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The Reaction Mechanism of Yeast Alcohol Dehydrogenase (ADH), Studied by Overall Reaction Velocities

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Previous authors have investigated the kinetics of the yeast ADH system at pH 7.9. This pH was unsuitable for distinguishing between different possible reaction mechanisms, because the maximum overall reaction velocities (V_f and V_r) in both directions happened to be similar. We have now found that V_f and V_r are greatly different at pH 7.15 and 6.0, and new kinetic data are presented. These data are consistent with the postulate that coenzyme and substrate form a ternary complex with the enzyme, in which a rate limiting intramolecular reaction takes place. The affinity of DPN for the enzyme was found to increase with increasing concentration of ethanol, and *vice versa*, whereas the affinity of DPNH decreased with increasing concentration of acetaldehyde and *vice versa*.

The reaction mechanism of Alberty postulating oxidation-reduction of the enzyme protein itself with the formation of enzyme-substrate, or enzyme-coenzyme intermediates was found unlikely from our experimental data. Our data exclude the reaction mechanism probably occurring in the liver ADH system, which required that a ternary complex, if formed, must be shortlived in comparison with the enzyme-coenzyme complexes.

An enzymatic method for preparing reduced diphosphopyridine nucleotide has been described.

Equations relating Michaelis constants, maximum velocities and the equilibrium constant have been developed [1,2,3] in kinetic approaches to the selection of possible mechanisms of enzyme action. The relationship between these constants presents a means of testing the possibility of a particular mechanism.

Such an approach to the mode of action of yeast alcohol dehydrogenase has been carried out by Negelein and Wulff [4] and by Hayes and Velick [5,6]. However, their data were obtained at pH 7.9 which did not allow clear distinction between different possible reaction mechanisms. This is evident from

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the fact that the relationship between the equations as developed by Alberty [2], is determined by the ratio V_f/V_r*, and this ratio was less than 2 at that pH.

In an effort to obtain a greater V_f/V_r ratio and thereby to distinguish more definitely between the different alternatives, we determined the overall velocity constants at pH 7.15 and 6.0. It was found that V_f/V_r was 8.1 and 10, respectively, at these pH values. These conditions therefore were much more favorable for distinguishing between various possible mechanisms.

MATERIALS AND METHODS

Crystalline yeast alcohol dehydrogenase (yeast ADH)

A commercial preparation from C. F. Behringer & Soehne, G.m.b.H., Mannheim, was used. The maximum turnover number of this preparation with acetaldehyde and DPNH was found to be 30 000 min^{-1} at pH 7.15, and 23 000 min^{-1} at pH 6.0 (23°). At pH 7.9 Negelein and Wulff [4] found 15 600 min^{-1} for their preparation. The experiments were carried out in the presence of M/100 versene, since the enzyme is readily poisoned by heavy metals. However, yeast ADH has recently been shown to contain zinc [7], which might be essential for the activity and removed by versene. We have therefore investigated the velocity at pH 7.15 in the presence and absence of 0.001 M versene. No effect of versene was observed under these conditions.

DPN AND DPNH

(Oxidized and reduced diphosphopyridine nucleotide).

DPN was prepared according to the method of Neilands and Åkeson [8], and this preparation was reduced enzymatically to obtain DPNH.

DPNH was prepared by modification of existing methods, the main difference being that boiling of the solution to inactivate the enzyme was avoided. To 25 ml of 0.1 M sodium carbonate/bicarbonate buffer pH 9.6 was added 0.4 ml ethanol, 300 μg yeast ADH, and 1 ml of DPN solution (20 mg/ml in H_2O). During the reaction period, three additional 300 μg portions of ADH, four 1 ml portions of DPN solution, and four portions of 0.4 ml ethanol were added. When about 90 % of the DPN was reduced, the solution was placed under vacuum for some 5 minutes, to remove acetaldehyde, the pH was adjusted to 10.0 and a last addition of enzyme finally made. 80 ml ethanol were added and the solution kept at $-10°$ C for 10 minutes. The precipitate containing the enzyme was removed by centrifugation and discarded. DPNH was precipitated by adding 320 ml ethanol + 400 ml ether at $-10°$. The precipitate flocculated in 5—10 minutes, was collected by centrifugation, washed with ether and dried. The yield was approximately 70 % of the theoretical. There remained 2—4 % unreduced DPN. A solution containing 60 mg DPNH per ml was only moderately yellow.

The oxidation of DPNH or reduction of DPN was followed by fluorescence measurements as described previously [9]. For concentrations of DPNH above 16 μM, a sensitive recording spectrophotometer was used. This apparatus was constructed at this institute and will be described elsewhere. The use of these instruments allowed more accurate determinations of the kinetic constants than can be obtained in an ordinary Beckman spectrophotometer.

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* V_f = maximum velocity for forward reaction.
V_r = » » » reverse »

All the kinetic constants presented in this paper have been determined twice and found to check within ± 10 % with the exception of those obtained at low concentration of the second reactant where the deviations were somewhat higher.

