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Original scientific paper

Isomerization of an enzyme-coenzyme complex in yeast alcohol dehydrogenase-catalyzed reactions

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Abstract: In this work, all the rate constants in the kinetic mechanism of the yeast alcohol dehydrogenase-catalyzed oxidation of ethanol by NAD^+, at pH 7.0, 25 ºC, have been estimated. The determination of the individual rate constants was achieved by fitting the reaction progress curves to the experimental data, using the procedures of the FITSIM and KINSIM software package of Carl Frieden. This work is the first report in the literature showing the internal equilibrium constants for the isomerization of the enzyme-NAD^+ complex in yeast alcohol dehydrogenase-catalyzed reactions.

Keywords: yeast alcohol dehydrogenase, kinetic mechanism of action.

INDRODUCTION

The kinetic mechanism of yeast alcohol dehydrogenase (YADH) (EC 1.1.1.1, cytoplasmic, constitutive) has been investigated by numerous workers.[1–11] These investigations established conclusively the kinetic mechanism of this enzyme with a variety of substrates.

The partial set of individual rate constants in the kinetic mechanism was reported by Dickinson and Dickenson,[4] obtained with initial rate methods. In this work, a novel approach was applied, in order to obtain a full complement of all the rate constants in the mechanism. The values of all rate constants, throughout the entire catalytic cycle, in the yeast alcohol dehydrogenase-catalyzed oxidation of ethanol with NAD^+, have been determined, including the isomerization step, at pH 7.0, 25 ºC. This was achieved by using the procedures for fitting the reaction progress curves to the experimental data, as described by Carl Frieden.[12–14]

EXPERIMENTAL

Materials

The kinetic measurements in this work were performed with a single batch of a yeast alcohol dehydrogenase preparation (lyophilized powder), donated by Dr. A. Karsten of Boehinger Mannheim. NAD^+

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(Grade V, 99 %) and NADH (Grade IV, dipotassium salt, 98 %) were obtained from Sigma. Ethanol and acetaldehyde were obtained from Sigma and distilled before use; their concentrations were determined enzymatically.[15] All other chemicals were of the highest grade purity obtainable from commercial sources.

Methods

The concentration of the enzyme protein in solution was determined according to Hayes and Velick[16] and the concentration of enzyme active sites by the fluorescent method of Leskovac *et al.*[17] Initial velocity studies were performed and reaction progress curves were recorded in a double-beam spectrophotometer fitted with thermostated cuvette holders; the reaction rates were determined from the initial linear phase of reaction progress curves at 25 ºC. The kinetic measurements were performed in 0.1 M sodium phosphate buffer, pH 7.0, supplemented with 0.5 mM EDTA and 0.325 mM glutathione. The initial rate data were collected at 4 or 5 different concentrations of a variable and of a constant substrate. The initial rate data in the forward direction, oxidation of alcohols, obtained by varying the concentration levels of both substrates, were fitted to Eq. (1) with the Fortran program of Cleland.[18]

$$\frac{v_0}{E_0} = \frac{V_1 AB}{K_{iA} K_B + K_B A + K_A B + AB} \quad (1)$$

where v_0 is the initial rate (M s^{-1}), E_0 the concentration of enzyme active sites (M), V_1 the catalytic constant in the forward direction (s^{-1}), K_A and K_B the Michaelis constants for NAD^+ and alcohols (M), K_{iA} the inhibitory constant for NAD^+ (M), and A and B the concentrations of NAD^+ and alcohols (M), respectively.

In the fully reversible, Steady-State Ordered Bi Bi mechanism, the rate equation in the reverse direction is analogous to Eq. (1):

$$\frac{v_0}{E_0} = \frac{V_2 PQ}{K_{iQ} K_P + K_P Q + K_Q P + PQ} \quad (2)$$

In the reverse direction, reduction of aldehydes, the kinetic constants are: V_2, the catalytic constant in the reverse direction (s^{-1}), K_Q and K_P the Michaelis constants for NADH and aldehydes (M), K_{iQ} the inhibitory constant for NADH (M), and Q and P the concentrations of NADH and aldehydes (M), respectively.[19]

For the estimation of the rate constants, the KINSIM and FITSIM software packages,[12–14] obtained from Professor Carl Frieden and revised by Dr. Quoc Dang, were implemented throughout this work. The KINSIM and FITSIM software packages may be obtained from the Carl Frieden's website, and downloaded directly from the same: http://biochem.wustl.edu/cflab/.

