

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

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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 [^3H]prostaglandin D_2 and $9\alpha,11\beta$ -prostaglandin F_2 suggest that the enzyme obeys the ping-pong mechanism. Using a fluorescence technique we obtained a binding constant of $3\ \mu\text{M}$ 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) $\text{F}_{2\alpha}$ 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 $9\alpha,11\beta$ -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- ^3H (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.

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 [^3H] PGD_2 and $9\alpha,11\beta$ -PGF $_2$ was assayed in a mixture containing 100 mM potassium phosphate buffer (pH 6.5), 0.2 μCi [^3H] PGD_2 , various concentrations of cold PGD_2 , 1 mM $9\alpha,11\beta$ -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 $9\alpha,11\beta$ -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 $9\alpha,11\beta$ -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.

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Abbreviation: PG, prostaglandin.

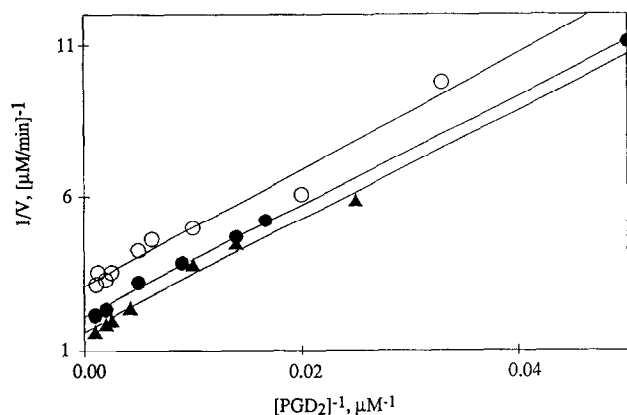


Fig. 1. $1/V$ versus $1/[PGD_2]$ under various NADPH concentrations. NADPH concentrations were: $3 \mu M$ ($\circ$), $10 \mu M$ ($\bullet$), and $80 \mu M$ ($\blacktriangle$).

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 $[^3H]PGD_2$ and $9\alpha,11\beta$ -PGF₂ in the absence of $NADP^+/NADPH$ was followed. Appearance of radio-label in PGF₂ under the action of enzyme would imply a ping-pong mechanism of catalysis. The PGF synthase spectrum taken at an enzyme concentration of $10 \mu M$ 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 $9\alpha,11\beta$ -PGF₂ was proportional to the enzyme concentration, but the exchange did not proceed in the absence of cold $9\alpha,11\beta$ -PGF₂. Accumulation of radio-label in $9\alpha,11\beta$ -PGF₂ was time-dependent. Radioisotope exchange initial velocity exhibited Michaelis dependence with respect to PGD_2 concentration giving a K_m of $174 \pm 33 \mu M$ when the $9\alpha,11\beta$ -PGF₂ 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

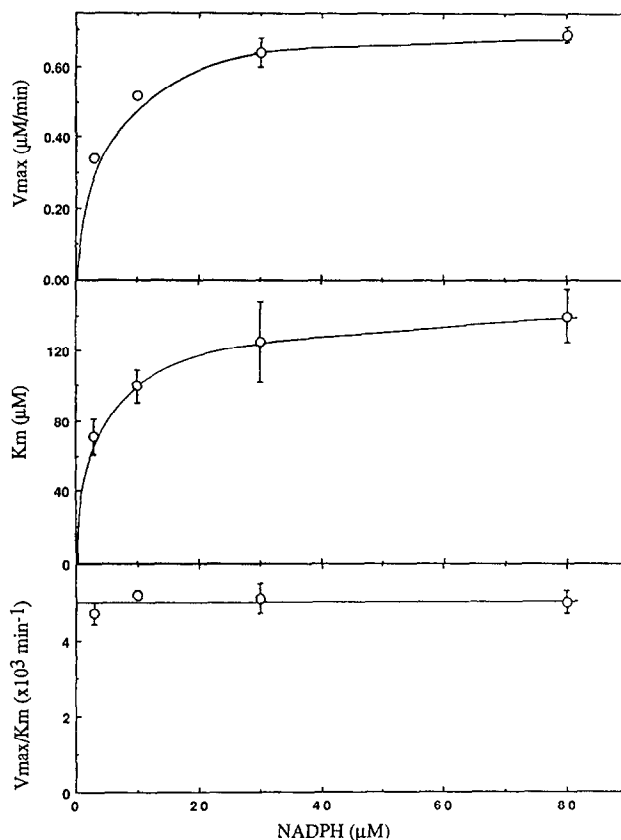


Fig. 2. Dependencies of maximal velocity (V_m) (upper panel), Michaelis constant (K_m) (middle panel), and V_m/K_m (lower panel) for PGD_2 on NADPH concentration. V_m , K_m , and V_m/K_m were calculated from the data of Fig. 1. The plot obtained at $30 \mu M$ NADPH was too close to that obtained at $80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

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 $3.4 \pm 0.6 \mu M$ for NADPH and $141 \pm 6 \mu M$ for PGD_2 were calculated; and the V_m/K_m for PGD_2 was independent on NADPH concentration.

