Acid-Base Catalysis in the Yeast Alcohol Dehydrogenase Reaction*

(Received for publication, August 99, 1974)

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SUMMARY

The effect of pH on steady state kinetic parameters for the yeast alcohol dehydrogenase-catalyzed reduction of aldehydes and oxidation of alcohols has been studied. The oxidation of \( \text{p-CH₃ benzaldehyde-1,1-} \) and \(-1,1-\text{d₂} \) by \( \text{NAD⁺} \) was found to be characterized by large deuterium isotope effects \( (k_{\text{H}}/k_{\text{D}} = 4.1 \pm 0.1) \) between pH 7.5 and 9.5, indicating a rate-limiting hydride transfer step in this pH range; a plot of \( k_{\text{cat}} \) versus pH could be fit to a theoretical titration curve, \( pK = 8.25 \), where \( k_{\text{cat}} \) increases with increasing pH. The Michaelis constant for \( \text{p-CH₃ benzyl alcohol} \) was independent of pH. The reduction of \( \text{p-CH₃ benzaldehyde} \) by \( \text{NADH} \) and reduced nicotinamide adenine dinucleotide with deuterium in the 4-A position (NADD) could not be studied below pH 8.5 due to substrate inhibition; however, between pH 8.5 and 9.5, \( k_{\text{cat}} \) was found to decrease with increasing pH and to be characterized by significant isotope effects \( (k_{\text{H}}/k_{\text{D}} = 3.3 \pm 0.3) \). In the case of acetaldehyde reduction by \( \text{NADH} \) and NADD, isotope effects were found to be small and essentially invariant \( (k_{\text{H}}/k_{\text{D}} = 2.0 \pm 0.4) \) between pH 7.2 and 9.5, suggesting a partially rate-limiting hydride transfer step for this substrate; a plot of \( k_{\text{cat}}/K_b \) (where \( K_b \) is the Michaelis constant for acetaldehyde) versus pH could be fit to a titration curve, \( pK = 8.25 \). The titration curve for acetaldehyde reduction has the same \( pK \) but is opposite in direction to that observed for \( \text{p-CH₃ benzyl alcohol} \) oxidation. The data presented in this paper indicate a dependence on different enzyme forms for aldehyde reduction and alcohol oxidation and are consistent with a single active site side chain, \( pK = 8.25 \), which functions in acid-base catalysis of the hydride transfer step.

Yeast alcohol dehydrogenase (EC 1.1.1.1) catalyzes the following interconversion of aldehydes and alcohols (1):

\[
\begin{align*}
R-C & \quad + \text{NADH} + H^+ = R-\text{CH₂OH} + \text{NAD⁺} \\
& \quad \text{(1)}
\end{align*}
\]

The equilibrium equation for this interconversion requires a proton, and it is likely that the alcohol product acquires its hydroxyl proton prior to leaving the enzyme. However, the uptake of the proton in the course of the forward reaction could occur directly from the solvent, or alternatively, a catalytic residue at the enzyme active site could function in acid-base catalysis. It is possible to postulate several mechanisms which involve such catalysis. According to Scheme 1A and B, proton uptake by alcohol dehydrogenase occurs prior to aldehyde reduction; in Scheme 2, proton uptake by alcohol dehydrogenase occurs subsequent to aldehyde reduction. The distinction between Scheme 1A and 1B concerns whether proton transfer from an enzyme catalytic residue to substrate takes place prior to or concomitant with the hydride transfer step (Scheme 1A), as opposed to after hydride transfer (Scheme 1B). Yeast alcohol dehydrogenase is a zinc-metallo enzyme (2) and a possible role for zinc is indicated in Schemes 1B and 2. An important feature distinguishing Scheme 1 from Scheme 2 and from a mechanism involving the direct uptake of proton from solvent is the requirement for different enzyme forms in the direction of aldehyde reduction and alcohol oxidation. Although it is frequently not possible to obtain unambiguous information concerning acid-base catalysis at enzyme active sites from the pH dependence of kinetic constants, the observation of opposite pH profiles for the forward and reverse directions of an enzyme reaction characterized by a pH-dependent equilibrium equation would provide strong evidence for acid-base catalysis and permit a determination of the \( pK \) of the catalytically active group.

