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Initial Velocity and Product Inhibition Studies on L-Iditol:NAD Oxidoreductase

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The mechanism of action of the enzyme L-iditol:NAD oxidoreductase (EC 1.1.1.14; sorbitol dehydrogenase) from sheep liver has been investigated. The catalyzed reaction:

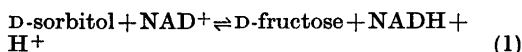
D-sorbitol + $NAD^+ \rightleftharpoons$ D-fructose + NADH + H^+

was followed from both sides in series of spectrophotometric measurements of initial velocity and product inhibition. All measurements were carried out at pH 7.0.

The results indicates that this two-substrate reaction follows a Rapid Equilibrium Random mechanism in which one of the possible ternary complexes — the combination of enzyme, D-sorbitol, and NADH — is a dead-end complex.

The kinetic constants of the reaction are given.

L-Iditol:NAD oxidoreductase (EC 1.1.1.14; sorbitol dehydrogenase) catalyzes tke reversible reaction shown in eqn. 1.



$$\text{D-sorbitol} + NAD^+ \rightleftharpoons \text{D-fructose} + NADH + H^+ \quad (1)$$

The enzyme is found in the livers of a variety of mammalian species and it has been purified from several sources. [1-6] Interest in it has hitherto been concentrated upon its clinical use in the diagnosis of liver diseases,[7,8] although the substrate specificity,[5,6,9-11] the effects of NAD analogs,[5] the pH and temperature optima,[4,6,10] and the effects of various inhibitors [6,9,11,12] have been investigated in earlier studies.

This paper reports the results of kinetic studies that were designed to determine the mechanism by which sheep liver sorbitol dehydrogenase exerts its catalytic action in reaction (1). The attempt to distinguish between various possible mechanisms has involved measurements of initial velocities, obtained in the absence of products, and a determination of the product inhibition pattern.[13,14] The results indicate that sorbitol dehydrogenase acts with a Rapid Equilibrium Random mechanism with one dead-end complex, the enzyme—sorbitol—NADH complex.

EXPERIMENTAL

Materials

L-Iditol:NAD oxidoreductase (sorbitol dehydrogenase) from sheep liver was obtained from Boehringer, Mannheim, 60 mg lyophilisate contained 10 mg enzyme and 50 mg maltose. When stored at −18 °C, the lyophilisate lost no activity over several months. Agarose gel electrophoresis in barbitone buffer 0.075 M, calcium lactate 0.002 M, pH 8.6 showed one protein band.

The specific activity was not enhanced by gel filtration on a Sephadex G-150 column (1.5 × 35 cm). The commercial product was therefore used directly. Enzyme stock solutions (0.1 or 1 mg lyophilisate per ml phosphate buffer) were prepared each day and were kept at 0−5 °C. Under these conditions the activity decreased approximately 2 % per hour.

NAD and Na_2-NADH, both grade III, were purchased from Sigma Chemical Company. Stock solutions (1 or 10 mM) were prepared on the day they were to be used.

D-Sorbitol and D-fructose were analytical grades from BDH Chemicals Ltd.

All solutions were made in 0.05 M phosphate buffer, pH 7.0, containing 10 μM EDTA to prevent influence from heavy metal ions.

Methods

The equilibrium constant and the initial velocities were determined spectrophotometri-

cally. The change in the concentration of NADH was followed by measuring the light absorbance at $\lambda = 340$ nm as a function of time. Quartz cuvettes with a light path length of 10 mm were used. A Zeiss PM QII spectrophotometer was connected through a linear amplifier and a log-converter, to a recorder with adjustable sensitivity and multiple-speed chart drive. Full scale sensitivities of the recorder ranged from 0.04 to 0.2 absorbance units. Background absorbances could be eliminated.