For the implementation of the KINSIM and FITSIM computer programs, two reaction progress curves in the forward direction (E1, E2) and three in the reverse direction (A1, A2, A3) were analyzed; the starting concentrations of substrates assured that reactions were predominantly ordered (Table I).

TABLE I. Starting concentrations of substrates used for recording the reaction progress curves

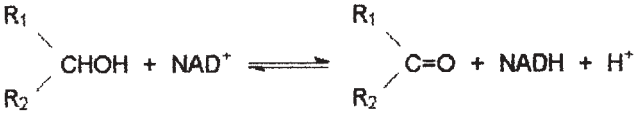
File number		E1	E2	A1	A2	A3
Enzyme	(nM)	2.0	2.034	1.55	0.2	0.28
Ethanol	(mM)	413.4	16.78	–	–	–
NAD^+	(μM)	488.45	495.6	–	–	–
Acetaldehyde	(mM)	–	–	0.1939	20.27	0.1987
NADH	(μM)	–	–	170.0	30.48	30.42

The experimental data were collected in the form of five files, each with 18 – 25 data points. The fitting procedure was accomplished in three steps, as described in the Results section.

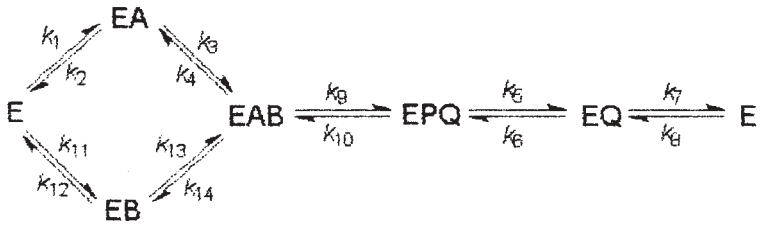
RESULTS

Kinetic mechanism of action of enzyme

Yeast alcohol dehydrogenase (EC 1.1.1.1, constitutive, cytoplasmic) catalyzes the following reversible reaction[11]:



Analysis of the steady-state kinetic data indicates that the yeast enzyme follows the *steady-state random* mechanism on the alcohol side, and the *steady-state ordered* mechanism on the aldehyde side of the catalytic cycle, with lower aliphatic alcohols and aldehydes, including propan-2-ol[11,20,21] (Scheme 1)



Scheme 1.

Table II shows the steady-state kinetic constants for yeast alcohol dehydrogenase-catalyzed oxidation of ethanol with NAD^+ at pH 7.0.

TABLE II. Steady-state kinetic constants of the yeast alcohol dehydrogenase catalyzed reaction: ethanol + $NAD^+ \rightleftharpoons$ acetaldehyde + NADH + H^+

Constant		This work pH 7.0 25 ºC	Dickinson and Monger[3] pH 7.0 25 ºC
V_1	(s^{-1})	350.5 ± 32	454
K_A	(μM)	239 ± 38	109
K_{iA}	(μM)	1137 ± 737	325
K_B	(mM)	18.7 ± 11.8	21.7
V_1/K_A	($mM^{-1}s^{-1}$)	1485 ± 105	4167
V_1/K_B	($mM^{-1}s^{-1}$)	18.75 ± 10.6	20.9
V_1K_{iA}/K_A	(s^{-1})	1667	1353
V_2	(s^{-1})	1997 ± 68	3846
K_Q	(μM)	92.4 ± 6.7	96
K_{iQ}	(μM)	16.8 ± 6.1	12.5
K_P	(mM)	0.49 ± 0.04	0.93
V_2/K_Q	($\mu M^{-1}s^{-1}$)	21.28 ± 0.87	40
V_2/K_P	($mM^{-1}s^{-1}$)	3992 ± 221	4135
V_2K_{iQ}/M_Q	(s^{-1})	358.7	501
K_{eq} ($\times 10^3$)[a]	–	0.068	0.195