Previous studies on the yeast alcohol dehydrogenase-catalyzed interconversion of aromatic substrates indicated a rate-limiting hydride transfer at pH 8.5 (3, 4). Thus, it appeared possible to study the effect of pH on the hydride transfer step for both aldehyde reduction and alcohol oxidation. In this paper the effect of pH on steady state kinetic parameters for the reduction of acetaldehyde and \( \text{p-CH₃ benzaldehyde} \) by \( \text{NADH} \) and NADD, and the oxidation of \( \text{p-CH₃ benzyl alcohol-1,1-} \) and \(-1,1-\text{d₂} \) by \( \text{NAD⁺} \) are reported. The determination of isotope effects as a function of pH was undertaken in order to evaluate the extent to which the hydride transfer step is rate-limiting at each pH. The effect of pH on the enzyme-catalyzed oxidation of ethanol was reported by previous authors to result in a 4-fold decrease in rate between pH 9.5 and 6.5 (5).

\[^{1}\text{The abbreviations used are: NAD⁺, reduced nicotinamide adenine dinucleotide with deuterium in the nicotinamide 4-A position; E BH, a protonated enzyme form; EB, a free base enzyme form.} \]
MATERIALS AND METHODS

All chemicals were reagent grade unless otherwise indicated. Yeast alcohol dehydrogenase was obtained as an ammonium sulfate suspension from Boehringer Mannheim, and was dialyzed prior to use as previously described (3). Liver alcohol dehydrogenase was obtained from Worthington in its lyophilized form. Determinations of pH were carried out on a Radiometer type PM26 equipped with an expanded scale attachment. Kinetic studies were carried out on a Cary 118B recording spectrophotometer; constant temperature was maintained at 25°. Nuclear magnetic resonance spectra were obtained on a Varian HA-100.15 spectrometer.

Coenzymes and Substrates—Except where indicated, substrates were prepared and assayed as described previously (3). NAD+, grade III, was purchased from Sigma and was used without further purification; solutions of NAD+ were assayed enzymatically. NADH and NADD were prepared either by ethanol precipitation as described by Rafter and Colowick (6) or by acetone precipitation as described by Oppenheimer et al. (7), followed by DEAE-column chromatography. Reduced coenzymes prepared by either method were identical with regard to their kinetic properties. p-CH₃ benzyl alcohol-1,1-ß₂ and p-CH₃ benzyl alcohol-1,1-ß₄ were prepared in parallel experiments by reduction of p-CH₃ benzyl chloride with either lithium aluminum hydride (Calbiochem) or lithium aluminum deuteride (Merck, Sharp and Dohme of Canada), 99 atom % deuterium (8). The kinetic properties of p-CH₃ benzyl alcohol (Aldrich) recrystallized from heptane to constant melting point agreed with those of p-CH₃ benzyl alcohol-1,1-ß₂ obtained by lithium aluminum hydride reduction. The isotopic purity of p-CH₃ benzyl alcohol-1,1-ß₄ was confirmed by the absence of methylene protons at C-1 of product (r = 4.8 ppm, for protonated product), as ascertained by nuclear magnetic resonance. Solutions of p-CH₃ benzyl alcohol were assayed at 25°, pH 8.5 in F₁ glycine buffer containing 1 mg/ml of 3-acetyl-
Table I

pH Dependence of steady state kinetic constants for oxidation of p-CH$_3$ benzyl alcohol-1,1-h$_3$ and -1,1-d$_3$ by NAD$^+$

<table>
<thead>
<tr>
<th>pH</th>
<th>$k_H$</th>
<th>$k_D$</th>
<th>$k_H'</th>
<th>$k_D'</th>
<th>$K_{a\text{H}}</th>
<th>$K_{a\text{D}}</th>
<th>$K_{b\text{H}}</th>
<th>$K_{b\text{D}}</th>
<th>$K_{\text{cat}}</th>
<th>$K_{\text{cat}}'</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>m$^{-1}$s$^{-1}$</td>
<td>m$^{-1}$s$^{-1}$</td>
<td>m$^{-1}$s$^{-1}$</td>
<td>m$^{-1}$s$^{-1}$</td>
<td>m$^{-1}$</td>
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<td>m$^{-1}$</td>
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<td>m$^{-1}$</td>
</tr>
<tr>
<td>7.5</td>
<td>0.77</td>
<td>0.057</td>
<td>4.0</td>
<td>0.60</td>
<td>0.40</td>
<td>1.0</td>
<td>9.2</td>
<td>11.0</td>
<td>0.87</td>
<td>0.32</td>
</tr>
<tr>
<td>8.1</td>
<td>0.60</td>
<td>0.75</td>
<td>-</td>
<td>0.46</td>
<td>-</td>
<td>-</td>
<td>13.1</td>
<td>-</td>
<td>-</td>
<td>0.19</td>
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<td>8.5</td>
<td>0.96</td>
<td>0.23</td>
<td>4.1</td>
<td>0.48</td>
<td>0.30</td>
<td>1.6</td>
<td>11.1</td>
<td>13.4</td>
<td>0.84</td>
<td>0.24</td>
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<tr>
<td>9.1</td>
<td>0.90</td>
<td>0.09</td>
<td>-</td>
<td>0.63</td>
<td>-</td>
<td>-</td>
<td>11.1</td>
<td>-</td>
<td>-</td>
<td>0.37</td>
</tr>
<tr>
<td>9.5</td>
<td>1.23</td>
<td>0.30</td>
<td>4.2</td>
<td>0.52</td>
<td>0.43</td>
<td>1.2</td>
<td>7.6</td>
<td>9.9</td>
<td>0.77</td>
<td>0.42</td>
</tr>
</tbody>
</table>

