Kinetic Cooperativity of Human Liver Alcohol Dehydrogenase $\gamma_2^{*}$

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Previous studies showed that natural human liver alcohol dehydrogenase $\gamma$ exhibits negative cooperativity (substrate activation) with ethanol. Studies with the recombinant $\gamma_2$ isoenzyme now confirm that observation and show that the saturation kinetics with other alcohols are also nonhyperbolic, whereas the kinetics for reactions with NAD$^+$, NADH, and acetaldehyde are hyperbolic. The substrate activation with ethanol and 1-butanol are explained by an ordered mechanism with an abortive enzyme-NADH-alcohol complex that releases NADH more rapidly than does the enzyme-NADH complex. In contrast, high concentrations of cyclohexanol produce noncompetitive substrate inhibition against varied concentrations of NAD$^+$ and decrease the maximum velocity to 25% of the value that is observed at optimal concentrations of cyclohexanol. Transient kinetics experiments show that cyclohexanol inhibition is due to a slower rate of dissociation of NADH from the abortive enzyme-NADH-cyclohexanol complex than from the enzyme-NADH complex. Fluorescence quenching experiments confirm that the alcohols bind to the enzyme-NADH complex. The nonhyperbolic saturation kinetics for oxidation of ethanol, cyclohexanol, and 1-butanol are quantitatively explained with the abortive complex mechanism. Physiologically relevant concentrations of ethanol would be oxidized predominantly by the abortive complex pathway.

Liver alcohol dehydrogenases (E.C. 1.1.1.1) catalyze the reversible oxidation of alcohols using NAD$^+$ as a cofactor. Class I alcohol dehydrogenases from human liver are homo- and heterodimers comprised of $a$, $b$, and $\gamma$ subunits (1). $Hs$ADH$^1_b$ and $Hs$ADH$^1_g$ have high catalytic efficiencies on ethanol and contribute significantly to its metabolism (1). Polymorphisms in the ADH$^3$ gene lead to the isoenzymes $Hs$ADH$^{1}_y$, which has Oln-271 and Val-349, and $Hs$ADH$^{2}_g$, with Arg-271 and Ile-349 (3). Genotyping indicates the allele frequency for ADH$^1_y$ is about 10% in East Asians and 43% in Europeans (4, 5). The $V_{\text{max}}$ for ethanol oxidation by $Hs$ADH$^1_y$ is 2.5 times higher than that for $Hs$ADH$^{2}_g$ (1, 6), and it was suggested that susceptibility to alcoholism and cirrhosis may be associated with the presence of $Hs$ADH$^{2}_g$ (1, 7). However, extensive studies have not established a correlation (4, 5, 8). Further insights into the possible roles of alcohol dehydrogenases in alcoholism require quantitative descriptions of the kinetics of the various enzymes involved, but the properties of $Hs$ADH$^1_y$ are a challenge because both isoenzymes exhibit negative cooperativity for ethanol oxidation (6), and the mechanism has not yet been described.

The negative cooperativity could arise from different mechanisms (9). Subunit interactions or “half-of-the-sites” reactivity (an extreme case of negative cooperativity) were reported for the horse liver E (ethanol active) enzyme (10, 11) but were later disputed (12–14). Other studies have found nonadditivity in the heterodimers of horse liver enzymes (15) and human liver enzymes (16), suggesting that the constituent subunits do not act independently. Nonhyperbolic kinetics may also involve mechanisms that do not include subunit interactions, such as a random mechanism (17), a mixture of isoenzymes (18), or an Ordered Bi Bi mechanism with an abortive complex pathway (19, 20). Negative cooperativity for ethanol oxidation was observed for a purified human liver alcohol dehydrogenase (18), which may have resulted from a mixture of isoenzymes in the preparation. Oxidation of cyclohexanol by horse liver alcohol dehydrogenase exhibits negative cooperativity (19), and oxidation of ethanol and benzyl alcohol show substrate inhibition (21–24). These results are explained by an ordered mechanism with alternative pathways, including abortive enzyme-NADH-alcohol and binary enzyme-NADH complexes, which differ in the rate of dissociation of NADH. Substrate activation and inhibition observed for recombinant $Hs$ADH$^{2}_g$ with different alcohols are explained by a similar mechanism.

