Biochimica et Biophysica Acta, 614 (1980) 14—24

BBA 69017

NAD^+-DEPENDENT 15-HYDROXYPROSTAGLANDIN DEHYDROGENASE FROM PORCINE KIDNEY

II. KINETIC STUDIES

DIANA T.-Y. KUNG-CHAO and HSIN-HSIUNG TAI *

Department of Biochemistry, North Texas State University Health Science Center/ Texas College of Osteopathic Medicine, Fort Worth, TX 76107 (U.S.A.)

(Received December 19th, 1979)

Key words: 15-Hydroxyprostaglandin dehydrogenase; NAD^+ dependence; (Porcine kidney)

Summary

The kinetic mechanism of porcine renal NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase (11α,15-dihydroxy-9-oxoprost-13-enoate:NAD^+ 15-oxidoreductase, EC 1.1.1.141) was investigated. Initial velocity studies gave intersecting double reciprocal plots that conform to a sequential mechanism. Product inhibition studies indicated that 15-keto-prostaglandin E_2 exhibited linear non-competitive inhibition with respect to either prostaglandin E_2 or NAD^+, and NADH yielded linear competitive inhibition with respect to NAD^+. Dead-end inhibition studies showed that adenosine-5′-diphosphoribose inhibited the enzyme competitively with respect to NAD^+ as expected, but inhibited the enzyme non-competitively with respect to prostaglandin E_2. Alternate substrate studies indicated that a mixture of 3-acetyl-NAD^+ and NAD^+ gave a concave upward double reciprocal plot, while a mixture of prostaglandin E_2 and prostaglandin $F_{2\alpha}$ yielded a linear plot. These results are consistent with an ordered Bi—Bi mechanism where NAD^+ is added first, followed by prostaglandin E_2, and 15-keto-prostaglandin E_2 is released, followed by NADH.

Introduction

NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase (11α,15-dihydroxy-9-oxoprost-13-enoate:NAD^+ 15-oxidoreductase, EC 1.1.1.141)

* To whom correspondence should be addressed at Department of Biochemistry, NTSU/TCOM, P.O. Box 13048, Denton, TX 76203, U.S.A.

catalyzes the oxidation of 15(S)-hydroxyl group of prostaglandins to a keto group [1]. This reaction has been considered a key step in the control of the biological inactivation of prostaglandins [2]. Mammalian kidneys have been shown to be a very active organ in catabolizing prostaglandins [3—5]. It will be of great interest to determine the relative importance of substrate, coenzyme and effectors in regulating prostaglandin catabolism. With the availability of a highly purified porcine renal enzyme as reported in the preceding paper [6], this study was initiated to elucidate the kinetic properties of this enzyme. The work reported here involves the determination of initial velocity, product inhibition and dead-end inhibition, as well as alternate substrate and coenzyme studies. The enzyme seems to be very similar to that from human placenta in its kinetic properties [7—9].

Materials and Methods

NAD^+, NADH, DL-dithiothreitol, adenosine-5′-diphosphoribose, 3-acetyl-NAD^+, α-ketoglutarate monosodium salt, bovine serum albumin, bovine liver L-glutamic dehydrogenase (L-glutamate:NAD^+ oxidoreductase (deaminating), EC 1.4.1.2, 0.5 U/mg) and β-galactose dehydrogenase (D-galactose:NAD^+ 1-oxidoreductase, EC 1.1.1.48, 5 U/mg) were obtained from Sigma Chemical Co. St. Louis, MO, U.S.A. Norit A (neutral) was supplied by Amend Drug and Chemical Co., Irvington, NJ, U.S.A. D-[1-^{3}H]Galactose (14.2 Ci/mmol) was obtained from the England Nuclear, Boston, MA, U.S.A. Prostaglandin E_2, prostaglandin $F_{2\alpha}$ and 15-keto-prostaglandin E_2, were kind gifts from Dr. John Pike of the Upjohn Co., Kalamazoo, MI, U.S.A. 15(S)-[15-^{3}H]-Prostaglandin E_2 and 15(S)-[15-^{3}H]-prostaglandin $F_{2\alpha}$ were prepared by stereospecific transfer of the tritium label of D-[1-^{3}H]galactose to prostaglandins by coupling 15-hydroxyprostaglandin dehydrogenase with β-D-galactose dehydrogenase as described by Tai [4].