Alberty [2] has introduced a new type of kinetic constant, called K_{AB}, which may be calculated by several different methods from kinetic data at concentrations of A and B, insufficient to saturate the enzyme (for instance, A = DPN, B = ethanol in our case).

K_{AB} is a function of ordinary and "apparent" Michaelis constants:

$$K_{AB} = K'_A K_B + [B_0](K'_A - K_A) = K_A K'_B + [A_0](K'_B - K_B) \quad (1)$$

where K_A and K_B = ordinary Michaelis constants; $[A_0]$ and $[B_0]$ = concentration of A or B at $t = 0$; and K'_A and K'_B = apparent Michaelis constants for A and B, respectively. The apparent Michaelis constant is determined in the same way as is the ordinary K_m, except that the concentration of the reactant which is kept constant (the second reactant) is made insufficient to saturate the enzyme, preferentially below K_m. We have determined K_{AB} in both ways indicated in the equation above and presented the average value in Table 1, column I, and in Table 2.

Our sensitive fluorimetric and spectrophotometric methods made it possible to determine with a fair degree of accuracy the initial velocities even in such cases where both A and B were low and the changes occurring in the concentration of DPNH therefore small and decreasing rapidly within a few seconds.

RESULTS AND DISCUSSION

Linear relationships have been obtained between reciprocal initial velocities and reciprocal concentrations of substrate and coenzyme over large concentration ranges, and no deviation from straight lines were observed at pH 6.0. However, at pH 7.15, deviation towards higher activity took place when the concentration of DPN and alcohol were very high (See Fig. 1). This occurred above 100 mM alcohol in the presence of 800 μM DPN, but was not observed in the presence of 38 μM DPN and 280 mM alcohol, nor in the presence of 6.2 mM alcohol and 800 μM DPN. There are of course several possible explanations for this activation. For example, the catalytic reaction of the ADH·DPN·alcohol complex may be increased due to changes in the charge distribution on the surface of the enzyme at alcohol concentrations high enough to influence the dielectric constant. The activation is not observed at low DPN concentrations because other steps may be rate limiting. In this connection it may be recalled that liver ADH was inhibited by concentrations of alcohol higher than 5 mM. Under the conditions studied, the dissociation of the ADH·DPNH complex was the rate-limiting reaction, and this complex may have been stabilized by the high alcohol concentrations due to changes in the dielectric properties of the medium. This is in agreement with the interpretation of the effect of salts on this reaction.

In the following calculations, all data relate only to the linear parts of the plots.

Table 1. Kinetic data from Table 2 inserted into four formulae representing different reaction sequences [2].

Buffer: phosphate, μ = 0.1 + versene, M/1000	I Ternary complex formed, relatively slow intramolecular transformation of the complex. General mech. of Alberty [2] $K_{eq}^{*} = \frac{V_f}{V_r} \cdot A^{**} \times 10^{11}$	II Special case of I. Affinity of ADH for DPN(H) not influenced by substrate, or *vice versa* $K_{eq} = \frac{V_f}{V_r} \cdot B^{***} \times 10^{11}$	III A group in the enzyme is oxidized/reduced by substrate (or coenzyme) with the formation of enzyme-substrate intermediates $K_{eq} = \left(\frac{V_f}{V_r}\right)^2 \cdot B \times 10^{11}$	IV Relative stable ADH · DPN(H) complexes, shortlived ternary complex formed $K_{eq} = \left(\frac{V_f}{V_r}\right)^3 \cdot B \times 10^{11}$
pH 7.15	1.1	17.9	2.2	0.27
pH 6.0	0.81	4.5	0.45	0.045

* $K_{eq} = 0.9 \times 10^{-11}$ over the whole pH range.

** $A = \frac{K_{ald} \cdot DPNH}{K_{alc} \cdot DPN}$; For the definition of these constants, see Alberty [2].

*** $B = \frac{K_{DPNH} \cdot K_{ald}}{K_{DPN} \cdot K_{alcohol}}$

Table 2. Michaelis constants and maximum reaction velocities for yeast alcohol dehydrogenase (23°). Buffer: phosphate, μ = 0.1 + versene, M/1000.

Concentration of second reactant		Kinetic Constants		
pH 7.15	pH 6.0		pH 7.15	pH 6.0
μM	μM		μM	μM
185	185	K'_m, DPNH	14	12
5000	5000	K'_m, DPNH	38	24
13.5	13.2	K'_m, aldehyde	250	140
150	80	K'_m, aldehyde	550	300
0.62×10^4	5.5×10^4	K'_m, DPN	200	167
84×10^4	84×10^4	K'_m, DPN	57	160
38	77	K'_m, ethanol	50000	96000
800	800	K'_m, ethanol	18000	100000
		$K_{DPNH} \cdot ald$ *	5500	1300
		$K_{DPN} \cdot alc$	4.2×10^6	16×10^6
		V_f, sec^{-1} **	61	38
		V_r, sec^{-1}	491	380
		V_f/V_r	0.124	0.100

* For definition of these constants, see Alberty [2]. The constants can be calculated in two ways, and average values have been used (see eq. (1)).

