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ARABINOSE (FUCOSE) DEHYDROGENASE FROM PIG LIVER

II. STEADY-STATE KINETICS

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SUMMARY

1. The steady-state kinetics of arabinose (fucose) dehydrogenase from pig liver have been investigated at pH 10, 30 °C.

2. The mechanism is a sequential one and effectively proceeds to completion. Kinetic constants have been determined with D-arabinose and L-fucose as substrates.

3. Evidence obtained from product inhibition studies and the method of alternate substrates supports an ordered mechanism.

INTRODUCTION

A number of NAD- and NADP-dependent arabinose (fucose) dehydrogenase (EC 1.1.1.115 and EC 1.1.1.116) has been isolated and purified. Breusch [1] has shown an arabinose dehydrogenase in cat liver, and Metzger and Wick [2] have reported the partial purification of a NADP-dependent ADH from rat liver. Palleroni and Doudoroff [3] have isolated a NAD-dependent enzyme from the bacteria, *Pseudomonas saccharophilia*, and Schiwara et al. [4, 5] have established the presence of at least two such NAD-requiring enzymes in pig liver.

In fact, two arabinose (fucose) dehydrogenases from pig liver have been isolated and purified by Schachter et al. [6] and Maijub et al. [7]. The latter enzyme [7] has a molecular weight of 245 000 and a pH maximum of 10, which is similar to that obtained for glucose dehydrogenase [8] from the same source.

Metzger and Wick [2] have established that this enzyme is capable of catalyzing the oxidation of D-arabinose to arabonolactone.

This particular enzyme does not react with L-galactose as a substrate. It should also be mentioned that L-fucose (6-deoxy-1-galactose) is a sugar component of blood group substances, plasma glycoproteins and cell membranes. Elevated protein-bound fucose levels have been reported to occur in the plasma of diabetic patients. Little is known of the pathway of catabolism of this sugar [9].

In this work we report the kinetic analysis of the second arabinose (fucose) dehydrogenase of molecular weight 245 000 from pig liver [7]. The steady-state kinetic nomenclature developed by Cleland [10–12] is used throughout this report.

MATERIALS AND METHODS

Chemicals

Arabinose (fucose) dehydrogenase was prepared and purified by the method of Maijub et al. [7]. Chemicals were purchased as follows: NAD, NADH from California, Biochemical Company D-(−)-arabinose from Nutritional Biochemicals, D-arabonic acid γ-lactone from Alfred Bader Chemicals and Sephadex from Pharmacia.

D-Arabonolactone was prepared by air oxidation of D-fructose in 50% methanolic KOH, isolation of D-arabonic acid as the calcium salt, and dehydration of the free acid [13, 14]. L-Fuconic acid was prepared by oxidation of L-fucose (Nutritional Biochemicals Corporation, $[\alpha]_D^{20}$ 124.1 → −75.6 °) with bromine water [15]. The acidic solution was neutralized with $NaHCO_3$, acidified with oxalic acid, evaporated to dryness and heated at 75 °C for 15 h to give L-fuconolactone. δ-D-Gluconolactone, m.p. 155 °C, was recrystallized from absolute ethanol.

Assay procedure

The assay procedure is as follows: 2.6–2.8 ml of 0.05 M NaOH–glycine (1.0 mM EDTA) buffer (pH 10.0), varying amounts of 1.2 mM NAD (0.05–0.1 ml) and 0.1 ml enzyme solution (1.0 mg/ml) were added to two identical cuvettes. 0.05–0.2 ml deionized water was added to the blank cuvette and both cuvettes were incubated for 5 min in the chamber of a Gilford DU spectrophotometer connected to a constant temperature bath and circulator set at 30±0.1 °C. At the end of the incubation period, varying amounts of 1 M sugar solution (0.05–0.2 ml) were added to the sample cuvette. During the product inhibition studies, 0.04–0.1 ml of either NADH (1.5 mM) or lactone (1–4 M) was added to both cuvettes. Since the formation of reduced NAD produces an increase in absorbance at 340 nm, the reaction was followed with absorbance at 30 and 90 s after addition of the sugar solution. All activities were recorded as change in absorbance of 0.001/min. The values were later adjusted to the more recent definition of 1 unit of activity being the amount of protein causing the formation of 1 μmole of NADH per min. Protein concentrations were determined by 280/260-nm measurements after Warburg and Christian [16]. NAD concentrations were determined by assaying with alcohol dehydrogenase.

BASIC KINETIC MODELS

The steady state equations for two substrate reactions are readily obtained by the King-Altman [17] method using the criteria outlined by Cleland [10] for the various mechanisms. The mechanisms of choice to be initially considered in this study include the ping-pong mechanism, rapid equilibrium random and an ordered mechanism. The ordered mechanism can further be divided into a Theorell–Chance [18] model and a sequential model with a central ternary complex. The latter mechanism can be depicted as follows:

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA + B \underset{k_4}{\overset{k_3}{\rightleftharpoons}} (EAB\text{–}EQP) \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EQ + P \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E + Q \qquad (1)$$

where E, A, B, P, Q represent enzyme ,NAD, arabinose or fucose, arabinolactone or fuconolactone, and NADH respectively.