Preparation of NAD^+-dependent-15-hydroxyprostaglandin dehydrogenase

The enzyme was purified from porcine kidney through the Affi-gel Blue affinity chromatography as described in the preceding paper [6]. NADH was removed from the purified preparation by chromatography on a Sephadex G-25 column prior to use.

Enzyme assay

Two standard assay methods were described in the following. The concentrations of NAD^+ and prostaglandin E_2 were varied depending on the type of kinetic study performed.

Method A. Enzyme activity was determined by measuring the transfer of tritium from 15(S)-[15-^{3}H]-prostaglandin E_2 to glutamate by coupling with glutamate dehydrogenase according to Tai [4]. The incubation mixture contained 5 μmol NH_4Cl, 1 μmol monosodium α-keto-glutarate, 1 μmol NAD^+, 1 nmol 15(S)-[15-^{3}H]-prostaglandin E_2 (20 000 cpm), 100 μg (excess) glutamate dehydrogenase and an appropriate amount of 15-hydroxyprostaglandin dehydrogenase in a final volume of 1 ml of 50 mM potassium phosphate buffer (pH 7.5). The reaction was initiated by the addition of

15-hydroxyprostaglandin dehydrogenase and allowed to proceed for 5 min at 37°C. The reaction was terminated by the addition of 0.2 ml of 10% charcoal suspension in 1% dextran solution. The reaction mixture was centrifuged at 1000 × *g* for 8 min after standing for 10 min at room temperature. The supernatant was decanted and the radioactivity was determined by liquid scintillation counting. The amount of substrate oxidized was calculated on the assumption that no kinetic isotopic effect was involved in the removal of 15(S)-tritium during oxidation of labeled substrate.

Method B. Enzyme activity was determined by following the formation of NADH spectrophotometrically. The reaction mixture contained 1 μmol NAD^+, 28 nmol prostaglandin E_2 and enzyme in a final volume of 1 ml of 50 mM potassium phosphate buffer (pH 7.5). The reaction mixture was incubated at 25°C and the NADH formed was recorded by the increase in absorbance at 340 nm, using a Gilford 250 spectrophotometer attached to a recorder.

Kinetic determinations

All kinetic measurements, including initial velocity, product inhibition (except NADH as a product), dead-end inhibition and alternate substrate studies were carried out by the rapid enzyme assay Method A. When NADH was used as a product to carry out product inhibition studies, Method B was employed since the reaction mixture in Method A contained a NAD^+ regenerating system. Each reaction was initiated by the addition of enzyme to the reaction mixture containing varying amounts of substrate or coenzyme with or without inhibitors. 1—2 μg of purified enzyme was used per assay.

Data processing

The nomenclature used herein is that of Cleland [10,11]. Reciprocal velocities were plotted graphically against the reciprocal of substrate concentrations. Reciprocal plots of initial velocity, product inhibition, and dead-end inhibition data were examined to determine the pattern (i.e. intersecting, competitive inhibition, etc), and the slope and intercepts were plotted graphically against either the reciprocal of the non-varied substrate concentration (for initial velocity experiments), or the inhibitor concentration (for inhibition experiments), to determine the linearity of these replots. Data conforming to a sequential initial velocity pattern, a linear competitive inhibition pattern, a linear uncompetitive, and a linear non-competitive inhibition pattern were fitted to Eqn. 1, 2, 3 and 4, respectively.

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \tag{1}$$

$$v = \frac{VA}{K(1 + I/K_{is}) + A} \tag{2}$$

$$v = \frac{VA}{K + A(1 + I/K_{ii})} \tag{3}$$

$$v = \frac{VA}{K(1 + I/K_{is}) + A(1 + I/K_{ii})} \tag{4}$$

$$F = \sum_{i=1}^{N} \left[\frac{Y_{i,\text{exp}} - Y_{i,\text{calcd}}}{\sigma_i} \right]^2 \tag{5}$$

In Eqn. 1, K_a and K_b are Michaelis constants for substrates A and B, respectively, and K_{ia} is the dissociation constant for substrate A. In Eqn. 2—4, K_{is} and K_{ii} are apparent inhibition constants for slope and intercept. Curve fitting to these equations was done by a FORTRAN program package VINIT, which utilizes the nonlinear regression subroutine STEPIT [12] to minimize a weighted sum of squares, F, defined by Eqn. 5, where $Y_{i,exp}$ is the measured velocity, $Y_{i,calcd}$ is the computed velocity, N is the number of points and σ_i is the standard deviation in measured velocity [13].

