D-Mannitol Dehydrogenase from *Absidia glauca*. Steady-State Kinetic Properties and the Inhibitory Role of Mannitol 1-Phosphate†

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ABSTRACT: Steady-state kinetic studies including initial velocity for mannitol oxidation and fructose reduction and product inhibition for mannitol oxidation using fructose and reduced nicotinamide adenine dinucleotide (NADH) are in accord with a reaction mechanism best described as ordered Bi-Bi with NAD^+ and NADH designated as the first substrate, last product, respectively, at pH 8.8. All replots of slopes and intercepts from product inhibition studies were linear. Dead-end inhibition studies using mannitol 1-phosphate gave slope-parabolic, intercept-linear noncompetitive inhibition for both NAD^+ and mannitol as substrates. The dead-end inhibitor is capable of binding multiply to the E, EA, and EQ forms of the enzyme to an extent that is controlled by the concentration of substrates. The EQ complex is inferred to undergo a conformational change, $E'Q \rightleftharpoons EQ$, since $(V_1/E_t) > (K_{iq}V_2)/(K_qE_t)$, and no evidence for dead-end complex formation with NADH can be adduced. This is interpreted to mean that the release of fructose from the central complex is faster than the isomerization of the E·NADH complex. When mannitol is saturating, the noncompetitive inhibition against NAD^+, as the variable substrate, becomes parabolic uncompetitive. A replot of the slopes of the parabola against mannitol 1-phosphate remains concave upward. This situation could arise if the conformational change we infer in the EQ complex opens up additional sites on the protein which can interact with the dead-end inhibitor.

Mannitol dehydrogenase (E.C. 1.1.1.67), prepared from the heterothallic fungus *Absidia glauca* (ATCC 7852 A), was assigned a regulatory role in the initial utilization of D-mannitol as the sole source of carbon on the basis of its associative properties and inhibitory response to mannitol 1-phosphate (Ueng et al., 1976). The preparation was shown to be enzymatically homogeneous, based on substrate specificity studies and activity staining following gel electrophoresis.

While a variety of polyol dehydrogenases have been reported (IUB, Enzyme Nomenclature, 1973), with but few exceptions (Nordlie and Fromm, 1959; Fromm and Nelson, 1962; Vidal-Leiria and Van Uden, 1973; Burleigh et al., 1974; Christensen et al., 1975) little effort appears to have been directed to an investigation of the steady- or transient-state behavior of these enzymes in spite of the diversity of function of their acyclic and alicyclic polyol substrates. The report of Varma and Kinoshita (1974) that sorbitol or galactitol formation is accelerated in the intact rat lens in the diabetic state, and that of Miller and Smith (1975) that threitol and sorbitol have been implicated as cryoprotective agents in the adult beetle *Upis ceramboides* are illustrative of this diversity. In addition, mannitol is used as a diluent for cocaine (Johnson and Gunn, 1972).

In this paper, we report the results of a series of steady-state kinetic investigations in which the mannitol dehydrogenase catalyzed reaction was followed in both directions using initial

$$\text{D-mannitol} + NAD^+ \rightleftharpoons \text{D-fructose} + NADH + H^+$$

velocity measurements, and in the direction of mannitol oxidation employing product and dead-end inhibition measurements.

Marked differences can be noted in the steady-state behavior of the three NAD^+-linked acyclic polyol dehydrogenases that have been studied to date. Thus, while ribitol dehydrogenase (Fromm and Nelson, 1962) from *Aerobacter aerogenes* appears to follow a sequential binding order similar to the mannitol dehydrogenase described here, it is also capable of forming kinetically significant abortive ternary complexes involving E·NAD and D-ribulose and E·NADH and ribitol. We find no evidence for dead-end inhibition by either fructose or mannitol. In contrast to both of these cases of ordered addition of substrates, Christensen et al. (1975) have reported that sorbitol dehydrogenase from sheep liver follows a rapid equilibrium random mechanism where E·sorbitol forms a dead-end complex with NADH.