EXPERIMENTAL PROCEDURES

Materials—The plasmid for the expression of $Hs$ADH$^{2}_g$ (3) was obtained from Dr. Jan-Olov Höög (Karolinska Institutet, Stockholm, Sweden). $Hs$ADH$^{2}_g$ was expressed and purified as described previously (25). Protein homogeneity was confirmed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The concentration of active sites was measured by titration with NAD$^+$ in the presence of 10 mM pyrazole (26). The turnover number of the purified enzyme in a standard assay at 25°C (27) was 0.5 s$^{-1}$. LINAD$^+$ (grade I) and NAD$^+$ (grade I) were obtained from Roche Molecular Biochemicals. Alcohols and carbonyl compounds were redistilled before use.

Kinetic Studies—Kinetic measurements were made in 50 mM sodium phosphate and 0.25 mM EDTA buffer, pH 7.5, at 25°C. Initial velocities were determined by monitoring the formation or oxidation of NADH with a Cary 118C spectrophotometer ($\varepsilon_{340} = 6.22 \text{mM}^{-1} \text{cm}^{-1}$) or an SLM Aminco 4800 fluorometer ($\lambda_{\text{ex}} = 340 \text{nm}$; $\lambda_{\text{em}} = 460 \text{nm}$) and fitting the progress curves to a line or a parabola, which calculates the initial slope. Very wide ranges of concentrations of alcohols were used as substrates in some experiments, and velocities at the lowest concentrations were used only if the background rates in the presence of NAD$^+$ and enzyme and in the absence of alcohol were less than 10% of the measured rate. Steady-state kinetic data were analyzed using Cleland’s programs (28). Data for the nonhyperbolic saturation curves were fit to the TOWNEO equation, Equation 1, which is the general form of the equation that describes either activation or partial inhibition by substrates.

$$v = \frac{V_{\text{sd}} B + B^2}{c + bB + B^2}, \quad K_b = \frac{b}{2} - d + \left[\left(\frac{b}{2} - d\right)^2 + c\right]^{1/2} \quad \text{(Eq. 1)}$$

$V$ is the velocity at saturating concentrations of the varied substrate, B, and $b$, $c$, and $d$ represent collections of rate constants from which $K_b$.

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The abbreviations used are: $Hs$ADH$^1_b$ and $Hs$ADH$^1_g$ human (Homo sapiens) liver alcohol dehydrogenase $\beta$ and $\gamma$ enzymes, respectively, otherwise named ADH$^1_B$ and ADH$^1_C$ (2); $Eq$ADHE, horse (Equus caballus) liver alcohol dehydrogenase E (ethanol active) enzyme.
Kinetic Cooperativity of Alcohol Dehydrogenase

may be calculated (28). Initial velocity data were fit with the SEQUEN program to the kinetic equation for a sequential Bi reaction in Equation 2,

\[ v = \frac{V_A B}{K_a + K_B + K_A + AB} \]  
(Eq. 2)

where \( v \) is the maximal velocity, \( A \) and \( B \) are coenzyme and substrate concentrations, \( K_a \) and \( K_b \) are the Michaelis constants, and \( K_{ab} \) is the dissociation constant for coenzyme. Data for inhibition by high concentrations of alcohol were fit with NONLIN (29) to the hyperbolic replot equation in Equation 3,

\[ v = \frac{V_{max} + V_{max B/K_d}}{1 + B/K_d} \]  
(Eq. 3)

where \( V_{max} \) and \( V_{max B} \) are the uninhibited and inhibited maximal velocities, respectively, and \( K_d \) is the dissociation constant for the alcohol, \( B \), from the inhibited form of the enzyme. Steady-state kinetics were simulated with KINSIM (30).

