Acid-Base Catalysis in the Yeast Alcohol Dehydrogenase Reaction*

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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 p-CH₃ benzyl alcohol-1,1-1₃ and -1,1-d₂ by NAD⁺ was found to be characterized by large deuterium isotope effects (kₜ/kₐ = 4.1 ± 0.1) between pH 7.5 and 9.5, indicating a rate-limiting hydride transfer step in this pH range; a plot of kₜ versus pH could be fit to a theoretical titration curve, pK = 8.25, where kₜ increases with increasing pH. The Michaelis constant for p-CH₃ benzyl alcohol was independent of pH. The reduction of p-CH₃ benzaldehyde by 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, kₜ was found to decrease with increasing pH and to be characterized by significant isotope effects (kₜ/kₐ = 3.3 ± 0.3). In the case of acetaldehyde reduction by NADH and NADD, isotope effects were found to be small and essentially invariant (kₜ/kₐ = 2.0 ± 0.4) between pH 7.2 and 9.5, suggesting a partially rate-limiting hydride transfer step for this substrate; a plot of kₜ 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 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):

\[
R-C \quad + \text{NADH} \quad + \text{H}^+ \quad = \quad R-\text{CH}_2\text{OH} \quad + \quad \text{NAD}^+ \quad (1)
\]

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1 The abbreviations used are: NADD, reduced nicotinamide adenine dinucleotide with deuterium in the nicotinamide 4-A position; EBH, 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 PM20 equipped with an expanded scale attachment. Kinetic studies were carried out on a Cary 118B recording spectrophotometer; constant temperature was maintained at 25°C. 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 NAD⁡⁺ 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-δ₂ and p-CH₃ benzyl alcohol-1,1-δ₄ 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-δ₂ obtained by lithium aluminum hydride reduction. The isotopic purity of p-CH₃ benzyl alcohol-1,1-δ₂ was confirmed by the absence of methylene protons at C-1 of product (δ = 4.6 ppm, for protonated product), as ascertained by nuclear magnetic resonance. Solutions of p-CH₃ benzyl alcohol were assayed at 25°C, pH 8.5 in FP₁ glycine buffer containing 1 mg/ml of 3-acetyl-

![Scheme 1. Proton uptake prior to aldehyde reduction.](image1)

![Scheme 2. Proton uptake subsequent to aldehyde reduction.](image2)
pyridine adenine dinucleotide and liver alcohol dehydrogenase. Assays were initiated by the addition of alcohol and monitored at 340 nm ($\epsilon_{340}$ mm$^{-1}$ cm$^{-1}$). Assays were initiated by the addition of alcohol and monitored at 340 nm ($\epsilon_{340}$ mm$^{-1}$ cm$^{-1}$). Assays were initiated by the addition of alcohol and monitored at 340 nm ($\epsilon_{340}$ mm$^{-1}$ cm$^{-1}$). Assays were initiated by the addition of alcohol and monitored at 340 nm ($\epsilon_{340}$ mm$^{-1}$ cm$^{-1}$). Assays were initiated by the addition of alcohol and monitored at 340 nm ($\epsilon_{340}$ mm$^{-1}$ cm$^{-1}$). Assays were initiated by the addition of alcohol and monitored at 340 nm ($\epsilon_{340}$ mm$^{-1}$ cm$^{-1}$). Assays were initiated by the addition of alcohol and monitored at 340 nm ($\epsilon_{340}$ mm$^{-1}$ cm$^{-1}$).