** V_f is maximum velocity of ethanol oxidation.
V_r " " " " aldehyde reduction.

Mol.wt. of enzyme is 150 000[5,6].

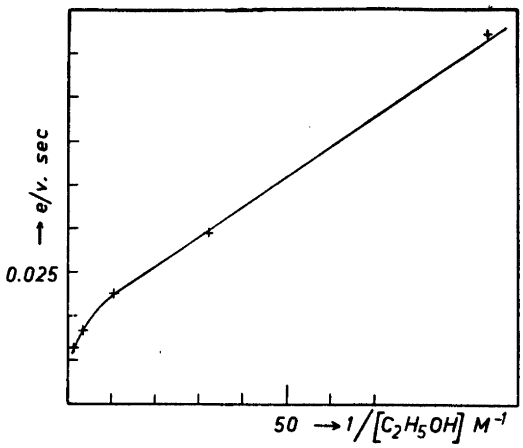

Fig. 1. Lineweaver-Burk plot of yeast alcohol dehydrogenase with varied alcohol concentration. Conc. of DPN: 770 μM. Buffer: phosphate, μ = 0.1 + M/1000 versene. pH = 7.15, 23° C.

It is apparent from Table 1 that case I agrees well with the experimental data. This equation was developed [2] by the steady state treatment of a reaction between enzyme and two substrates (one of the substrates may be coenzyme) to form a ternary complex, in which an intramolecular reaction occurs to form another ternary complex. The velocity of this reaction is assumed to be slow compared with the rates of formation and breakdown of binary and ternary complexes. From this relationship it is not possible to decide whether two or more intermediary complexes are formed, or whether the substrate and the coenzyme combine and dissociate in a certain order. However, the investigations of Hayes and Velick [5] have shown that binary complexes of the composition ADH·$(DPN)_4$ and ADH·$(DPNH)_4$ can be formed in the absence of substrate. Whether binary complexes of the type ADH·alcohol or ADH·acetaldehyde can be formed is not known. It has been reported that some acetaldehyde is nonspecifically attached to yeast ADH [4]. Liver ADH contains bound ethanol in non dialyzable form [10].

Case II represents the general mechanism described above with the additional assumption that the affinity of the coenzyme for the enzyme is uninfluenced by the concentration of substrate, and *vice versa*. Negelein and Wulff [4] and Hayes and Velick [5,6] in kinetic studies of yeast ADH at pH 7.9 found the equation presented in column II to agree well with their experimental data. However, it is clear from our data that yeast ADH does not follow this reaction mechanism at pH 7.1 and 6.0. First, the relationship between kinetic constants does not give the correct equilibrium constant (see Table 1). Second, as shown in Table 2, the apparent Michaelis constant, K_m, of DPN and alcohol, as well as DPNH and acetaldehyde were in most cases affected by changes in the concentration of the second reactant. K_m for alcohol and DPN decreased with increasing concentrations of the second reactant at pH 7.1, whereas at pH 6.0 there was no effect. K_m for acetaldehyde and DPNH (both at pH 7.1 and 6.0) increased with increasing concentration of the second reactant.

Case III presumes an oxidation-reduction of the enzyme itself with the formation of enzyme-substrate intermediates. The involvment of thiol groups of DPN-linked enzymes in hydrogen transfer has been suggested by several authors [11,12], but strong arguments have been presented against such a mechanism for yeast ADH [13,14]. The results obtained with deuterium-labeled substrate exclude all mechanisms of yeast alcohol dehydrogenase which allow hydrogen exchange with the medium. Such an exchange would probably have taken place if thiol groups were directly involved in the hydrogen transfer. Case III is therefore improbable from previous experiments. Our own data may not be accurate enough to eliminate this reaction scheme. In the unlikely case that 10 % error in the determination of each of the different kinetic constants added up in the same direction, the error in K could give a deviation from 0.9 to 0.44 or from 0.9 to 2.2 (See Table 1).

In case IV the assumption has been made that the ternary complex is extremely short-lived and dissociates into products much more rapidly than do the binary complexes (ADH·DPN and ADH·DPNH).

In some recent experiments [9] we have confirmed that liver ADH appears to follow Case IV. Considering the great difference in the properties of yeast and liver ADH [5,6] it is not surprising that the two enzymes could catalyze the same reaction in different manners. A further difference was revealed by the reaction velocities in the presence of sodium chloride. This salt had no effect on V_f and V_r for the yeast enzyme, whereas V_f was increased and V_r was decreased with the liver enzyme [9]. At relative low concentration of the second reactant, yeast ADH was competively inhibited by 0.15 M NaCl using alcohol, DPN and DPNH as variable reactants. The effect of formate [9], however, was qualitatively the same with both enzymes: strong inhibition was observed except in the presence of high alcohol concentrations.

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