The equilibrium constant was determined from experiments in which all four substrates were present in known concentrations at zero time. The reaction was measured until equilibrium was reached. Substrate equilibrium concentrations were calculated and the equilibrium constant, K_{eq}, was determined from eqn. 2.

$$K_{eq} = [\text{D-fructose}]_{eq}[\text{NADH}]_{eq}[\text{H}^+]/([\text{D-sorbitol}]_{eq}[\text{NAD}]_{eq}) \quad (2)$$

In each initial velocity experiment one substrate, the product inhibitor (if any) and a suitable amount of buffer were mixed in a 1×8 cm pyrex test tube. The tubes for a set of experiments (16–48 experiments with 4–8 concentrations of the varied substrate and 4–6 concentrations of inhibitor or fixed substrate) were placed in a thermostat at 23.5 °C. For each experiment, enzyme was added to one tube and exactly 10 min after this the reaction was initiated by adding the other substrate. After vigorous mixing the content was transferred to a cuvette and the absorbance was measured for 5 min. The slope of the tangent to the progress curve at zero time was taken as the initial velocity. A standard assay of enzyme activity was performed at least three times within each set of experiments and the results of these assays were used to correct the initial velocities for the loss of enzyme activity in the stock solution. Values of initial velocities were scaled up to the enzyme concentration 1 mg/l, the unit thus being μmol mg^{-1} min^{-1}. All experiments were run twice; mean values of the initial velocities were used in data processing.

Reciprocal velocities were plotted graphically against the reciprocals of the substrate concentrations. The data, which gave linear primary plots, were fitted to eqn. 3 using a least squares method assuming a normal distribution and equal variances of initial velocities.

$$s/v = s/V + K/V \quad (3)$$

Slopes ($S = K/V$) and intercepts ($I = 1/V$) (Lineweaver-Burk plots) were replotted against either inhibitor concentration or the reciprocal of the fixed substrate concentration to determine the type of inhibition.[14] When the intercept values for all inhibitor concentrations in a product inhibition experiment were equal to within the 67 % confidence limit, the inhibition was considered to be competitive. Data from the apparently linear replots were fitted to a linear equation, using a least squares method in which the weighting factors were the reciprocals of the squares of the standard errors. Values of the kinetic constants and estimates

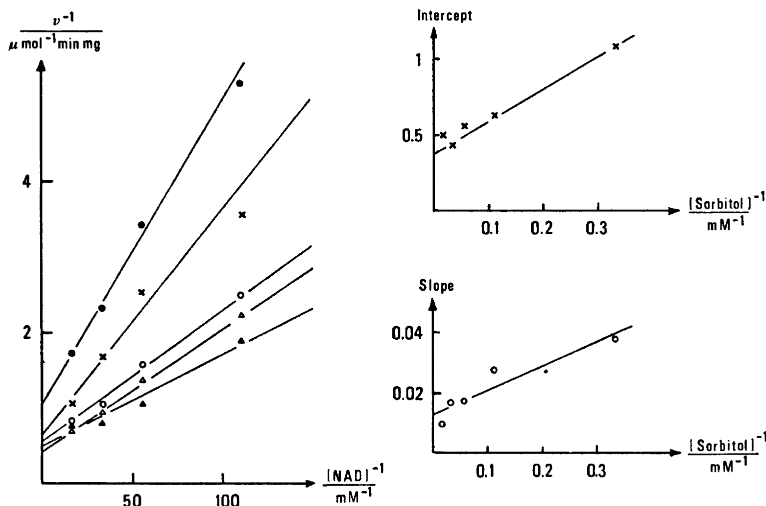


Fig. 1. Double reciprocal plot of the results of the initial velocity experiments with D-sorbitol and NAD as substrates. Intercept and slope replots are also shown. Concentrations were: sorbitol dehydrogenase 1 mg/l; NAD 9 μM, 18 μM, 30 μM and 60 μM; and D-sorbitol (●) 3 mM, (×) 9 mM, (○) 18 mM, (△) 30 mM and (▲) 60 mM.