Transient kinetic experiments used a BioLogic SFM3 stopped-flow instrument with a dead time of 2.5 ms. Data were analyzed with the BioKine Software. The rate constant for association of \( \text{NAD}^+ \) was obtained as described previously (31) using pyrazole to trap the enzyme-NAD\(^+\) complex and form the ternary complex (26), which absorbs at 294 nm (\(\Delta_{294} = 8400 \text{ M}^{-1} \text{ cm}^{-1}\)). The transients were measured at varied concentrations of \( \text{NAD}^+ \) (0.09–0.27 mM) and pyrazole (0.5–5 mM) in 50 mM sodium phosphate, 0.25 mM EDTA, pH 7.5, at 25 °C. The rate constants obtained from the exponential phase of each of the traces were fit to the SEQUEN equation in Equation 2. The rate constant for binding of NADH to free enzyme was determined by following quenching of protein fluorescence with \(\Delta_{355} = 294 \text{ nm and } \Delta_{308} > 330 \text{ when } 2 \mu l \text{ enzyme was mixed with } 2–10 \mu l \text{ NADH in 50 mM sodium phosphate, } 0.25 \text{ mM EDTA, } \text{pH 7.5, at } 25 °C. \) The rate constant for binding of NADH to the enzyme-alcohol binary complex was determined as described above in the presence of either ethanol (100–400 mM) or cyclohexanol (1–100 mM). The rate of dissociation of NADH from enzyme was measured by trapping free enzyme with 40 mM AMP (a concentration determined to be saturating) and monitoring the absorbance increase at 355 nm (\(\Delta_{355} = 3800 \text{ M}^{-1} \text{ cm}^{-1}\)) that results from the shift in spectra for free and enzyme-bound NADH (32).

**RESULTS**

**Steady-state Kinetics**—The earlier report of negative cooperativity (substrate activation) for ethanol saturation kinetics with natural \( \text{HsADH}_{2g} \) isolated from human liver (6) was confirmed with homogeneous recombinant enzyme (Fig. 1A). Ethanol saturation fit well to the TWOONE equation (Equation 1), and the “concave-up” curvature of the Eadie-Hofstee plot (9) clearly indicates the negative cooperativity (Fig. 1A). Because these results were obtained for the recombinant enzyme, isoenzyme heterogeneity is not likely to be the origin of the observed phenomenon. When the data from Fig. 1A are replotted as in Fig. 1B, some substrate inhibition is also apparent with ethanol concentrations above 100 mM. The dashed and solid lines in Fig. 1 will be discussed later.

Saturation kinetics of \( \text{HsADH}_{2g} \) with other alcohols were also measured (Table I). Substrate inhibition, rather than activation, was observed for cyclohexanol (Fig. 1C) and 1-hexanol, and mechanisms for these alcohols seem to be similar. The saturation data for cyclohexanol fit well to the TWOONE equation. The kinetic data for 1-hexanol (0.004–8.5 mM) saturation could also be described by the TWOONE equation but were best fit to the equation describing classical substrate inhibition (SEQUEN, 28), although the highest concentration of alcohol decreased the velocity only to 80% of the maximum. Interestingly, 1-butanol (Fig. 1D) and \( \text{R-2-butanol} \) exhibit both activation and inhibition. Oxidation of 1-butanol and \( \text{R-2-butanol} \) reaches maximal rates of 0.72 s\(^{-1}\) and 0.19 s\(^{-1}\), respectively, before the inhibition is observed. The steady-state kinetics of methanol oxidation were hyperbolic, exhibiting no indication of activation or inhibition.

The initial velocity kinetics were studied in more detail by collecting data for the forward and reverse reactions with concentrations of the coenzymes and substrates varied over wide ranges. Kinetics for ethanol and cyclohexanol saturation showed nonhyperbolic behavior, even at saturating concentrations of \( \text{NAD}^+ \), which suggests that the activation and inhibition by the alcohols are not likely to be the result of a random Bi Bi mechanism (19). In contrast, the saturation kinetics for \( \text{NAD}^+ \), acetaldehyde, cyclohexanone, and NADH were hyperbolic.