Experimental Section

Materials. D-Mannitol dehydrogenase was prepared as previously described (Ueng et al., 1976). Dithiothreitol (DTT), D-mannose 6-phosphate, NAD^+, and NADH were obtained from Sigma Chemical Co. D-Mannitol and D-fructose were obtained from Pfanstiehl Laboratories. D-Mannitol 1-phosphate was prepared as described (Wolff and Kaplan, 1956).

Methods. Initial rate measurements, carried out by following the change in absorbance of the cofactor at 340 nm arising from the polyol oxidation or ketose reduction, were made on a thermostated Beckman Acta spectrophotometer at 25 °C. A typical reaction mixture contained D-mannitol (5–40 mM), NAD^+ (0.05–0.4 mM), NaOH–glycine buffer (0.05 M, pH 8.8, with NaCl added such that the final contents of the cuvette were adjusted to an ionic strength of 0.1) and enzyme (0.01–0.1 ml) sequentially added to a final volume of 3.00 ml in the cuvette. For the reverse reaction, D-fructose (30–120 mM) and NADH (0.08–0.30 mM) replaced mannitol and NAD^+. For product inhibition studies, either D-fructose (0–240 mM) or NADH (0–0.08 mm) was incorporated into the reaction mixture. For dead-end inhibition studies, mannitol 1-phosphate (0–10 mm) was incorporated into the reaction mixture.

Each reaction was initiated by the addition of enzyme to the cuvette, then followed with a recorder trace set for full-scale

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[1] Abbreviations used are: NAD^+, nicotinamide adenine dinucleotide; NADH, reduced NAD^+; DTT, dithiothreitol.

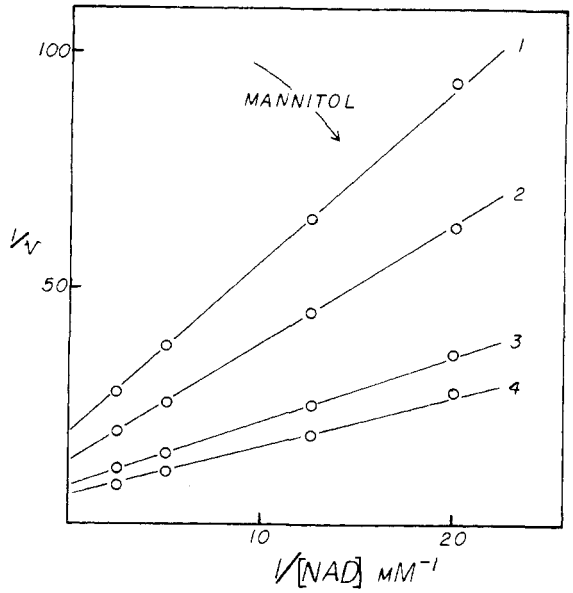


FIGURE 1: Reciprocal plots with NAD^+ as the variable substrate and D-mannitol as the changing-fixed substrate. Polyol concentrations were: (1) 5, (2) 8, (3) 20, and (4) 40 mM, respectively. The reaction rate (v) is expressed as ΔA_{340nm}/min. Data were fitted to eq 2.

deflection corresponding to an absorbance change of 0.1 unit. The initial velocity was determined from the tangent drawn to that portion of the recorder trace extrapolated to the time of addition of the enzyme to the cuvette. The change in concentration of NAD^+ (or NADH) was followed for a period of time such that no more than 5% of the original reactants was consumed.

Data Processing. Preliminary initial velocity experiments were carried out to establish the boundary conditions for the forward and reverse reactions from estimates of K_a, K_b, K_p, and K_q by fitting the experimental data to the equation

$$v = \frac{VA}{K + A} \tag{1}$$

using least-squares analysis and assuming equal variances for the velocities according to Wilkinson (1961). All subsequent rate data were examined graphically using Lineweaver–Burk plots (1934), followed by pertinent replots of these slope and intercept values, to facilitate the choice of the appropriate rate equation. Data fitting to a given rate equation, carried out on a Control Data (Model 3100) computer employing available Fortran programs (Cleland, 1963a), was used to generate values for the various kinetic constants and the standard errors of these values.