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 $9\alpha,11\beta$ -PGF₂ in the absence of $NADP^+/NADPH$. $[^3H]PGD_2$, $0.2 \mu Ci$; PGD_2 , $30 \mu M$; $9\alpha,11\beta$ -PGF₂, 1 mM.

Condition	Velocity (nM/min)
Without enzyme	36
Enzyme 0.04 mg/ml	99
Enzyme 0.11 mg/ml	182
Without $9\alpha,11\beta$ -PGF ₂ , enzyme 0.11 mg/ml	28

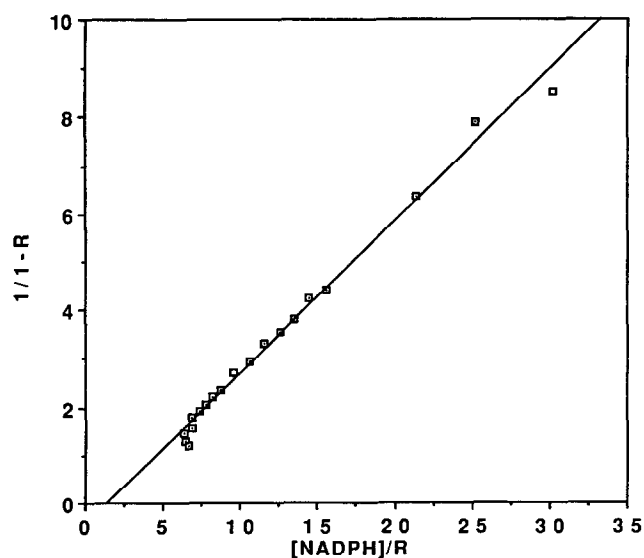


Fig. 3. Linear transformation of data on NADPH binding to PGF synthase. Experimental conditions and data processing are described in section 2.

dinates obtained at two concentrations of nitroacetophenone (2 and 60 μM) also exhibited a parallel pattern as in the case with PGD₂ (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 $3.0 \pm 0.1 \mu\text{M}$.

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 $119 \pm 1 \text{ kJ/mol}$ for nitrobenzaldehyde and $96 \pm 12 \text{ kJ/mol}$ 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 ($3.0 \pm 0.1 \mu\text{M}$) is quite close to the kinetically determined intrinsic Michaelis constant ($3.4 \pm 0.6 \mu\text{M}$). The K_m value for PGD₂ obtained in a radioisotope exchange experiment ($174 \pm 33 \mu\text{M}$) is a good approximation for PGD₂ binding constant, provided that the concentration of 9 α ,11 β -PGF₂ 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 PGD₂) is much less than that of most enzymes, including the previously mentioned flavin-dependent oxidoreductases (e.g. $3,400 \text{ min}^{-1}$ 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.

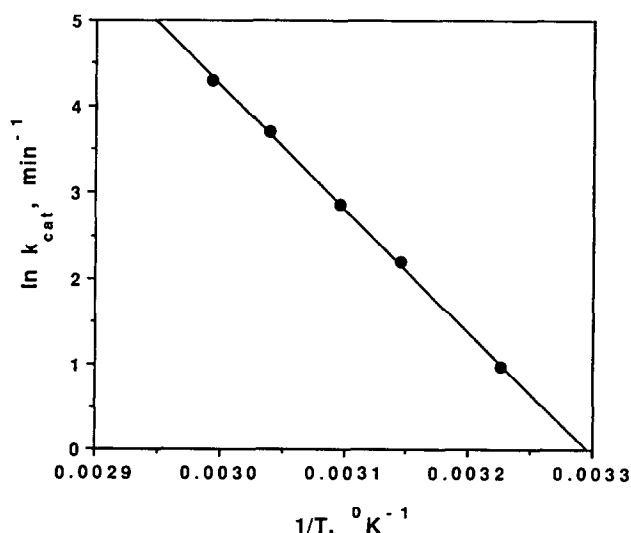


Fig. 4. Temperature dependence of catalytic constant for 4-nitrobenzaldehyde in Arrhenius coordinates. Experimental conditions: nitrobenzaldehyde, 1 mM; NADPH, 80 μM ; PGF synthase, 6.2 $\mu\text{g/ml}$.

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