**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. In Table I values for $k_{cat}$, $K_a$, $K_b$, and $K_a$ are summarized for the oxidation of p-CH$_3$ benzyl alcohol-1,1-$\text{H}$ and -1,1-$\text{D}_2$ between pH 7.5 and 9.5. The data indicate large invariant isotope effects on $k_{cat}$ in the pH range investigated. In Fig. 1A, the pH dependence of $k_{cat}$ for p-CH$_3$ benzyl alcohol oxidation. The data are seen to conform to a theoretical titration curve, $pK = 8.25$. Since values for $K_a$ are independent of pH, the pH dependence of $k_{cat}/K_a$ is the same as the dependence of $k_{cat}$. In Table II values for $k_{cat}$, $K_a$, $K_b$, and $K_a$ are summarized for p-CH$_3$ benzaldehyde and acetaldelyde reduction by NADH and NADD. Due to an increase in substrate inhibition with decreasing pH, the range of pH studied for p-CH$_3$ benzaldehyde reduction was limited to 8.5 to 9.5. The data indicate larger isotope effects for p-CH$_3$ benzaldehyde reduction than acetaldelyde reduction; the isotope effects on $k_{cat}$ and $K_m$ previously reported by this author for acetaldelyde reduction at pH 8.5 (3) were found to be too large upon reinvestigation (4), and the corrected isotope effects are given in Table II. As is illustrated in Fig. 1B, the pH dependence of $k_{cat}/K_b$ for acetaldelyde reduction can be fit by a theoretical titration curve, $pK = 8.25$.

**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$_3$ benzyl alcohol oxidation by yeast alcohol dehydrogenase. The solid line is a theoretical titration curve for a group of pH 8.25. The reference point for $k_{cat}$ is pH 8.1, and the reference point for $k_{cat}/K_b$ is pH 8.5. The value for $k_{cat}/K_b$ at pH 8.1 was omitted from this figure as the deviation of this point from the line exceeded 4 times the average standard deviation of all other data points.

![Fig. 1. A. pH dependence of p-CH$_3$ benzyl alcohol-1,1-$\text{H}$ (○--○) and -1,1-$\text{D}_2$ (●--●) oxidation by NAD$.+$ catalyzed by yeast alcohol dehydrogenase. The solid line is a theoretical titration curve for a group of pH 8.25. Values for $k_{cat}$ and $k_D$ plotted in this figure are relative to $k_0$ and $k_D$ at pH 9.5, which was taken as the reference point. B. pH dependence of $k_{cat}/K_b$ for acetaldelyde reduction by NAD$(+)$(○--○) and NADD (●--●) catalyzed by yeast alcohol dehydrogenase. The solid line is a theoretical titration curve for a group of pH 8.25. The reference point for $k_{cat}/K_b$ is pH 8.1, and the reference point for $k_{cat}/K_b$ is pH 8.5. The value for $k_{cat}/K_b$ at pH 8.1 was omitted from this figure as the deviation of this point from the line exceeded 4 times the average standard deviation of all other data points.](http://www.jbc.org/)

![Table I. pH Dependence of steady state kinetic constants for oxidation of p-CH$_3$ benzyl alcohol-1,1-$\text{H}$ and -1,1-$\text{D}_2$ by NAD$^+$](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>pH</th>
<th>$k_{H}$</th>
<th>$k_{D}$</th>
<th>$k_{H}/k_{D}$</th>
<th>$K_{a}^{(H)}$</th>
<th>$K_{a}^{(D)}$</th>
<th>$K_{b}^{(H)}$</th>
<th>$K_{b}^{(D)}$</th>
<th>$K_{i}^{(H)}$</th>
<th>$K_{i}^{(D)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5</td>
<td>0.77</td>
<td>0.057</td>
<td>4.5</td>
<td>0.60</td>
<td>0.40</td>
<td>1.0</td>
<td>1.0</td>
<td>0.40</td>
<td>0.40</td>
</tr>
<tr>
<td>8.1</td>
<td>0.60</td>
<td>-</td>
<td>-</td>
<td>0.46</td>
<td>-</td>
<td>13.0</td>
<td>-</td>
<td>0.46</td>
<td>-</td>
</tr>
<tr>
<td>8.5</td>
<td>0.96</td>
<td>0.23</td>
<td>4.2</td>
<td>0.48</td>
<td>0.30</td>
<td>1.6</td>
<td>1.6</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>9.1</td>
<td>0.90</td>
<td>-</td>
<td>-</td>
<td>0.63</td>
<td>-</td>
<td>11.6</td>
<td>-</td>
<td>0.63</td>
<td>-</td>
</tr>
<tr>
<td>9.5</td>
<td>1.23</td>
<td>0.30</td>
<td>4.1</td>
<td>0.52</td>
<td>0.63</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</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 P$_i$, $\mu = 0.22$. 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, $\mu = 0.22$. The general form of the rate equation for a two-substrate enzyme is given by