Results

Initial velocity studies

The initial velocity patterns for the forward reaction are shown in Figs. 1 and 2. When NAD^+ was plotted as the variable substrate, with different concentrations of prostaglandin E_2 as the changing fixed substrate (Fig. 1), an intersecting pattern in the double reciprocal plot was obtained. When prostaglandin E_2 was plotted as the variable substrate, with different concentrations of NAD^+ as the changing fixed substrate, an intersecting pattern was again observed (Fig. 2). When the data were fitted To Eqn. 1, the Michaelis constants for NAD^+ and prostaglandin E_2 were found to be 38.4 ± 6.4 μM and 1.58 ± 0.19 μM, respectively. The dissociation constant for NAD^+ was 81.6 ± 8.37 μM. That the dissociation constant is higher than the Michaelis constant for NAD^+ is indicated by the fact that the intersecting point is above the horizontal axis.

Product inhibition studies

With NAD^+ as the variable substrate, 15-keto-prostaglandin E_2 gave non-competitive inhibition (Fig. 3). Slope and intercept replots showed linear relationship (plots not shown). K_{is} and K_{ii} were calculated to be 32 ± 2.4 μM and 31 ± 2.37 μM, respectively. With prostaglandin E_2 as the variable substrate, 15-keto-prostaglandin E_2 also gave non-competitive inhibition (Fig. 4). Slope and intercept replots also showed linear relationship (plots not shown). K_{is} and K_{ii} were determined to be 21 ± 1.6 μM and 19.6 ± 1.5 μM, respectively. With NAD^+ as the varied substrate, NADH gave competitive inhibition (Fig. 5). Slope replot exhibited a linear relationship. K_{is} was determined to be 13.5 ± 1.1 μM. Since the available data from product inhibition studies posed at least two possibilities with respect to the order of addition of substrates and the order of release of products, dead-end inhibition and alternate substrate studies were performed.

Dead-end inhibition studies

Adenosine-5′-diphosphoribose (ADP-Rib), a moiety of NAD^+ and an inhibitor of a number of dehydrogenases was selected as the dead-end inhibitor. With NAD^+ as the variable substrate, ADP-Rib gave competitive inhibition (Fig. 6). Slope replot showed a linear function of ADP-Rib. With prostaglandin E_2 as the variable substrate, ADP-Rib gave non-competitive inhibition (Fig. 7). Slope and intercept replots were both found to be linear function of ADP-Rib. A summary of the kinetic constants for product and dead-end inhibitions and their kinetic patterns is shown in Table I.

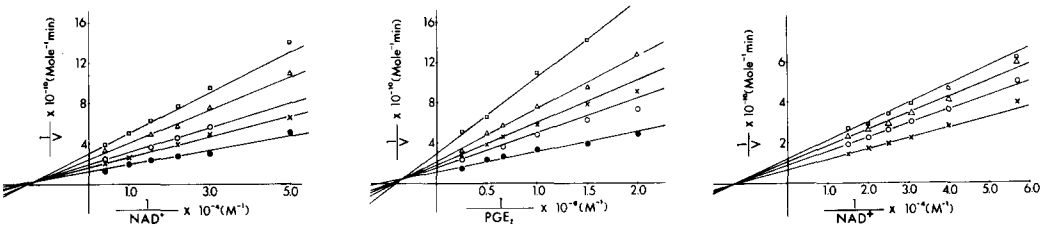


Fig. 1. Double reciprocal plot of initial velocity study with NAD^+ as the varied substrate at different fixed concentrations of prostaglandin E_2. The concentrations of NAD^+ was varied between 0.02 and 0.25 mM. The concentrations of prostaglandin E_2 were (μM) 0.75 (□), 1.0 (△), 1.5 (○), 2.0 (X) and 4.0 (●).

Fig. 2. Double reciprocal plot of initial velocity study with prostaglandin E_2 as the varied substrate at different fixed concentrations of NAD^+. The concentration of prostaglandin E_2 (PGE_2) was varied between 0.5 and 4.0 μM. The concentrations of NAD^+ were (mM) 0.020 (□), 0.033 (△), 0.045 (X), 0.065 (○) and 0.25 (●).