For initial velocity studies, Eqn 2 is used.

$$\frac{1}{v} = \frac{K_b}{V_1}\left(1 + \frac{K_{ia}}{A}\right)\frac{1}{B} + \frac{1}{V_1}\left(1 + \frac{K_a}{A}\right) \tag{2}$$

where K_a, K_b are Michaelis constants and K_{ia} is the NAD dissociation constant [10].

For the studies of product inhibition, the rate equation leads to Eqn 3 and 4 [10].

$$\frac{1}{v} = \frac{K_b}{V_1}\left(1 + \frac{K_{ia}}{A}\right)\left(1 + \frac{K_q P}{K_{iq}K_p}\right)\frac{1}{B} + \frac{1}{V_1}\left(1 + \frac{K_a}{A} + \frac{P}{K_{ip}}\right) \tag{3}$$

$$\frac{1}{v} = \frac{K_b}{V_1}\left[1 + \frac{K_{ia}}{A}\left(1 + \frac{Q}{K_{iq}}\right)\right]\frac{1}{B} + \frac{1}{V_1}\left[1 + \frac{K_a}{A}\left(1 + \frac{Q}{K_{iq}}\right)\right] \tag{4}$$

where K_q, K_p are Michaelis constants, K_{iq} and K_{ip} are inhibition constants [10].

RESULTS AND DISCUSSION

Initial velocity measurements in the absence of products

The dependence of the reaction rate on the concentration of D-arabinose and L-fucose was examined at several fixed levels of NAD. The concentrations of NAD used were 20, 25, 32.5 and 40 μM. For each level of NAD, the D-arabinose and L-fucose concentration was varied (2.67, 3.33, 4.33, 5.33, and 6.67 mM). Substrate saturation curves established the existence of substrate inhibition at concentrations greater than 10 mM in L-fucose and 50 mM in D-arabinose.

The Lineweaver–Burk [19] plots or reciprocal velocity versus reciprocal substrate concentration are shown in Figs 1 and 2 for D-arabinose, L-fucose and NAD respectively as the variable substrates. The fact that the lines intersect indicate that one of the possible mechanisms is ordered sequential or rapid equilibrium random with dead-end inhibitors [10–12, 20–22]. In any case, the ping-pong mechanism is completely ruled out by the intersecting lines in Figs 1 and 2. The data from each experiment (duplicate sets) was processed on an IBM 360 computer using a program

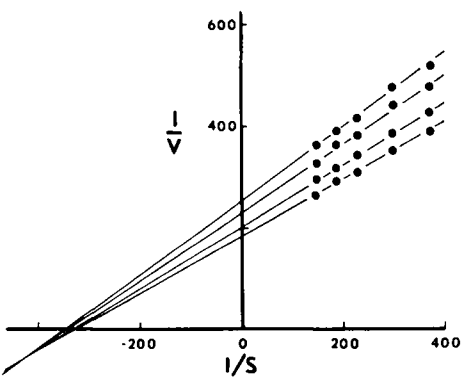

Fig. 1. $1/V$ (min/μM) vs 1/S (M^{-1}) plot for L-fucose reaction. NAD concn varied from 20 to 40 μM. L-Fucose concn varied from 2.67 to 6.67 mM.

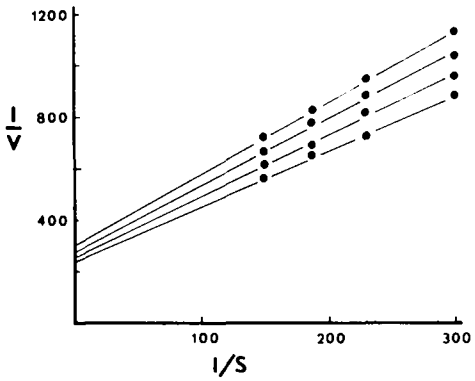

Fig. 2. $1/V$ (min/μM) vs $1/S$ (M^{-1}) plot for D-arabinose reaction. NAD concn varied from 20 to 40 μM. D-Arabinose concn varied from 2.67 to 6.67 mM.

developed by Cleland [23]. All data points were given equal weight after they were observed to fall within a 5% error limit during a test of linearity.

The overall results are given in Table I and are consistent with a sequential mechanism. To further check this hypothesis, we used a technique developed by Fromm [24] in which alternate substrates are used to choose between various mechanisms. In our case, this approach is an ideal one as both L-fucose and D-arabinose are active substrates.