Using this sequence, initial velocity data were fitted to eq 2, linear competitive inhibition data to eq 3, linear noncompetitive inhibition to eq 4, linear uncompetitive inhibition to eq 5, slope parabolic[2] noncompetitive inhibition to eq 6, and parabolic competitive inhibition to eq 7.

$$v = \frac{VAB}{K_{ia}K_b + K_bA + K_aB + AB} \tag{2}$$

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

[2] The reader is reminded that the term parabola is reserved for equations of the general type, $y = kx^2$; but curves of the general type $y = kx^n$, where n is a positive number are designated parabolic curves (Daniels, 1956). Whether the concept is to be expressed as a noun or in an adjectival sense follows subsequent to experimental interpretation.

TABLE I: Product Inhibition Patterns and Kinetic Constants.

Inhibitor	Substrates (mM) NAD^+	Substrates (mM) D-Mannitol	Pattern	Inhibition Constants K_{is}	Inhibition Constants K_{ii}
NADH	Var[a]	10.0	C[b]	112 ± 7 μM	
NADH	0.2	Var	NC	53 ± 3 μM	125 ± 11 μM
D-Fructose	Var	10.0	NC	60 ± 8 mM	212 ± 29 mM
D-Fructose	0.2	Var	NC	106 ± 5 mM	226 ± 18 mM
D-Fructose	Var	240	UC		234 ± 23 mM

[a] Var designates the variable substrate. [b] C, NC, and UC designate, respectively, competitive, noncompetitive, and uncompetitive inhibition. Data were derived from fits of experimental values to appropriate equations described in the text.

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

$$v = \frac{VA}{K + A(1 + I/K_{ii})} \tag{5}$$

$$v = \frac{VA}{K(1 + I/K_{isi} + I^2/K_{is2}) + A(1 + I/K_{ii})} \tag{6}$$

$$v = \frac{VA}{K(1 + I/K_{isi} + I^2/K_{is2}) + A} \tag{7}$$

Results

Initial Velocity Studies. When NAD^+ and mannitol were examined as variable and changing-fixed substrates, respectively, the pattern shown (Figure 1) was obtained. Intersecting plots were also obtained when mannitol and NAD^+ were examined as variable and changing-fixed substrates, and for the reverse reaction, when D-fructose and NADH were similarly manipulated as substrates. Maximum velocities for the forward (V_1) and reverse (V_2) reactions were 43.9 ± 1.6 μM/min and 7.38 ± 0.29 μM/min, respectively. Michaelis (and inhibition) constants for the variable substrates were: NAD^+, 202 ± 15 μM (174 ± 16 μM); D-mannitol, 21.8 ± 1.6 mM; NADH, 79.1 ± 8.8 μM (65.2 ± 7.7 μM); D-fructose, 79.2 ± 5.8 mM. A series of preliminary experiments, carried out at pH 9.6, the optimum for the forward reaction, also showed a sequential addition of reactants, but the slow rate of fructose reduction at this pH precluded an examination of the reverse reaction under these conditions.

Product Inhibition Studies. D-Fructose gave linear noncompetitive inhibition against NAD^+ and mannitol as the variable substrates. When NADH was used with NAD^+ and mannitol as the variable substrates, the resulting inhibition patterns were competitive and noncompetitive, respectively. All replots of slopes and intercepts were linear. When the inhibition with fructose against NAD^+ as the variable substrate was examined at the level of 240 mM of mannitol (i.e., 11 × K_b), the intersecting pattern was transformed into a parallel pattern with a horizontal, slope = 0, replot. A summary compilation of these inhibition patterns and the corresponding constants is presented in Table I.

No indication of substrate inhibition up to a mannitol concentration of 693 mM (32 × K_b) was evident. NAD^+, however, gave rise to a progressive inhibition, starting at 2.5 mM (12 × K_a) and terminating in complete inhibition at 6.5 mM.

Dead-End Inhibition. Both reciprocal plots of NAD^+ (Figure 2) and mannitol, as variable substrates against in-

TABLE II: Dead-End Inhibition Patterns and Constants for Mannitol 1-Phosphate.