Fig. 3. Double reciprocal plot of product inhibition of 15-keto-prostaglandin E_2 with NAD^+ as the varied substrate. The concentration of NAD^+ was varied from 17.5 to 67.5 μM. The concentration of prostaglandin E_2 was kept constant at 2 μM. The concentrations of 15-keto-prostaglandin E_2 were none (X), 10.4 μM (○), 17.6 μM (△), 24.0 μM (□).

Alternate substrate studies

3-Acetyl-NAD^+ exhibited some coenzyme activity, as indicated in coenzyme specificity studies. When 3-acetyl-NAD^+ was mixed with NAD^+ at the same concentration (1 mM) and prostaglandin E_2 was varied in a wide range of concentrations, a concave upward curve with two different slopes was observed in a double reciprocal plot (Fig. 8). Extrapolation of two lines with different slopes

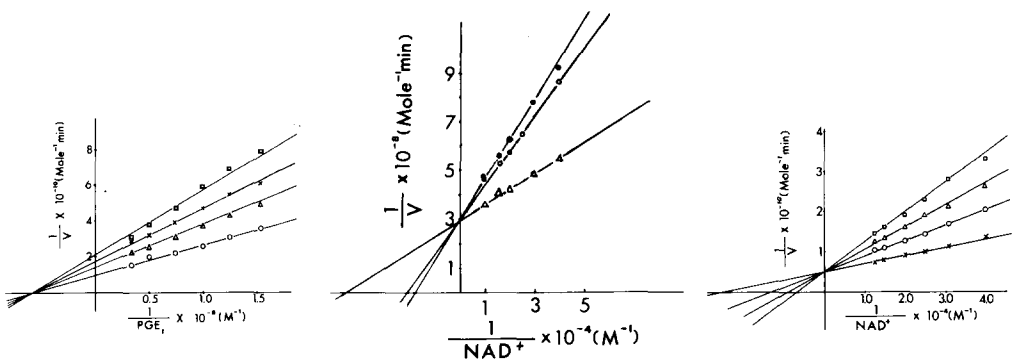


Fig. 4. Double reciprocal plot of product inhibition by 15-keto-prostaglandin E_2 with prostaglandin E_2 as the varied substrate. The concentration of prostaglandin E_2 (PGE_2) was varied from 0.65 to 3.0 μM. The concentration of NAD^+ was kept constant at 1 mM. The concentration of 15-keto-prostaglandin E_2 were none (○), 8.8 μM (△), 15.0 μM (X), 22.4 μM (□).

Fig. 5. Double reciprocal plot of product inhibition by NADH with NAD^+ as the varied substrate. The concentration of prostaglandin E_2 was kept constant at 28.3 μM. The concentrations of NADH were none (△), 15.6 μM (○), 22.2 μM (●). The enzyme was assayed under the conditions of Method B.

Fig. 6. Double reciprocal plot of dead end inhibition by ADP-Rib with NAD^+ as the varied substrate. The concentration NAD^+ was varied between 25 and 80 μM. The concentration of prostaglandin E_2 was kept constant at 2 μM. The concentrations of inhibitor used were none (X), 0.2 mM (○), 0.4 mM (△), 0.6 mM (□).

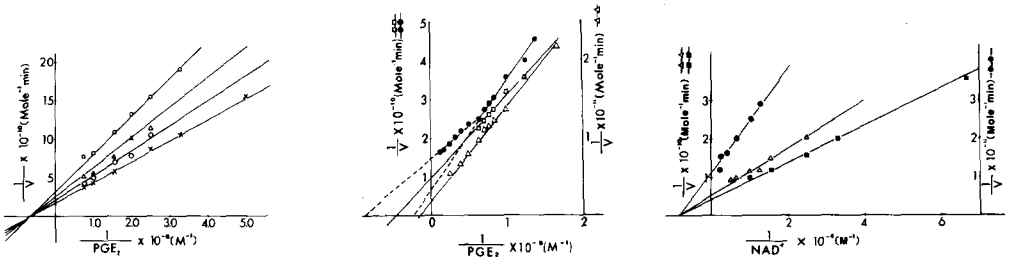


Fig. 7. Double reciprocol plot of inhibition by ADP-Rib with prostaglandin E_2 as the varied substrate. The concentration of prostaglandin E_2 (PGE_2) was varied between 0.2 and 1.35 μM. The concentration of NAD^+ was kept constant at 1.0 mM. The concentration of inhibitor used were none (X), 0.5 mM (○), 1.2 mM (△), 1.8 mM (□).

