ/mol for menadione (data not shown).

4. DISCUSSION

The radioisotope exchange and kinetic data are both consistent with the ping-pong mechanism for the ketoreductase activity of PGF synthase. The K_s value for NADPH (3.0 ± 0.1 μM) is quite close to the kinetically determined intrinsic Michaelis constant (3.4 ± 0.6 μM). The K_m value for PGD_2 obtained in a radioisotope exchange experiment (174 ± 33 μM) is a good approximation for PGD_2 binding constant, provided that the concentration of 9α,11β-PGF_2 used is close to saturation. (In the radioisotope exchange intrinsic K_m is equal to K_s due to the simplification of the kinetic scheme.)

A ping-pong mechanism implies that there is a group in the enzyme molecule capable of changing its oxidation state in the course of the reaction. NADH/NADPH-dependent oxidoreductases obeying the ping-pong mechanism usually contain flavin or metal cofactors in their structure. Dihydropyrimidine dehydrogenase from pig liver and glutamate synthase from *Azospirillum brasilense*, which contain FAD, FMN, and iron-sulfur centers [5,6], and FMN-dependent NADH-quinone reductase from *E. coli* [7] are examples of such enzymes. However, it is remarkable that no such cofactor was reported for PGF synthase. Addition of FAD or FMN to a concentration of up to 100 μM did not alter the enzyme activity (data not shown). At the same time the turnover number of PGF synthase (4.1 min^{-1} for PGD_2) is much less than that of most enzymes, including the previously mentioned flavin-dependent oxidoreductases (e.g. 3,400 min^{-1} for glutamate synthase [6], 90 min^{-1} for dihydropyrimidine dehydrogenase [5]).

Rather high activation energies were determined from the temperature dependencies of the 4-nitrobenzaldehyde and menadione maximal velocities (119 and 96 kJ/mol, respectively). From this we infer that high activation energy of the redox process is characteristic of the PGF synthase reaction mechanism and probably accounts for the low catalytic constant observed for this enzyme.

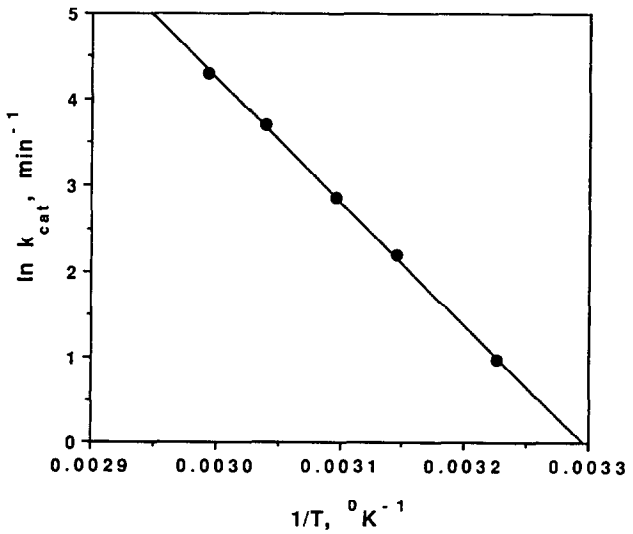

Fig. 4. Temperature dependence of catalytic constant for 4-nitrobenzaldehyde in Arrhenius coordinates. Experimental conditions: nitrobenzaldehyde, 1 mM; NADPH, 80 μM; PGF synthase, 6.2 μg/ml.

Acknowledgements: We are grateful to Professor Osamu Hayaishi for active discussion of the results and critical reading of this manuscript. This investigation was supported in part by research grants from the Ministry of Education, Science, and Culture of Japan; the Japan Foundation for Applied Enzymology; and the Japan Society for the Promotion of Science. Oleg Barski is a recipient of the Japan Society for the Promotion of Science fellowship.

REFERENCES

[1] Watanabe, K., Yoshida, R., Shimizu, T. and Hayaishi, O. (1985) J. Biol. Chem. 260, 7035–7041.
[2] Watanabe, K., Fujii, Y., Nakayama, K., Ohkubo, H., Kuramitsu, S., Kagamiyama, H., Nakanishi, S. and Hayaishi, O. (1988) Proc. Natl. Acad. Sci. USA 85, 11–15.
[3] Watanabe, K., Fujii, Y., Ohkubo, H., Kuramitsu, S., Kagamiyama, H., Nakanishi, S. and Hayaishi, O. (1991) Biochem. Biophys. Res. Commun. 181, 272–278.
[4] Birdsall, B., King, R.W., Wheeler, M.R., Lewis, C.A., Goode, S.R., Dunlap, R.B. and Roberts, G.C.K. (1983) Anal. Biochem. 132, 353–361.
[5] Podschun, B., Cook, P.F. and Schnackerz, K.D. (1990) 265, 12966–12972.
[6] Vanoni, M.A., Nuzzi, L., Rescigno, M., Zanetti, G. and Curti, B. (1991) Eur. J. Biochem. 202, 181–189.
[7] Hayashi, M., Hasegawa, K., Oguni, Y. and Unemoto, T. (1990) Biochim. Biophys. Acta 1035, 230–236.