Variable Substrate	Fixed Substrate	Inhibition Patterns[a]	Replots		Inhibition Constants[f]		
			Slopes[d]	Intercepts	K_{is1}	K_{is2}	K_{ii}
NAD^+	Mannitol (10 mM)	NC[b]	Parabolic	Linear	1.1 ± 0.2	4.9 ± 0.7	9.4 ± 1.3
Mannitol	NAD^+ (0.20 mM)	NC[b]	Parabolic	Linear	4.3 ± 1.6	6.8 ± 0.9	9.1 ± 1.7
Mannitol	NAD^+ (2.1 mM)	C[c]	Parabolic		2.8 ± 0.3	15.5 ± 1.3	
NAD^+	Mannitol (693 mM)	UC		Parabolic[e]			

[a] C, NC, and UC designate competitive, noncompetitive, and uncompetitive inhibition, respectively. [b,c] Experimental data points were fitted to eq 6 and 7, respectively. [d] Replots of the slopes of these parabolic (i.e., concave-upward) curves against the inhibitor concentration gave straight lines. [e] When these slopes (from the secondary plot) were plotted against mannitol 1-phosphate, they gave a concave-upward (i.e., tertiary) replot. [f] Units of K_i values are: mM (K_{is1} and K_{ii}) and $(mM)^2$ (K_{is2}).

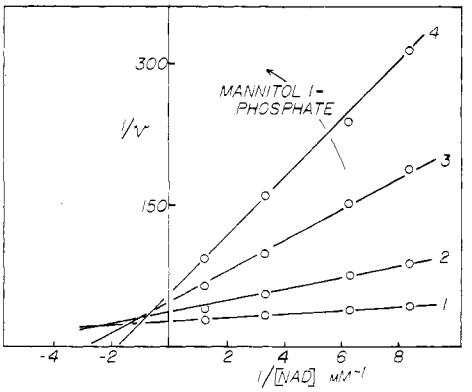


FIGURE 2: Reciprocal plots with NAD^+ as the variable substrate and D-mannitol 1-phosphate as the inhibitor. D-Mannitol concentration was 10 mM. Mannitol 1-phosphate concentrations were: (1) 0, (2) 2, (3) 5, and (4) 7 mM, respectively. Data were fitted to eq 6.

creasing amounts of mannitol 1-phosphate (0–10 mM) as inhibitor, gave straight lines crossing at different points in the $-x$, $+y$ quadrant. When the level of mannitol was raised to saturation (693 mM, $32 \times K_b$), the plot for variable NAD^+ (Figure 2) was transformed into a parallel pattern indicative of uncompetitive inhibition. When mannitol was used as the variable substrate and the level of NAD^+ as constant second substrate was raised from 0.2 to 2.1 mM, the reciprocal plot was transformed into a plot pattern indicative of competitive inhibition.

Computer-assisted analyses of the noncompetitive inhibition patterns were in agreement with the graphic analyses, which showed slope-parabolic, intercept-linear noncompetitive inhibition for both cases where the constant second substrate was held at a nonsaturating level. Similar analysis of the competitive inhibition pattern manifest at a saturating level of NAD^+ confirms that the slopes replot is parabolic. In contrast, when the slopes of the intercepts replot arising from the uncompetitive inhibition shown at a saturating level of mannitol are themselves replotted, the resulting curve is nonlinear concave upward, indicative of a higher degree of binding (i.e., more than twice) by mannitol 1-phosphate to the E·NADH complex. A summary of these inhibition patterns is shown in Table II.

Discussion

The results of the initial velocity experiments, when interpreted together with the array of product inhibition patterns (one competitive, three noncompetitive), are taken to indicate that the reaction proceeds by an ordered sequential mechanism with NAD^+ and NADH identified as the initial reactant and final product, respectively, in the direction of mannitol oxidation. When the product inhibition experiment with fructose against NAD^+ as the variable substrate is carried out at a saturating level of mannitol, the transformation from an intersecting to a parallel plot pattern provides confirmatory evidence for an ordered Bi-Bi reaction with NAD^+ as the leading substrate.