TABLE I

KINETIC CONSTANTS

Constant*	Substrate	
	D-Arabinose	L-Fucose
$V_1(\times 10^6)$	14.0 (± 2.1)	28.2 (± 3.3) μmoles/min
K_a	8 (± 5) μM	25 (± 6) μM
K_b	6.02 (± 1.7) mM	3.5 (± 0.9) mM
K_{ia}	19 (± 6) μM	17 (± 6) μM
K_{iq}	21 (± 8) μM	25 (± 7) μM
E_o	0.135 μM	0.135 μM
$K_{ib} = K_{ia} K_b/K_a$	13.9 (± 8.7) mM	2.4 (± 0.8) mM
k_1	$4.3 \cdot 10^4$ (± $1.2 \cdot 10^3$)$min^{-1} \cdot M^{-1}$	$2.8 \cdot 10^4$ (± $1.4 \cdot 10^3$)$min^{-1} \cdot M^{-1}$
k_2	0.082 (± 0.026) min^{-1}	0.048 (± 0.022)min^{-1}

* For details, see Cleland, ref. 10.

The first test in this technique is the variation of NAD while maintaining a constant ratio of L-fucose and D-arabinose. In this particular experiment, the NAD concentration was varied from 18 to 42 μM. The concentrations of each sugar were either 1.67 or 3.33 mM. The results at pH 10 and 30 °C are given in Fig. 3a. The linear plots indicate that an ordered pathway prevails and that further consideration of a rapid equilibrium random mechanism can be kept at a minimum.

The next step in this test is to reprocess our original data in terms of a Theorell–Chance mechanism and evaluate the rate constant, k_3, for each substrate separately.

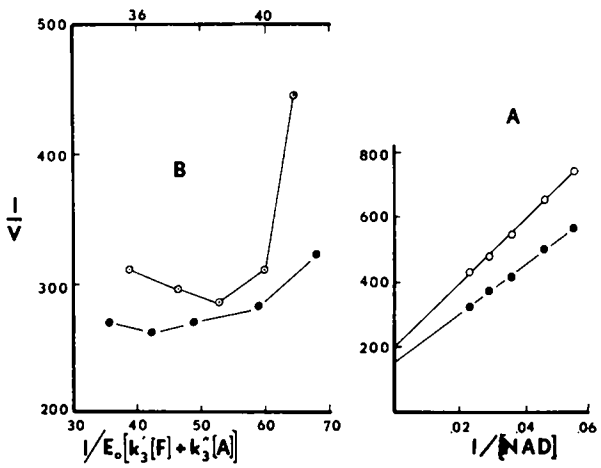

Fig. 3. A. Mixed Substrate, (●), 3.33 mM in D-arabinose and L-fucose; (○), 1.67 mM in D-arabinose and L-fucose. NAD concn varied from 18 to 42 μM. B. Plot of Eqn 5 for L-fucose (○) and D-arabinose (●) concn at 6.67 mM. NAD concn is 40 μM in each case. In (○), D-arabinose is varied from 2.67 to 6.67 mM whereas in (●), L-fucose is varied from 2.67 to 6.67 mM.

These were determined to be $2.77 \cdot 10^{-4}$ $\mu M^{-1} \cdot min^{-1}$ for D-arabinose and $10.5 \cdot 10^{-4}$ $\mu M^{-1} \cdot min^{-1}$ for L-fucose. Having accomplished this, each substrate is then varied at a fixed level of the other. In our experiment, the L-fucose concentration was 6.67 mM, the NAD concentration was 40 μM and the D-arabinose was varied from 2.67 to 6.67 mM. The same experiment was repeated in which the D-arabinose concentration was 6.67 mM, the NAD concentration was 40 μM and the L-fucose was varied from 2.67 to 6.67 mM. Eqn 5 was then tested

$$\frac{1}{V} = 1/E_0 \, [k_3' \text{ (fucose)} + k_3'' \text{ (arabinose)}] \tag{5}$$

for linearity as shown in Fig. 3b. In this figure the non-linearity indicates that the Theorell–Chance mechanism is inappropriate. In view of the above, we will assume for the present that the ordered pathway given by Eqn 1 is representative of this sytem.

PRODUCT INHIBITION

NADH inhibition

When NADH was used as the product inhibitor, noncompetitive inhibition [11] was observed with NAD at an unsaturating level as shown in Figs 4 and 5. A computer analysis [23] was also consistent with these observations, as the data gave inconsistent results when analyzed as any other form of inhibition. Furthermore, when NAD was present at a saturating level (500 μM), NADH was not inhibitory. These results are consistent with the prediction of Eqn 4 and identifies NADH as the second product released from enzyme. A note of caution should be inserted at this point, as these same results would also be obtained if a rapid-equilibrium-random mechanism with dead-end complexes were operative.