*The symbols a and b refer to NAD$^+$ and alcohol, respectively. In the pH range 7.2 to 8.1, the buffer contained 80 to 112 mM Pi, $\mu = 0.22$. At pH 8.5 and above, the buffer contained 26 to 90 mM PP$_i$, 61 to 140 mM glycine and 0 to 5 mM KCl, $\mu = 0.22$. pyridine adenine dinucleotide and liver alcohol dehydrogenase. Assays were initiated by the addition of alcohol and monitored at 234 nm ($\epsilon$$_{234}$ mm$_{-1}$ cm$^{-1}$ = 6.2).

Kinetic Measurements—Kinetic measurements were carried out at 25$^\circ$, $\mu = 0.22$, by measuring either the disappearance or appearance of NADH or NADD at 340 nm. In order to compare kinetic data on different days the activity of dialyzed enzyme was determined using a standard assay and normalized to a specific activity = 300 units per mg. Kinetic constants reported here are corrected for activity losses due to handling and dialysis and are larger by a factor of 3 than previously reported constants (3, 4). The general form of the rate equation for a two-substrate enzyme is given by

$$V = \frac{V_{\text{max}} \cdot B}{K_a + K_B + AB}$$

(2)

where according to the nomenclature of Cleland (9), $V$ = maximum velocity; $K_a$ = limiting Michaelis constant for $A$; $K_B$ = limiting Michaelis constant for $B$; and $K_{a\alpha}$ = inhibition constant. Primary reciprocal plots were analyzed for $1/V$ and $K/\nu$ by a weighted least squares computer program described by Cleland (10). Secondary reciprocal plots were analyzed by an unweighted least squares method. The data have been calculated from $V$ assuming four active sites per mole.

RESULTS

With the exception of substrate inhibition in the case of p-CH$_3$ benzaldehyde reduction, linear double reciprocal plots have been observed in these studies. The data are summarized for the oxidation of p-CH$_3$ benzyl alcohol-1,1-h$_3$ and -1,1-d$_3$ between pH 7.5 and 9.5. The data indicate large invariant isotope effects on $k_{\text{cat}}$ in the pH range investigated. In Fig. 1a, the pH dependence of $k_{\text{cat}}$ is plotted for p-CH$_3$ benzaldehyde oxidation. The data are seen to conform to a theoretical titration curve, $pK = 8.25$. Values for $k_{\text{cat}}$ have been calculated from $V$ assuming four active sites per mole.

DISCUSSION

It was previously concluded from substituent and isotope effect studies that hydride transfer is the rate-limiting step for the yeast alcohol dehydrogenase-catalyzed interconversion of p-substituted benzaldehydes and benzyl alcohols at pH 8.5 (3, 4). On the basis of isotope effects reported here (Tables I and II) hydride transfer is concluded to be rate-limiting for p-CH$_3$ benzaldehyde oxidation between pH 7.5 and 9.5 and for p-CH$_3$ benzaldehyde reduction between pH 8.5 and 9.5. The larger isotope effects observed in the direction of alcohol oxidation are consistent with the deuterium at C-1 of the alcholic substrate, and most likely reflect secondary isotope effects superimposed on the primary
kinetic isotope effect (11). Although the range of pH studied for p-CH₃ benzaldehyde reduction is limited, values of $k_{cat}$ for the reduction of this aldehyde decrease with increasing pH. This effect is in contrast to the effect of pH on alcohol oxidation, Fig. 1A, suggesting a role for an active site side chain, pK = 8.25, in acid-base catalysis of the hydride transfer step as illustrated in Scheme I.