[a] $K_{eq} = V_1K_{iQ}K_P/V_2K_{iA}K_B$

Determination of rate constants

In order to determine the rate constants in the kinetic mechanism of the yeast enzyme, two dozens reaction progress curves were recorded with YADH-catalyzed reaction: ethanol + $NAD^+ \rightleftharpoons$ acetaldehyde + NADH + H^+, at pH 7.0, 25 °C, a dozen in the forward and a dozen in the reverse direction, with widely different starting concentrations of the substrates.

The kinetic mechanism for this enzyme at neutral pH and with the above substrates, is steady-state random from the alcohol side and strictly ordered from the aldehyde side of the catalytic cycle (Scheme 1). Therefore, five reaction progress curves were chosen, with starting concentrations of substrates which assured a predominantly ordered mechanism for the best part of reaction progress curves (Scheme 2).

Scheme 2

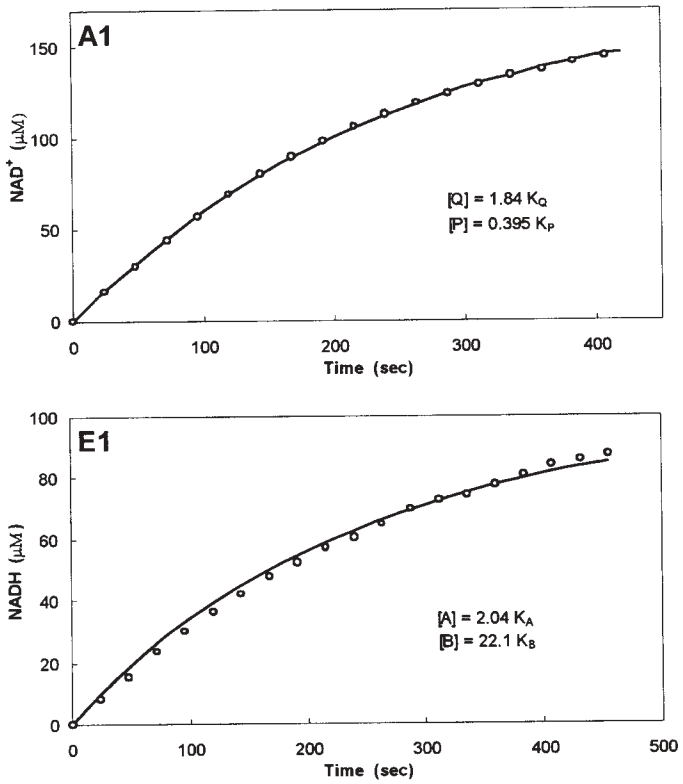


Fig. 1. Reaction progress curves for the two experiments from Table I, one in the forward direction (E1) and the other in the reverse direction (A1). The figures show the experimental points (open circles) and the calculated reaction progress curve (full line) estimated by computer programs from the mechanism in Scheme 2, and assuming that the rate constants have the values listed in Table IV.

The inclusion of the isomerization equilibrium k_{31}/k_{41} was justified by the direct proof for the isomerization step obtained recently by Northrop.[22,23] Thus two reaction progress curves in the forward direction (E1, E2) and three in the reverse direction (A1, A2, A3) were analyzed; the starting concentrations of substrates assured that reactions were predominantly ordered (Table I). The experimental data were collected in the form of five files, each with 18 – 25 data points. The fitting procedure was accomplished in three steps.