Fig. 8. Double reciprocal plot of mixed coenzyme studies. The concentrations of NAD^+ and 3-acetyl-NAD^+ used were both 1.0 mM (●———●). The concentration of prostaglandin E_2 (PGE_2) was varied between 0.6 and 10 μM. [15-^{3}H]-prostaglandin E_2 was 20 000 cpm/ml. When 1 mM NAD^+ was used as substrate, prostaglandin E_2 concentration was varied between 0.8 and 2 μM (□———□). When 1 mM 3-acetyl-NAD^+ was used as substrate, the concentration of prostaglandin E_2 was varied between 0.6 and 4 μM (△———△).

Fig. 9. Double reciprocal plot of mixed prostaglandin substrates study. The prostaglandin E_2 and prostaglandin $F_{2\alpha}$ concentrations were both at 1 μM. The specific activity of [15-^{3}H]-prostaglandin E_2 and [15-^{3}H]-prostaglandin $F_{2\alpha}$ were both at 20 000 cpm/nmol. When prostaglandin E_2 was used as substrate, NAD^+ concentration was varied between 15 and 100 μM (■———■). When prostaglandin $F_{2\alpha}$ was used as substrate NAD^+ concentration was varied between 80 and 500 μM (●———●). When prostaglandin E_2 and prostaglandin $F_{2\alpha}$ were mixed as substrates, NAD^+ concentration was varied between 40 and 200 μM (△———△).

intersected with the horizontal axis at two points which gave apparent K_m values of 4.0 μM and 1.1 μM for prostaglandin E_2, respectively.

When prostaglandin E_2 and prostaglandin $F_{2\alpha}$ were mixed at the same concentration (1 μM) and NAD^+ was varied in different concentrations, a linear plot was observed. The apparent K_m for NAD^+ determined with mixed substrates was identical with the apparent K_m for NAD^+ obtained by either using prostaglandin E_2 or prostaglandin $F_{2\alpha}$ as substrate as evidenced by three lines intersecting at the same point on the horizontal axis (Fig. 9).

TABLE I

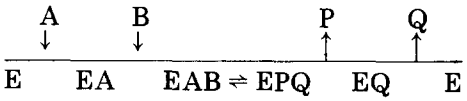
PRODUCT AND DEAD-END INHIBITION STUDIES

types: NC, non-competitive; C, competitive. Constants are expressed in μM. PG, prostaglanding

Varied	Inhibitors					
	15-keto-PGE_2		NADH		ADP-Rib	
	Type	Constants	Type	Constants	Type	Constants
NAD^+	NC	K_{is} = 31 ± 2.3 K_{ii} = 32 ± 2.4	C	K_{is} = 13.5 ± 1.1 —	C	K_{is} = 225.9 ± 30.1 —
PGE_2	NC	K_{is} = 19.6 ± 1.5 K_{ii} = 21.0 ± 1.6	—	— —	NC	K_{is} = 19.6 ± 1.5 K_{ii} = 21.0 ± 1.6

Discussion

The results of kinetic studies on the initial velocity, product inhibition, dead-end inhibition, and alternate substrates, are consistent with the following ordered Bi—Bi mechanism:



where NAD^+ (A) is added first, followed by prostaglandin E_2 (B) and 15-keto-prostaglandin E_2 (P) is then released, followed by NADH (Q).

The study of initial velocity patterns in which one substrate is varied at different fixed levels of the second substrate provides a means of distinguishing between sequential and non-sequential kinetic mechanisms. In sequential mechanisms, addition of substrates may be ordered or random, but all must be enzyme-bound before product release can occur. Mechanisms such as the 'ping-pong' type are non-sequential, and the first product is released before the second substrate is added. Double reciprocal plots which yield intersecting lines in the second quadrant are indicative of sequential mechanisms while plots which give parallel lines are consistent with the non-sequential mechanism [14].

Most sequential reactions conform to Eqn. 1, as formulated in the Materials and Methods section. Taking the reciprocal form of Eqn. 1, one obtains Eqns. 6 and 7. B is the changing fixed substrate and A is varied.

$$\frac{1}{v} = \frac{K_a}{V}\left(1 + \frac{K_{ia}K_b}{K_aB}\right)\left(\frac{1}{A}\right) + \left(1 + \frac{K_b}{B}\right)\frac{1}{V} \tag{6}$$

A is the changing fixed substrate when B is varied.

$$\frac{1}{v} = \frac{K_b}{V}\left(1 + \frac{K_{ia}}{A}\right)\frac{1}{B} + \frac{1}{V}\left(1 + \frac{K_a}{A}\right) \tag{7}$$

The slope of the two equations is

$$\frac{K_a}{V}\left(1 + \frac{K_{ia}K_b}{K_aB}\right) \quad \text{and} \quad \frac{K_b}{V}\left(1 + \frac{K_{ia}}{A}\right)$$

respectively, for A or B as the variable substrate.