An examination of kinetic parameters for acetaldehyde reduction reveals comparatively smaller isotope effects and a relatively weak pH dependence of $k_{cat}$, suggesting that hydride transfer is only partially rate-limiting for acetaldehyde reduction in the pH range 7.2 to 9.5. The kinetic mechanism for the interconversion of acetaldehyde-ethanol has been described as a preferred ordered mechanism (12, 13), and can be represented by the following scheme (14):

$$
A \\ B \\
E \rightarrow EAB \rightarrow E' \rightarrow A'B' \\
$$

where A is NAD⁺, B is ethanol, $B'$ is acetaldehyde, and $A'$ is NADH. Although the kinetic equation for $k_{cat}$ for acetaldehyde reduction will contain the rate constant for the release of NAD⁺ from enzyme, $k_{cat}/K'$, where $K'$ is the Michaelis constant for acetaldehyde) is independent of this step.*

* An interpretation of $k_{cat}/K'$, where $K'$ refers to the Michaelis constant for NADH (D), depends on the extent to which the kinetic mechanism is compulsory ordered with coenzyme binding first, since in such a situation $k_{cat}/K'$ equals the reciprocal of the rate constant for the addition of coenzyme to enzyme.

** The isotope effect at pH 8.5 is obtained from the difference in intercept between Hammett plots for $k_b$ versus $k_p$ (3).

* Previously reported isotope effects for acetaldehyde reduction at pH 7.6 $k_7/k_6 = 1.8$ (19) and $k_H/k_IOT = 2.1$ (20).

** See Ref. 21.

$$
\frac{k_{cat}}{K'} = \frac{k_B k_{eh}}{k_6 + k_{eh} + k_6} \\
$$

Thus, to the extent that product dissociation is rate-limiting for acetaldehyde reduction, $k_{cat}/K'$ is a simplification of the rate expression and a better approximation of the hydride transfer step. In this context it should be noted that isotope effects on $k_{cat}/K'$ are greater than isotope effects on $k_{cat}$ for acetaldehyde reduction at pH 8.1, 8.5, and 9.1 (Table II). An examination of Equation 4 also indicates that when the release of acetaldehyde and ethanol from ternary complex is fast relative to the interconversion of ternary complex ($k_{eh} > k_{eh} + k_{eh}$), $k_{cat}/K'$ equals the ratio of $k_{eh}$ to $k_6$, the dissociation constant for the release of acetaldehyde from ternary complex: pH effects on substrate binding, which may diminish or eliminate a pH dependence of $k_{cat}$, are therefore also contained in $k_{cat}/K'$.

As illustrated in Fig. 1B, values of $k_{cat}/K'$ for acetaldehyde reduction conform to a theoretical titration curve. This curve is characterised by the same pK (8.25) but is opposite in direction to that observed for p-CH₃ benzyl alcohol oxidation, confirming a dependence on different enzyme forms for acetaldehyde reduction (EBH) and alcohol oxidation (EB). The data also indicate that the enzyme-catalyzed interconversion of both slow and fast substrates is dependent on an active site side chain with the same pK (8.25).
site of lactate dehydrogenase by x-ray crystallography (16). Although it is not possible to exclude a direct coordination of an active site zinc to the aldehyde carbonyl (Scheme 1B), the obligatory uptake of a proton prior to hydride transfer for aldehyde reduction supports a scheme in which a single active site side chain functions as both acid and base catalyst in the hydride transfer step (Scheme 1A). The similarity of the pK of zinc-bound water, pK = 8.7 (17), to the pH dependence of the yeast alcohol dehydrogenase-catalyzed interconversion of ternary complex, pK = 8.25, indicates a possible catalytic role for zinc-bound water-hydroxide; in a recent study, Sloan et al. (18) report distances between substrate and metal in cobalt-substituted liver alcohol dehydrogenase which are consistent with a catalytic role for a metal-bound hydroxide ion. However, the data presented here on the yeast alcohol dehydrogenase mechanism are also consistent with the participation of an active site amino acid side chain, e.g. imidazole, cysteine, or lysine, in acid-base catalysis.

Acknowledgment—The author wishes to thank Ms. Yvonne Li for excellent technical assistance.

REFERENCES
Additions and Corrections

Vol. 250 (1975) 2569-2573

In Klinman, Judith P. Acid-Base Catalysis in the Yeast Alcohol Dehydrogenase Reaction

Page 2572, Equation (4) should read:

$$\frac{k_{cat}}{K_v} = \frac{k_1 k_2 K_v}{k_3 + k_4 k_5 + k_7 k_8}$$

and second column, line 9 should read:

$$(k_7 k_8 > k_3 k_5 + k_7 k_8)$$

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