First step. Four rate constants were initially estimated from the steady-state kinetic data; in an ordered mechanism, some of the rate constants can be determined directly from the kinetic constants (Table II). In an ordered bisubstrate mechanism, $k_7 = V_2K_{iQ}/K_Q$ and $k_8 = V_2/K_Q$.[24] Further, with the yeast enzyme, the rate limiting step in the reverse direction is predominantly the dissociation of the E.NAD$^+$ complex; thus k_2 is approximately equal to V_2. The dissociation constant of the E.NAD$^+$ complex at pH 7.0 (300 μM) was determined independently[10]; from this value, the rate constant k_1 can be estimated from the relationship $k_1 = k_2/K_{E.NAD^+}$. Thus the initial estimate of the rate constants k_1, k_2, k_7 and k_8 was accomplished. The initial estimates of the rate constants k_5, k_6, k_9 and k_{10} were taken from the work of Dickinson and Dickenson.[4]

Initially, each file was fitted visually using the FITSIM program, changing always only one rate constant at a time. This procedure was repeated several hundred times, with each file in turn, until a satisfactory visual fit was obtained for each file with a single set of rate constants. In this way, a crude estimate of all the rate constants was obtained.

Second step. In the second step, all five files were introduced into the KINSIM program together with all the rate constants estimated as described above. Then, all the rate constants were kept fixed, except for one pair, such as k_1/k_2 or k_3/k_4, *etc.*; then, the sum of the squared differences between the calculated and experimental data points (sum of squares of SSQ) was determined. This procedure was repeated over one hundred times with many different combinations of rate constants until an absolute minimum in SSQ was obtained. This was the best statistical fit, and is labeled in Table III as the "best fit".

TABLE III. Rate constants in an Ordered Bi Bi mechanism (Scheme 2)

Rate constant		Best fit	Calculated
k_1	($\mu M^{-1}s^{-1}$)	7.0	7.0 ± 0.2
k_2	(s^{-1})	2100	2100 ± 57
k_3/k_4	(mM)	158.8	158.5
k_9	(s^{-1})	4000	3978 ± 97
k_{10}	(s^{-1})	35000	35036 ± 869
k_5	(s^{-1})	11000	10896 ± 158
k_6	($\mu M^{-1}s^{-1}$)	5.0	4.99 ± 0.04
k_7	(s^{-1})	395	388 ± 5
k_8	($\mu M^{-1}s^{-1}$)	28.5	28.1 ± 0.5
K_{E,NAD^+}	(μM)	300	300
$K_{E,NADH}$	(μM)	13.8	13.8

Third step. In the third step, all five files were introduced into the KINSIM program, together with all rate constants labeled as the "best fit". Then, again, all the rate constants were kept fixed, except for one pair, such as k_1/k_2 or k_3/k_4, *etc.*; the value for each pair of rate constants plus the standard error was calculated and introduced into Table III as the "calculated" value. The individual rate constants k_3 and k_4 could not be calculated, but only their ratio.

Recently, the value of the equilibrium constant for the isomerization equilibrium (k_{42}/k_{31}) was reported to be 75.[22,23] From this value, it was possible to estimate the equilibrium constant k_{41}/k_{32} (Table IV).

TABLE IV. Thermodynamics of the yeast alcohol dehydrogenase reaction, at pH 7.0, 25 ºC

Rate constant				Dissociation constant			ΔG^0 (kJ/mol)[a]
k_1	($\mu M^{-1}s^{-1}$)	7 ± 0.2	(11)[b]	k_2/k_1	(μM)	300	–20.08
k_2	(s^{-1})	2100 ±57	(3900)[b]				
k_4/k_3	(μM)	158500	(–)[b]	k_{41}/k_{31}	–	75[c]	10.70
				k_{42}/k_{32}	(μM)	2110[d]	–15.26
k_9	(s^{-1})	3980 ± 97	(4000)[b]	k_{10}/k_9		8.75	5.37
k_{10}	(s^{-1})	35040 ± 870	(35000)[b]				
k_5	(s^{-1})	10900 ± 160	(11000)[b]	k_5/k_6	(μM)	2180	15.18
k_6	($\mu M^{-1}s^{-1}$)	5.0 ± 0.04	(4.3)[b]				
k_7	(s^{-1})	388 ± 5	(480)[b]	k_7/k_8	(μM)	13.80	27.73
k_8	($\mu M^{-1}s^{-1}$)	28.1 ± 0.5	(44)[b]				
						Total	23.64
				$K_{eq} = 0.000068$[a]			23.7

[a] The Gibbs free energy was calculated from the relationship $\Delta G^0 = -RT \ln K_{eq}$.; [b] Data in parentheses are from the steady-state kinetic measurements of Dickinson and Dickenson[4], at pH 7.0, 25 ºC.; [c] Taken from Northrop.[22,23]; [d] Calculated from the equilibrium constant, $k_4/k_3 = (k_{41}/k_{31})/(k_{42}/k_{32})$; [e] Calculated from the Haldane relatioship, $K_{eq} = V_1K_{iQ}K_P / V_2K_{iA}K_B$.