$$v = V \cdot A \cdot B$$

where $V$, $A$, and $B$ are the maximum velocity; $K_a$ = limiting Michaelis constant for $A$; $K_b$ = limiting Michaelis constant for $B$; and $K_{ia}$ = inhibition constant. Primary reciprocal plots were analyzed for $1/v$ and $K/v$ by a weighted least squares computer program described by Cleland (10). Secondary reciprocal plots were analyzed by an unweighted least squares fit for $1/v$, $K_a$, $K_b$, and $K_{ia}$. Values for $k_{cat}$ have been calculated from $V$ assuming four active sites per mole.

![Fig. 1. A. pH dependence of p-CH$_3$ benzyl alcohol-1,1-$\text{H}$ (○--○) and -1,1-$\text{D}_2$ (●--●) oxidation by NAD$.+$ catalyzed by yeast alcohol dehydrogenase. The solid line is a theoretical titration curve for a group of pH 8.25. Values for $k_0$ and $k_D$ plotted in this figure are relative to $k_{cat}$ and $k_{D}$ at pH 9.5, which was taken as the reference point. B. pH dependence of $k_{cat}/K_b$ for acetaldelyde reduction by NAD$(+)$(○--○) and NADD (●--●) catalyzed by yeast alcohol dehydrogenase. The solid line is a theoretical titration curve for a group of pH 8.25. The reference point for $k_{cat}/K_b$ is pH 8.1, and the reference point for $k_{cat}/K_b$ is pH 8.5. The value for $k_{cat}/K_b$ at pH 8.1 was omitted from this figure as the deviation of this point from the line exceeded 4 times the average standard deviation of all other data points.](http://www.jbc.org/)
The rate constant for the release of NAD$^+$ from enzyme, $k_{cat}/K'$, 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 ($k_{cat} > k_{cat} + k_{cat}$), $k_{cat}/K'$ equals the ratio of $k_{cat}$ to $k_{cat}$, 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 characterized by the same pK (8.25) but is opposite in direction to that observed for p-CH$_3$ benzyl alcohol oxidation, confirming a dependence on different enzyme forms for aldehyde reduction and oxidation of p-CH$_3$ benzyl alcohol which is demonstrated here, are previous reports on lactate dehydrogenase which have implicated different enzyme forms in the formation of ternary complexes, with pyruvate and lactate binding preferentially to the binary complex of coenzyme and EBH and EB, respectively (15). The pK for this process is 6.8, and involves the uptake or release of a proton to histidine-195, shown to be at the active site of the enzyme (16).
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.

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REFERENCES
Additions and Corrections

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In KLINMAN, JUDITH P. Acid-Base Catalysis in the Yeast Alcohol Dehydrogenase Reaction

Page 2572, Equation (4) should read:

\[
\frac{k_{\text{cat}}}{K_v} = \frac{\frac{k_1 k_2 K_v}{k_4 k_5 + k_6 k_7 + k_3 k_8}}{k_3 k_4 + k_5 k_6 + k_7 k_8}
\]

and second column, line 9 should read:

\[(k_4 k_8 > k_5 k_7 + k_6 k_6)\]
Acid-base catalysis in the yeast alcohol dehydrogenase reaction.
J P Klinman


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