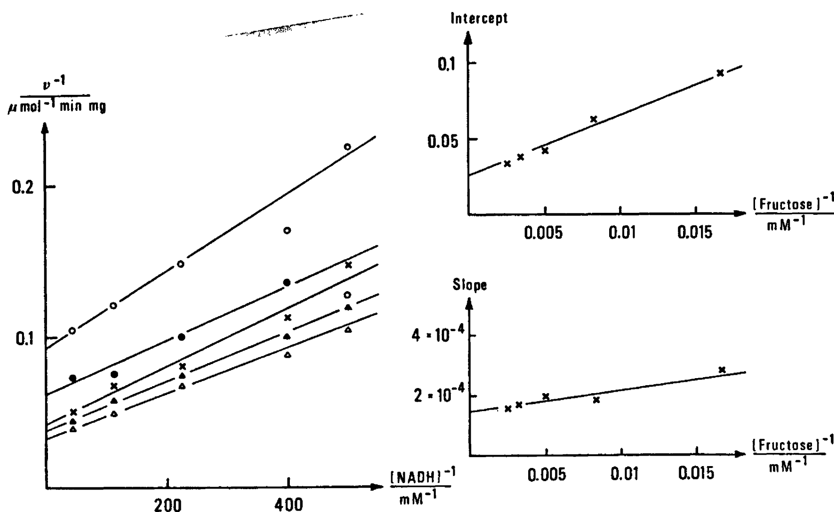


Fig. 2. Double reciprocal plot of the results of the initial velocity experiments with D-fructose and NADH as substrates. Intercept and slope replots are also shown. Concentrations were: sorbitol dehydrogenase 1 mg/l; NADH 2 μM, 2.5 μM, 4.5 μM, 9 μM and 22.5 μM; and D-fructose (○) 60 mM, (●) 120 mM, (×) 200 mM, (▲) 300 mM and (△) 400 mM.

of their accuracy were calculated from the slopes (IS = the slope of the intercept replot; SS = the slope of the slope replot) and the intercepts (II = the intercept of the intercept replot; SI = the intercept of the slope replot) and their estimated standard errors. The results given in Tables 3 and 4 are the best such estimates and their estimated standard errors, $y \pm S_y$.

RESULTS AND DISCUSSION

The results of a series of initial velocity experiments, in which sorbitol dehydrogenase was reacted with D-sorbitol and NAD and in which no products were present at zero time, are shown in Fig. 1. The results of a series of similar experiments on the reaction with D-fructose and NADH is illustrated in Fig. 2. The results are consistent with the initial rate equation (eqn. 4).

$$v = V/(1 + K_A/A + K_B/B + K_{iA}K_B/AB) \quad (4)$$

Eqn. 4 is consistent with most sequential mechanisms, but not with a Ping Pong mechanism.[14]

Table 1 shows the qualitative results of the product inhibition experiments; the results of one of these is shown in Fig. 3. The only simple sequential mechanism which fits the product inhibition pattern (Table 1) is the Rapid Equilibrium Random mechanism with one dead-end complex, Fig. 4.[14] The steady state rate equation for this mechanism is

Table 1. The product inhibition pattern of sorbitol dehydrogenase.

Fixed substrate	Varied substrate	Inhibitor	Resulting inhibition
D-Sorbitol	NAD	NADH	Competitive
D-Sorbitol	NAD	D-fructose	Competitive
NAD	D-sorbitol	D-fructose	Competitive
NAD	D-sorbitol	NADH	Noncompetitive
D-Fructose	NADH	D-sorbitol	Noncompetitive
D-Fructose	NADH	NAD	Competitive

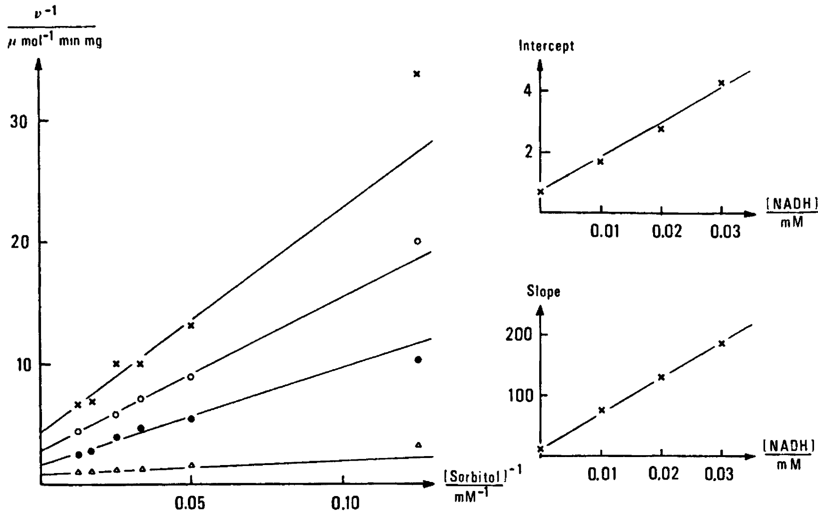
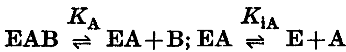