These slopes are a function of the non-varied substrate. Therefore, intersecting reciprocal plots will be observed. Figs. 1 and 2 show intersecting patterns for varying either NAD^+ or prostaglandin E_2, and thus they are compatible with a sequential mechanism for this enzyme.

Eqn. 1 is given by any sequential mechanism which include Ordered, Theorell-Chance and Rapid Equilibrium Random. To elucidate the precise kinetic mechanism, it is necessary to conduct further kinetic investigations. Product inhibition, dead-end inhibition, and alternate substrate studies have been the basic tools to achieve the objective. For any sequential Bi—Bi mechanism, the rate equation can be derived from the basic rate equation for the

ordered mechanism, which is shown in Eqn. 8:

$$v = V(AB - PQ/K_{eq}) \; / K_{ia}K_b + K_bA + K_aB + AB$$

$$+ \frac{K_{ia}K_bQ}{K_{iq}} + \frac{K_{ia}K_bK_qP}{K_{iq}K_p} + \frac{K_{ia}K_bPQ}{K_pK_{iq}} + \frac{K_bK_qAP}{K_{iq}K_p} + \frac{K_aBQ}{K_{ip}} + \frac{ABP}{K_{ip}} + \frac{K_{ia}K_bBPQ}{K_pK_{iq}K_{ib}} \tag{8}$$

The rate equation for the Theorell-Chance mechanism (where there is no kinetically significant ternary complex) is the same except in that it lacks the denominator terms in *ABP* and *BPQ*, while that for Rapid Equilibrium Random (where the order of addition of A and B is not obligatory, but the rate limiting step is solely the conversion of EAB to EPQ) lacks both of the *AP* and *BQ* terms as well [14].

For product inhibition studies, the rate equations can be derived from Eqn. 8 by setting either *P* or *Q* equal to zero. After rearranging, we obtain the following three equations for the product inhibition studies reported in this paper.

Varying *A*, inhibit with *P*:

$$\frac{1}{v} = \frac{K_a}{V}\left(1 + \frac{K_{ia}K_b}{K_aB}\right)\left[1 + \frac{P}{\frac{K_pK_{iq}}{K_q}\left(1 + \frac{K_aB}{K_{ia}K_b}\right)}\right]\frac{1}{A} + \frac{1}{V}\left(1 + \frac{K_b}{B}\right)$$

$$\times \left[1 + \frac{P}{\left(1 + \frac{K_b}{B}\Big/\frac{1}{K_{ip}} + \frac{K_qK_b}{K_pK_{iq}B}\right)}\right] \tag{9}$$

Varying *B*, inhibit with *P*:

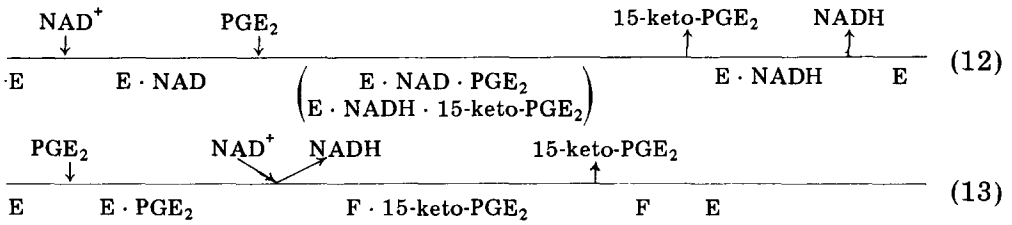
$$\frac{1}{v} = \frac{K_b}{V}\left(1 + \frac{K_{ia}}{A}\right)\left[1 + \frac{P}{\frac{K_pK_{iq}}{K_q}}\right]\frac{1}{B} + \frac{1}{V}\left(1 + \frac{K_a}{A}\right)\left[1 + \frac{P}{K_{ip}\left(1 + \frac{K_a}{A}\right)}\right] \tag{10}$$