The noncompetitive inhibition pattern arising from mannitol 1-phosphate inhibition against mannitol as variable substrate is indicative of a competitive effect (inhibitor binds to EA) and a noncompetitive contribution arising from the interaction of mannitol 1-phosphate with free enzyme (E). When the concentration of NAD^+ is raised from 0.2 to 2.1 mM, this latter effect is wiped out, since the level of E is reduced to negligible proportions, relative to the level of EA, and the competitive inhibition between mannitol and mannitol 1-phosphate is seen.

A noncompetitive inhibition pattern, arising when a dead-end inhibitor (mannitol 1-phosphate) is examined against the leading substrate (NAD^+) as variable substrate in an ordered Bi-Bi reaction, is indicative of a summation effect containing a competitive (inhibitor binds to E) and an uncompetitive (inhibitor binds to EA) component. In a similar way to the case when mannitol is the variable substrate, we should be able to experimentally extract the competitive contribution (between NAD^+ and mannitol 1-phosphate) to the noncompetitive inhibition by raising the level of mannitol to saturation, thereby wipng out the uncompetitive contribution that would occur when the increasing concentration of NAD^+ as variable substrate delivers the enzyme into the EA(E·NAD) form. Not only is the competitive effect not seen, but the resulting plot pattern is indicative of uncompetitive inhibition. This effect is a manifestation of the fact that mannitol 1-phosphate is binding, in dead-end fashion, to the EQ(E·NADH) complex. If we allow for the inherent limitations and experimental uncertainties associated with a crossover point analysis (Cleland, 1975; Janson and Cleland, 1974), an estimate of R from our data appears not to be at variance with the idea that the ratio of central complexes to all other forms present, including any conformers of E·NAD and E·NADH, is relatively low. This is in contrast to a rapid equilibrium ordered Bi-Bi reaction, where in the absence of added Q the level of EQ would be negligible and the value of R is 1. Thus, if we view the enzyme as oscillating in sequence under steady-state conditions

between the EA and EQ forms at high levels of mannitol, then, as the level of NAD^+ is increased in the presence of mannitol 1-phosphate, the reaction is driven rapidly through the central complexes, thereby increasing the concentration of E·NADH available to react with the inhibitor. If the level of E·NAD and E·NADH were insignificant relative to free enzyme, we should reasonably have expected to see the competitive contribution to the noncompetitive inhibition.

The parabolic slope effect with NAD^+ as the variable substrate (Table II) is taken to indicate that mannitol 1-phosphate binds multiply to the free (E) form of the enzyme at low levels of NAD^+, whereas the linearity of the intercepts replot is taken to indicate that mannitol 1-phosphate binds predominantly to E·NAD(EA), since the level of E is reduced to negligible proportions when NAD^+ is saturating. When these combined slope-parabolic, intercept-linear effects with NAD^+ as the variable substrate are taken together, they are interpreted to mean that mannitol 1-phosphate binds twice to free enzyme (E) and once to the binary complex (E·NAD).

The parabolic-slope replot arising when mannitol is the variable substrate suggests several possibilities for interaction of mannitol 1-phosphate with the enzyme. The inhibitor could bind twice (1) to E or EA, or (2) to both E and EA. Alternately, it could bind (3) to E twice and to EA once, or (4) once to E and twice to EA. This linear intercepts replot tells us that while the level of E is sufficient to bind mannitol 1-phosphate, it binds only once. When these effects (parabolic slope, linear intercept) are taken together, they tell us the inhibitor binds once to E and twice to EA (case 4) for the experimental conditions cited.

The parabolic-slope replot resulting from the interaction with enzyme when NAD^+ is very high (2.1 mM) and mannitol is the variable substrate is indicative of exclusive multiple binding (i.e., binds twice) by mannitol 1-hosphate to EA under these experimental conditions. This is in accord with the fact that K_{ia} (0.174 mM) is significantly less than K_{ii} (9.1 mM, which is interpreted as a measure of the degree of dissociation of mannitol 1-phosphate from the free enzyme form, E); that is, NAD^+ has a greater affinity for E than does mannitol 1-phosphate.