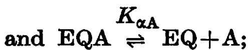
Fig. 3. Double reciprocal plot, and intercept and slope replots of the product inhibition experiments on sorbitol dehydrogenase with NAD as fixed substrate, D-sorbitol as varied substrate and NADH as inhibitor. Concentrations were: sorbitol dehydrogenase 1 mg/l; NAD 20 μM; D-sorbitol 8 mM, 20 mM, 30 mM, 40 mM, 60 mM, and 80 mM, and NADH (△) 0 μM, (●) 10 μM, (○) 20 μM and (×) 30 μM.

$$v = \frac{V_1 - V_2 \, PQ \, K_{iA}K_B/(ABK_{iP}K_Q)}{1 + \frac{K_A}{A} + \frac{K_B}{B} + \frac{Q \, K_B}{B \, K_{\alpha Q}} + \frac{K_{iA} \, K_B}{AB}\left(1 + \frac{P}{K_{iP}} + \frac{Q}{K_{iQ}} + \frac{PQ}{K_{iP}K_Q}\right)} \quad (5)$$

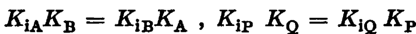
K_A, K_{iA} and $K_{\alpha A}$ are the equilibrium constants of the reactions:



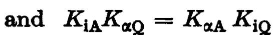
$\mathrm{EAB} \overset{K_A}{\rightleftharpoons} \mathrm{EA+B}; \mathrm{EA} \overset{K_{iA}}{\rightleftharpoons} \mathrm{E+A}$



and $\mathrm{EQA} \overset{K_{\alpha A}}{\rightleftharpoons} \mathrm{EQ+A}$;



$K_{iA}K_B = K_{iB}K_A$, $K_{iP} \, K_Q = K_{iQ} \, K_P$



and $K_{iA}K_{\alpha Q} = K_{\alpha A} \, K_{iQ}$ (6)

Eqn. 5 reduces to eqn. 4 when no product is present, P=Q=0.

The steady state initial rate equations for the product inhibitions can be obtained from eqn. 4 by setting either P or Q equal to zero.

If Q inhibits, P=0

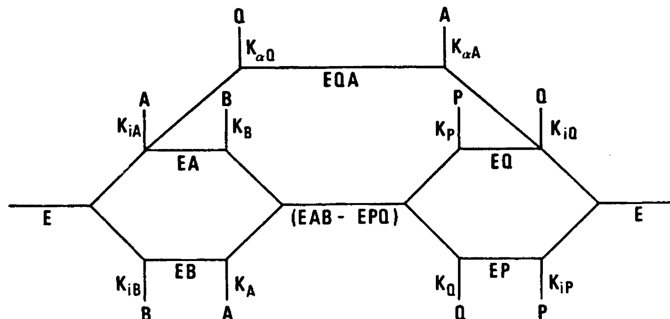


Fig. 4. Rapid Equilibrium Random mechanism with one dead-end complex. The conversion of the central complexes, EAB⇌EPQ, is the only rate-determining step in both reaction directions. All other steps are fast and therefore reach equilibrium rapidly after initiation of the reaction. Here E=sorbitol dehydrogenase, A=D-sorbitol, B=NAD, P=D-fructose, and Q=NADH.

$$v = \frac{V_1}{1 + \frac{K_A}{A} + \frac{K_B}{B}\left(1 + \frac{Q}{K_{\alpha Q}}\right) + \frac{K_{iA}K_B}{AB}\left(1 + \frac{Q}{K_{iQ}}\right)} \quad (7)$$