Varying *A*, inhibit with *Q*:

$$\frac{1}{v} = \frac{K_a}{V}\left(1 + \frac{K_{ia}K_b}{K_{aB}}\right)\left(1 + \frac{Q}{K_{iq}}\right)\frac{1}{A} + \frac{1}{V}\left(1 + \frac{K_b}{B}\right) \tag{11}$$

Eqns. 9 and 10 predict a linear non-competitive inhibition, while Eqn. 11 predicts a linear competitive inhibition. The linear non-competitive inhibition patterns as seen by 15-keto-prostaglandin E_2 with respect to NAD^+ (Fig. 3) and prostaglandin E_2 (Fig. 4) conform to Eqns. 9 and 10. These data clearly rule out Rapid Equilibrium Random mechanism, since no competitive inhibition patterns was observed. The linear competitive inhibition pattern as observed by NADH with respect to NAD^+ (Fig. 5) conforms to Eqn. 11. The results of these product inhibition studies, however, do not distinguish between an Ordered

Bi—Bi mechanism (12) and and Iso-Theorell-Chance mechanism (13) where PG refers to prostaglandin).



Both mechanism, (12) and (13), will be expected to give linear non-competitive inhibition with respect to either substrate when 15-keto-prostaglandin E_2 is used as a product, and linear competitive inhibition with respect to NAD^+ when NADH is used as a product.

To differentiate between these two possible mechanisms, dead-end inhibition and alternate substrate studies were carried out. The use of dead-end inhibitor has been known to be particularly useful for determining the order of addition of substrates in cases where product inhibition studies cannot or do not give an unequivocal answer. When ADP-Rib was used as a dead-end inhibitor, it demonstrated a linear competitive inhibition with respect to NAD^+ (Fig. 6) and a linear non-competitive inhibition with respect to prostaglandin E_2 (Fig. 7). These patterns are consistent only with ordered addition of NAD^+ followed by prostaglandin E_2, as shown in the mechanism described by (12), since reverse order of addition of substrates will predict an uncompetitive inhibition with respect to prostaglandin E_2. The kinetic mechanism actually can be further supported by the use of dead-end inhibitors, competitive to prostaglandin E_2. This kind of inhibitor should yield uncompetitive pattern with respect to NAD^+. Unfortunately, no such kind of inhibitor has been found.

Furtherr substantiation of the kinetic mechanism of porcine renal enzyme being an Ordered Bi—Bi mechanism was provided by alternate substrate studies. The use of alternate substrate to elucidate the kinetic order of addition of substrates was first suggested by Wong and Hanes [15]. If a mixture of two A substrates is used, namely A_1 and A_2, and A is the first substrate on the enzyme, B will no have two enzymic forms (A_1E) and (A_2E) with which to react. The result will be a mechanism now second degree in B, and the standard plots will be curved unless A_1 and A_2 coincidentally have identical effect of K_m^B. If. however, A is the second substrate, B reacts only with free enzyme and no increase in degree results. This behavior permits the experimental distinction of the first and the second substrate. When a mixture of 3-acetyl-NAD^+ and NAD^+ was used, the double reciprocal plot of $1/v$ vs. 1/prostaglandin E_2 appeared to be concave upward; K_m values for prostaglandin E_2 determined from two apparent slopes were significantly different from that obtained by the presence of either coenzyme alone. Apparently, the affinity for prostaglandin E_2 is significantly altered in the presence of both nucleotides. In contrast, when a mixture of prostaglandin E_2 and prostaglandin $F_{2\alpha}$ was used, the double reciprocal plot of $1/v$ vs. $1/NAD^+$ did not depart from linearity. K_m values for NAD^+ were the same irrespective of the presence of prostaglandin E_2 and

prostaglandin $F_{2\alpha}$, either alone or in a mixture. Obviously, the affinity for NAD^+ is not affected by the presence of different substrates. These results clearly suggest that NAD^+ is the first substrate added, followed by prostaglandins.