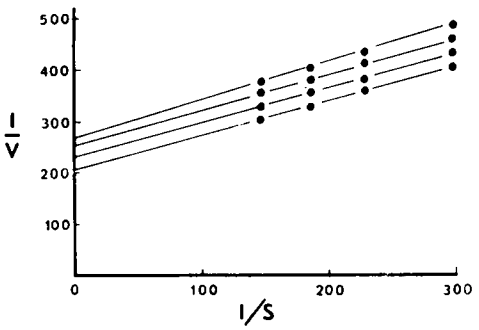

Fig. 4. NADH inhibition of L-fucose reaction. NAD concn is 40 μM, NADH concn varied from 20 to 50 μM, L-fucose concn varied from 16.7 to 67 mM, enzyme concn is 0.135 μM.

Figs 4 and 5 yield virtually identical values for K_{iq} indicating that E–NADH binding is the same in each system. This result is paralleled by the K_{ia} value for each substrate. There are slight differences in k_1 and k_2, however these merely reflect experimental error rather than any conformation or energetic change.

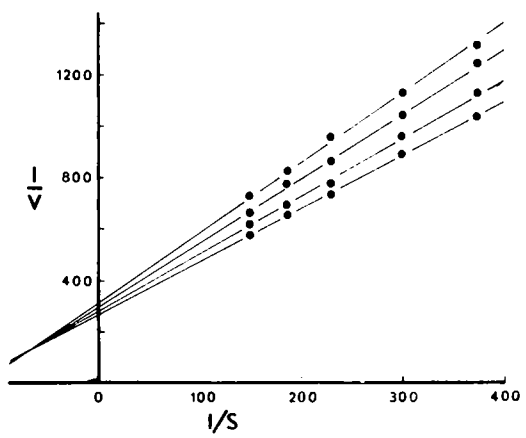

Fig. 5. NADH inhibition of D-arabinose reaction. NAD concn is 40 μM, NADH concn varied from 20 to 50 μM, D-arabinose concn varied from 16.7 to 67 mM, enzyme concn is 0.135 μM.

D-Arabinolactone inhibition

When D-arabinolactone (16.7–267 mM) or L-fuconolactone (16.7–267 mM) was present in the incubation mixture, no inhibition was observed with NAD at either an unsaturating or saturating level with either D-arabinose or L-fucose as the variable substrate.

One test was performed by using hydroxylamine [25] in the mixture of buffer and lactone to establish whether or not the lactone hydrolyzed immediately to the acid at pH 10.0. The results showed that there is only a slow hydrolysis of either lactone (5% hydrolysis after 1 h) in this pH range.

From Eqn 3, no inhibition suggests that both the terms K_q/K_{iq} K_p and $1/K_{ip}$

approach zero. These can be arranged to $K_{iq}K_p \gg K_q$ and $K_{ip} \gg 1$ and upon substituting the corresponding rate constants the following two relationships are indicated

$$k_7(k_4 + k_5) \gg k_4k_6 \tag{6}$$

$$k_5 + k_7 \gg k_6 \tag{7}$$

Equilibrium and reverse reaction study

An additional experiment was performed to check the possible reversibility of the reaction. The Haldane equilibrium constant, K_{eq}, is defined as [P][Q]/[A][B] [26] We attempted to measure K_{eq} by fixing the concentration of NAD and varying the concentration of D-arabinose or L-fucose. The results indicated that all of the NAD was reduced to NADH, i.e. $k_1k_3k_5k_7 \gg k_2k_4k_6k_8$. There was no apparent equilibrium.

Experiments involving the reverse reaction were carried out to verify the results of the equilibrium experiment. No significant reaction was observed and the reaction appears to be irreversible.

Utilizing the values of K_{ia} and K_{iq} in Table I, the relationship $k_1k_3k_5k_7 \gg k_2k_4k_6k_8$ can be narrowed to

$$k_3k_5 \gg k_4k_6 \tag{8}$$

As a final check on the rate constant k_5, it is possible to compare the values for each reaction by referring to the points of intersection in Figs 1 and 2. Lueck et al. [27] point out that at the intersection point,

$$E_0/V_1 = \frac{1}{k_5} + \frac{1}{k_7} - \frac{1}{k_2} \tag{9}$$

The sum of the terms $(1/k_5 + 1/k_7)$, equals 14.4 min for L-fucose and 29.0 min for D-arabinose. Assuming that k_7 is equal for each case leads to the conclusion that k_5 for L-fucose is greater than k_5 for D-arabinose. In short, L-fucose is more rapidly converted to the lactone under saturating conditions.

CONCLUSIONS

The kinetic data presented here are clearly consistent with an ordered sequential mechanism involving a ternary complex of enzyme–NAD–sugar. It is an apparently irreversible reaction at pH 10.0. At present we have no explanation for the significance of these enzymes, however their general occurrence indicates a metabolic role of some importance.

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