If P inhibits, Q=0

$$v = \frac{V_1}{1 + \frac{K_A}{A} + \frac{K_B}{B} + \frac{K_{iA}K_B}{AB}\left(1 + \frac{P}{K_{iP}}\right)} \quad (8)$$

Eqn. 7 predicts linear noncompetitive inhibition when A is varied and linear competitive inhibition when B is varied. Eqn. 8 predicts linear competitive inhibition in both cases. The kinetic expressions of the linear coefficients derived from eqns. 7 and 8 are shown in Table 2. The results of the experiments (Table 1) exhibit the product inhibition pattern predicted by eqn. 7 and 8. The noncompetitive inhibition is between D-sorbitol and NADH, and this identifies A as D-sorbitol, B as NAD, P as D-fructose and Q as NADH (Fig. 4, eqns. 7 and 8). Table 3 gives the values of the kinetic constants calculated from the results shown in Figs. 1, 2 and 3, the kinetic expressions (Table 2) and eqns. 6. Table 4 shows the quantitative results of the experiments and, where an experimental value was not used in a determination, the corresponding values calculated from the kinetic constants. Most pairs of experimental and theoretical values are identical within the estimated standard errors.

The value of the equilibrium constant of reaction (1) is $K_{eq} = 3.71 \times 10^{-9} \pm 8 \times 10^{-11}$ M (mean from five experiments). The Haldane

Table 3. The kinetic constants of sorbitol dehydrogenase.

V_1	=	2.7 ± 0.14 μmol min^{-1} mg^{-1}
K_S	=	5.9 ± 0.26 mM
K_{NAD}	=	19.0 ± 0.92 μM
K_{iS}	=	7.7 ± 0.55 mM
K_{iNAD}	=	25 ± 1.8 μM
$K_{\alpha S}$	=	45 ± 1.6 mM
V_2	=	38.5 ± 0.85 μmol min^{-1} mg^{-1}
K_F	=	150 ± 6.4 mM
K_{NADH}	=	11.1 ± 0.30 mM
K_{iF}	=	7.0 ± 0.39 mM
K_{iNAD}	=	0.53 ± 0.023 μM
$K_{\alpha NADH}$	=	3.1 ± 0.11 μM

relation for the Rapid Equilibrium Random mechanism with one dead-end complex is

$$K_{eq} = \frac{V_1 K_{iF} K_{NADH}\,[H^+]}{V_2\, K_{iS}\, K_{NAD}} \quad (9)$$

This gives $K_{eq} = 3.8 \times 10^{-9} \pm 3.7 \times 10^{-9}$ M, identical with the experimental and in agreement with the value $K_{eq} = 2.4 \times 10^{-9} \pm 1.3 \times 10^{-10}$ M reported by Blakley.[1]

No Ordered BiBi or Ping Pong mechanism fits the results. The product inhibition pattern, the identity between the calculated and experimental value of the equilibrium constant and the agreement of experimental and theoretical linear coefficients of the initial velocity and product inhibition data show that sorbitol

Table 2. The kinetic expressions of the linear coefficient derived from the rate equation.

Fixed substrate	Varied substrate	Inhibitor	II	IS	SI	SS
A	B	—	$1/V_1$	K_A/V_1	K_B/V_1	$K_{iA}\ K_B/V_1$
A	B	Q	$(1+K_A/A)/V_1$	0	$K_B(1+K_{iA}/A)/V_1$	$K_B(1/K_{\alpha Q} + K_{iA}/A\ K_{iQ})/V_1$
A	B	P	$(1+K_A/A)/V_1$	0	$K_B(1+K_{iA}/A)/V_1$	$K_{iA}K_B/A\ K_{iP}\ V_1$
B	A	P	$(1+K_B/B)/V_1$	0	$K_A(1+K_{iB}/B)/V_1$	$K_{iA}K_B/B\ K_{iP}\ V_1$
B	A	Q	$(1+K_B/B)/V_1$	$K_B/B\ K_{\alpha Q}\ V_1$	$K_A(1+K_{iB}/B)/V_1$	$K_{iA}K_B/B\ K_{iQ}\ V_1$

II=intercept of intercept replot; IS=slope of intercept replot; SI=intercept of slope replot and SS=slope of slope replot.