The fitting procedure described above, permitted the estimation of all the rate constants in the mechanism, and the calculated data were compared with experimental results. The goodness of the fit is shown in Fig. 1.

Figure 1 shows the reaction progress curves for the two experiments from Table I, one in the forwared direction (E1) and the other in the reverse direction (A1). The figure shows the experimental points (open circles) and the calculated reaction progress curves (full line) calculated by computer programs from the mechanism in Scheme 2, and assuming that the rate constants have the values listed in Table IV. In Fig. 1, a satisfactory fit between the measured data and the computer fit to the same data, was achieved.

DISCUSSION

In this work, the kinetic properties of two different preparations of yeast alcohol dehydrogenase are compared. The first one was the home-made preparation of Dickinson,[3,4] and the second one was the commercial preparation of the yeast enzyme investigated in this work; the steady-state kinetic parameters for both preparations are shown in Table II. The individual rate constants in the mechanism were estimated by steady-state kinetic methods for the home-made preparation, and by procedures for fitting the reaction progress curves for the commercial preparation (Table IV).

The individual rate constants in the mechanism are shown in Table IV; despite the differences in enzyme preparation, the rate constants are almost identical for both preparations, except those which are connected with the binding and release of coenzymes.

From Table II, it can be seen that the maximum rate relationship,[21] $V_1K_{iA}/K_A < V_2$, is satisfied for the redox pair ethanol/acetaldehyde at pH 7.0; this is consistent with an ordered addition of products in the reverse direction, reduction of aldehyde. On the other hand, the V_2K_{iQ}/K_Q function is almost equal V_1 for ethanol and acetaldehyde at pH 7.0. In the past, this equality was usually interpreted as a fact implying that the dissociation of the E.NADH complex is the rate-limiting step in the forward direction, oxidation of alcohols.[4,25]

The mechanism shown in Scheme 1 is compatible with deuterium kinetic isotope effects reported for ethanol, $^{D}V_1 = 1.8$, $^{D}(V_1/K_A) = 1.8$, and $^{D}(V_1/K_B) = 3.2$,[5] around neutrality. With ethanol, the effect on $^{D}(V_1/K_A)$ was smaller than on $^{D}(V_1/K_B)$, suggesting that NAD^+ binds before ethanol; the still significant size of $^{D}(V_1/K_A)$ is probably due to dissociation of NAD^+ from the ternary complex.[6]

This work presents the first complete report in the literature of internal equilibria in the yeast enzyme-catalyzed oxidation of an alcohol by NAD^+, including the isomerization of the enzyme-NAD^+ complex.

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ИЗВОД

ИЗОМЕРИЗАЦИЈА ЕНЗИМ-КОЕНЗИМ КОМПЛЕКСА У РЕАКЦИЈАМА КОЈЕ КАТАЛИШЕ АЛКОХОЛ-ДЕХИДРОГЕНАЗА КВАСЦА

ВЛАДИМИР ЛЕСКОВАЦ[1], СВЕТЛАНА ТРИВИЋ[2] и ДРАГИЊА ПЕРИЧИН[1]

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У овом раду, ми смо одредили све константе брзине у кинетичком механизму алкохол-дехидрогеназе квасца, која каталише оксидацију етанола са NAD^+, на pH 7,0, 25 ºC. Појединачне константе брзине смо израчунали из крива прогреса реакције, уз употребу FITSIM и KINSIM компјутерских програма Карла Фридена. У овом раду су, први пут у литератури, објављене интерне равнотежне константе за изомеризацију ензим-NAD^+ комплекса у реакцијама које каталише алкохол-дехидрогеназа квасца.

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