Schlegel and Greep [8] and Rückrich et al. [9] have separately employed product inhibition studies to deduce the kinetic mechanism of human placental 15-hydroxyprostaglandin dehydrogenase. They found that NADH exerted a linear competitive inhibition with respect to NAD^+, and that 15-keto-prostaglandins E_1 and $F_{2\alpha}$ showed a linear non-competitive inhibition with respect to the corresponding prostaglandins. Based on these studies, they suggested an Ordered Bi—Bi mechanism in which NAD^+ was added first, followed by prostaglandins for human placental 15-hydroxyprostaglandin dehydrogenase. Critically, these product inhibition studies do not distinguish between an Ordered Bi—Bi mechanism (12), and an Iso-Theorell-Chance mechanism (13), as pointed out by Hansen [16]. Jarabak and Braithwaite [7] conducted a more detailed kinetic analysis of the mechanism of human placental 15-hydroxyprostaglandin dehydrogenase. They carried out product inhibition studies at two different pH values, and also employed alternate substrate studies. They have concluded that human placental 15-hydroxyprostaglandin dehydrogenase proceeds by a single displacement mechanism. Addition of the substrates is ordered, with NAD^+ binding first. The life-time of the ternary complex is affected by the pH of the reaction mixture. At pH 7.0 a kinetically significant ternary complex is formed, while at pH 9.0 the ternary complex is not kinetically significant (Theorell-Chance mechanism). The results of the present study on porcine renal 15-hydroxyprostaglandin dehydrogenase (conducted at pH 7.5) appeared to agree well with the mechanism proposed for human placental 15-hydroxyprostaglandin dehydrogenase at pH 7.0.

The significance of these kinetic studies can be manifold. The K_m values for prostaglandins were found to be much higher than the prostaglandin concentrations present in animal kidneys [17,18]. It is anticipated that renal 15-hydroxyprostaglandin dehydrogenase should operate at a reaction rate far below the maximum velocity with respect to prostaglandins. The K_m values for NAD^+ were much lower than the actual concentration of NAD^+ [19]. The potential reaction rate would be at near-maximal rate with respect to NAD^+. Regulation of renal 15-hydroxyprostaglandin dehydrogenase reaction rate by the renal levels of prostaglandins is much more likely than a regulation by the NAD^+/NADH relation. Furthermore, because of the high K_i of 15-keto-prostaglandin E_2, neither inhibition of the enzymatic reaction by 15-keto-prostaglandins nor a possible backward reaction seems to be of physiological significance.

Acknowledgements

We are indebted to Dr. John Pike of the Upjohn Company for his kind gifts of prostaglandins. We are also grateful to Dr. Ben Harris and Dr. Richard Thompson for their kind advice in the use of computer programs. This study was supported in part by grants from U.S. Public Health Service (GM-25247) and American Heart Association (78-865).

References

1 Anggard, E. and Samuelsson, B. (1964) J. Biol. Chem. 239, 4097—4102
2 Anggard, E. (1966) Acta Physiol. Scand. 66, 509—510
3 Anggard, E., Larsson, C. and Samuelsson, B. (1971) Acta Physiol. Scand. 81, 396—404
4 Tai, H.H. (1976) Biochemistry 15, 4586—4592
5 Moore, P.K. and Hoult, J.R.S. (1977) Biochim. Biophys. Acta 528, 276—287
6 Kung-Chao, D.T.Y. and Tai, H.H. (1980) Biochim. Biophys. Acta 614, 1—13
7 Jarabak, J. and Braithwaite, S.S. (1976) Arch. Biochem. Biophys. 177, 245—254
8 Schlegel, W. and Greep, R.O. (1975) Eur. J. Biochem. 56, 245—252
9 Rückrich, M.W., Wendel, A., Schlegel, W., Jackish, R. and Jung, A. (1975) Hoppe-Seyler's Z. Physiol. Chem. 356, 799—809
10 Cleland, W.W. (1963) Biochim. Biophys. Acta 67, 104—137
11 Cleland, W.W. (1963) Biochim. Biophys. Acta 67, 173—187
12 Chandler, J.P. (1971) STEPIT, Quantum Chemistry Program Exchange, Indiana University, Chemistry Department Program 66
13 Thompson, R.E., Spivey, H.O. and Katz, A.J. (1976) Biochemistry 15, 862—867
14 Cleland, W.W. (1963) Biochim. Biophys. Acta 67, 188—196
15 Wong, J.T. and Hanes, C.S. (1962) Can. J. Biochem. Physiol. 40, 763—804
16 Hansen, H.S. (1976) Prostaglandins 12, 647—679
17 Karim, S.M.M., Hillier, K. and Devlin, J. (1968) J. Pharm. Pharmacol. 20, 749—753
18 Van Dorp, D.A. (1971) Ann. N.Y. Acad. Sci. 180, 181—199
19 Villee, C.A. (1962) Biochem. J. 191—194