Table 4. The experimental (E) and theoretical (T) values of the linear coefficients. The theoretical values were calculated from the kinetic expressions in Table 2 using the kinetic constants in Table 3. The unit of initial velocity is μmol min^{-1} mg^{-1} and the units of concentration and the kinetic constants are mM.

Fixed substrate	Varied substrate	Inhibitor		II	IS	SI	SS
D-Sorbitol	NAD	—	E	$3.7\times10^{-1}\pm1.2\times10^{-2}$	2.2 ± 2 10^{-1}	$1.2\times10^{-2}\pm2.5\times10^{-3}$	$8\times10^{-2}\pm2\times10^{-2}$
			T		2.16 ± 6.5 10^{-2}	$7.0\times10^{-3}\pm5\times10^{-4}$	$5.4\times10^{-2}\pm5.9\times10^{-3}$
D-Sorbitol 30 mM	NAD	NADH	E	$4.3\times10^{-1}\pm4\times10^{-2}$	0	$8.1\times10^{-3}\pm5\times10^{-4}$	$3.2\pm8\times10^{-1}$
			T	$4.4\times10^{-1}\pm1\times10^{-2}$	0	$8.8\times10^{-3}\pm6\times10^{-4}$	$5.6\pm6.2\times10^{-1}$
D-Sorbitol 40 mM	NAD	D-fructose	E	$4.2\times10^{-1}\pm1.3\times10^{-2}$	0	$7.7\times10^{-3}\pm3\times10^{-4}$	$1.9\times10^{-3}\pm4\times10^{-4}$
			T		0	$8.4\times10^{-3}\pm6\times10^{-4}$	$2\times10^{-4}\pm2.2\times10^{-5}$
NAD 0.02 mM	D-sorbitol	D-fructose	E	$6.4\times10^{-1}\pm5\times10^{-2}$	0	$6.6\pm6.4\times10^{-1}$	$7\times10^{-1}\pm1\times10^{-1}$
			T	$7.2\times10^{-1}\pm1.4\times10^{-2}$	0	$4.9\pm3\times10^{-1}$	$3.9\times10^{-1}\pm4.3\times10^{-2}$
NAD 0.02 mM	D-sorbitol	NADH	E	$7.2\times10^{-1}\pm1.4\times10^{-2}$	114 ± 2.3	$4.9\pm3\times10^{-1}$	$5.6\pm4.5\times10^{-1}$
			T				$5.1\pm5.6\times10^{-1}$
D-Fructose	NADH	—	E	$2.6\times10^{-2}\pm6\times10^{-4}$	$3.9\pm1.4\times10^{-1}$	$1.5\times10^{-1}\pm2\times10^{-2}$	$6.6\times10^{-3}\pm3.5\times10^{-3}$
			T			$2.88\times10^{-1}\pm6\times10^{-3}$	$2.1\times10^{-3}\pm2.2\times10^{-4}$
D-Fructose 400 mM	NADH	NAD	E	$3.9\times10^{-2}\pm8\times10^{-4}$	$2.18\times10^{-4}\pm1.5\times10^{-6}$	$3.11\times10^{-1}\pm5\times10^{-3}$	$6.7\times10^{-7}\pm4\times10^{-8}$
			T	$3.6\times10^{-2}\pm1.3\times10^{-4}$			$6.7\times10^{-7}\pm7.4\times10^{-8}$
D-Fructose 400 mM	NADH	NAD	E	$3.8\times10^{-2}\pm3.6\times10^{-3}$	0	$3.1\times10^{-1}\pm1.8\times10^{-2}$	$6\times10^{-4}\pm1.4\times10^{-4}$
			T	$3.6\times10^{-2}\pm1.3\times10^{-3}$	0	$3.11\times10^{-1}\pm5\times10^{-3}$	$2\times10^{-4}\pm2.2\times10^{-5}$

dehydrogenase most probably follows a Rapid Equilibrium Random mechanism with one dead-end complex, the enzyme-sorbitol-NADH complex.

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