For an ordered Bi-Bi reaction, the rate constant (k_7) for the dissociation of the EQ complex should be equal to $(K_{iq}V_2)/(K_qE_t)$ and greater than V_1/E_t, where V_2 is the maximum velocity for fructose reduction and K_q and K_{iq} correspond to Michaelis and inhibition constants, respectively, for NADH. From the data presented, it is evident that $(K_{iq}V_2)/(K_qE_t) < V_1/E_t$. This disparity is due either to dead-end inhibition involving E and NADH, or the EQ complex undergoes an isomerization reaction. Since all replots of slopes and intercepts from initial velocity and product inhibition experiments were linear, dead-end inhibition arising from cofactor interaction with the various enzyme forms is unlikely and the presence of an isomerization step is a more reasonable interpretation of this disparity.

While isomerization of noncentral transitory complexes (in this case EQ) will not alter the form of the rate equation when expressed in kinetic constants (Cleland, 1963b), it will alter the makeup of these constants (except for K_{ia} in this case) in terms of their component rate constants. Thus, the derivation of the rate equation for an ordered Bi-Bi reaction, incorporating the step

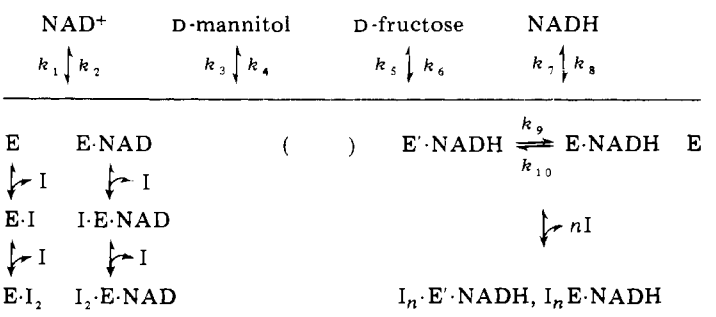
$$E'Q \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} EQ$$

leads to the statements that $(K_{iq}V_2)/(K_qE_t) = (k_7k_9k_{10})/((k_9 + k_{10})(k_7 + k_{10}))$, and $V_1/E_t = (k_5k_7k_9)/(k_7k_9 + k_5k_{10} + k_5k_7 + k_5k_9)$. Since $V_1 > (K_{iq}V_2)/K_q$, it must follow that $k_{10} < k_5$. That is, only when the release of fructose from the central complex is faster than the isomerization of E·NADH can this disparity be seen. While it is reasonable to suggest that the EA complex might be expected to undergo some sort of conformational change, we can adduce no evidence from our data for a kinetically discernible isomerization step involving this transitory complex.

Finally, the higher order concave-upward intercepts replot (last entry, Table II) arising when mannitol is saturating and at the limit of solubility (693 mM) and NAD^+ is the variable substrate could only occur if mannitol 1-phosphate binds more than twice to one (or both) of the EQ conformers. It is tempting to speculate that this situation could arise as a consequence of the fact that the conformational change we infer in this transitory complex opens up additional sites on the protein, which are thus rendered susceptible to interaction with the dead-end inhibitor.

The picture that emerges from this study indicates that mannitol oxidation proceeds by an ordered Bi-Bi reaction (Mechanism I) with NAD^+ and D-fructose as the first reac-

MECHANISM I.



tant-in, product-out, respectively. Since the rate-limiting step in the slow direction is associated with the release of NAD^+ from the E·NAD complex, and the values of the dissociation constants K_{ia} and K_{iq} (0.174 and 0.065 mM) are indicative of tight binding, it appears that the E·NAD and E·NADH complexes constitute the predominant enzyme forms. From an analysis of the kinetic data it is inferred that the E·NADH complex undergoes a conformational change that might possibly be the locus of the rate-limiting step in the forward direction, but no evidence on this point is yet available. It will be recalled (Ueng et al., 1976) that the organism is grown in a culture media containing a high level of mannitol (275 mM) as the sole source of carbon. It would be counterproductive, therefore, if substrate inhibition by mannitol were operative as a control mechanism for this enzyme. In fact, no evidence of substrate inhibition was observed up to the limit of solubility for mannitol. The enzyme is, however, uniquely susceptible to metabolic control by mannitol 1-phosphate acting as a dead-end inhibitor (see Figure 1 of Ueng et al., 1976). This polyol phosphate binds twice and nonpreferentially to the E and/or EA forms of the enzyme when the levels of mannitol and NAD^+ are relatively low, but multiply (i.e., more than twice) and predominantly to one (or both) of the EQ conformers when the level of mannitol is sufficiently high to drive the reaction rapidly through the central complexes. Thus, the degree to which mannitol is directed to catabolic pathways or diverted to fabrication of key metabolic precursors of cell wall material is governed, to a great extent, by the presence of mannitol 1-phosphate.

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Kinetics of Native and Activated Isozymes of Horse Liver Alcohol Dehydrogenase†

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ABSTRACT: The major isozymes of horse liver alcohol dehydrogenase (EC 1.1.1.1), EE, ES, and SS, have been separated by chromatography on phosphocellulose. Product inhibition studies showed that the kinetic behavior of EE and SS isozymes was consistent with the ordered BiBi mechanism. The different primary structures of the E and S subunits were expressed with higher Michaelis constants for ethanol and acetaldehyde and lower activity for the SS isozyme when compared with the EE isozyme. The differences for SS isozyme are reflections of slower rates of association and dissociation of coenzymes and slower rates of hydrogen transfer, not of affinities for the substrates. The contributions of each subunit in ES isozyme to the kinetic constants were not additive, indicating that the subunits may not act independently. Activation of the isozymes by amidination and alkylation suggested that lysine residues were present at the active sites of both E and S subunits. Kinetic studies indicated that isonicotinimidylation increased enzyme activity of the three isozymes by increasing the rates of dissociation of the enzyme–coenzyme complexes.

Horse liver alcohol dehydrogenase (EC 1.1.1.1) is heterogeneous; 12 isozymic forms have been reported [(Lutstorf et al., 1970; Pietruszko and Theorell, 1969; Pietruszko and Ryzewski, 1976); reviews: Pietruszko, 1975; Brändén et al., 1975]. Three of the major isozymes are dimers of two distinct subunits (Pietruszko et al., 1969; Pietruszko and Theorell, 1969). Jörnvall (1970) showed that the E subunit (active toward ethanol) and the S subunit (ethanol and steroid substrates) differed by at least six amino acid substitutions. The structures of the remaining isozymes have not been determined.

Kinetic studies have shown that the major isozymes (EE, ES, and SS) differ in Michaelis constants and specificities for various coenzymes, alcohols, and aldehydes and in pH dependencies (Lutstorf et al., 1970; Gurr et al., 1972; Theorell et al., 1970; Pietruszko, 1973), but the kinetic mechanisms of the ES and SS isozymes have not been determined. The E and S subunits act independently of each other in the binding of NADH[1] (Theorell et al., 1970), but they may interact with one another in catalyzing the oxidation of ethanol and cyclohexanol (von Wartburg et al., 1974).

The predominant EE isozyme is generally purified by modifications of the methods of Dalziel (1958, 1960), Theorell et al. (1966), and Taniguchi et al. (1967). Seven isozymes have been separated by chromatography on CM-cellulose and DEAE-cellulose (Lutstorf et al., 1970). Affinity chromatography on N^6-(6-aminohexyl)-AMP-Sepharose can be used to separate purified EE and SS isozymes (Andersson et al., 1974) and to purify SS isozymes (Andersson et al., 1975).

In order to study the ES and SS isozymes further, we used a simple method to isolate the major isozymes. The kinetics

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[1] Abbreviations used: NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; EDTA, ethylenediaminetetraacetic acid; CM, carboxymethyl; DEAE, diethylaminoethyl; AMP, adenosine monophosphate; Tris, tris(hydroxymethyl)aminomethane; NADD, 4-deuterio-NADH.