Initial velocity data collected for physiologically relevant concentrations of ethanol fit well to the SEQUEN equation (Equation 2), and good estimates of the steady-state kinetic constants could be obtained (Table II). Similarly, by using the noninhibitory concentrations of cyclohexanol, a good fit to the SEQUEN equation was obtained. The \( K_{eq} \) values, calculated by the Haldane relationship from the steady-state kinetic constants for both ethanol and acetaldehyde or cyclohexanol and cyclohexanone reactions, are in good agreement with other reported values (33, 34), which indicates that the kinetic constants obtained from these initial velocity studies are self-consistent. The values for the Michaelis and inhibition constants for the ethanol and acetaldehyde reactions are somewhat smaller (3–4-fold) than those previously for \( \text{HsADH}_{2g} \) and clearly different from the values for the other human liver class I enzymes (6). Interestingly, the kinetic constants for the respective substrates are similar to those for \( \text{EqADHE} \) acting on ethanol (15) or cyclohexanol (19, 34), except that \( V_2 \) and \( V_2 \) for the horse liver enzyme are larger than those of \( \text{HsADH}_{2g} \). For ethanol oxidation by \( \text{HsADH}_{2g} \), the values for \( V_2 \) and \( K_{2} \) in Table II agree well with the values obtained from the TWOONE analysis (Table I). Thus, the values obtained from the initial velocity studies describe the activity in the activation region of the ethanol saturation curve.

**The Abortive Complex Pathway**—On the basis of the experimental results described herein, the participation of an abortive complex pathway (Scheme 1, \( A = \text{NAD}^+, \text{B} = \text{alcohol}, P = \text{substrate} \)
Kinetic Cooperativity of Alcohol Dehydrogenase

Initial velocities were measured in 50 mM sodium phosphate and 0.25 mM EDTA buffer, pH 7.5, at 25 °C with varied concentrations of alcohols and a saturating level of 2 mM NAD⁺. Values for V and Kᵣ were obtained from fits to the TWOONE equation (Equation 1), except for the data for methanol, which were fitted to the Michaelis-Menten equation, and for 1-hexanol, which were fitted to the SUBIN equation, \( \nu = V/K_k + B + B/K_k \), yielding \( K_k = 28 \) mM. The value of \( V \) for cyclohexanol represents the value extrapolated to high, inhibitory concentrations of alcohol. The errors were less than 15% of the values, which indicates that the fits are good (28).

### Table I

| Alcohol          | Saturation pattern | \( V \), \( s^{-1} \) | \( K_s \), \( \mu M \) | \( V/K_s \), \( s^{-1} \mu M \) |
|------------------|--------------------|------------------------|------------------------|-------------------------------|
| Ethanol          | Activation         | 0.55                   | 110                    | 5.0                           |
| Methanol         | Hyperbolic         | 0.093                  | 150,000                | 0.00062                       |
| Cyclohexanol     | Inhibition         | 0.12                   | 2.2                    | 55                            |
| 1-Hexanol        | Inhibition         | 0.31                   | 9.2                    | 34                            |
| 1-Butanol        | Activation & inhibition | 0.72               | 110                    | 6.5                           |
| R-2-Butanol      | Activation & inhibition | 0.19               | 640                    | 0.30                          |

### Table II

Steady-state kinetic constants for human liver alcohol dehydrogenase \( \gamma \)

| Constant | Ethanol/acetaldehyde | Cyclohexanol/cyclohexanone |
|----------|----------------------|----------------------------|
| \( K_{eq} \) (\( \mu M \)) | 2.8                  | 5                          |
| \( K_a \) (\( \mu M \))   | 140                  | 110                        |
| \( K_{pq} \) (\( \mu M \)) | 190                 | 1500                       |
| \( K_{eq} \) (\( \mu M \)) | 7.6                 | 2.0                        |
| \( K_{eq} \) (\( \mu M \)) | 65                  | 67                         |
| \( K_{pq} \) (\( \mu M \)) | 0.55                | 0.54                       |
| \( V_s \), \( s^{-1} \)  | 0.45                 | 0.35                       |
| \( V_c \), \( s^{-1} \)  | 14                   | 3.2                        |
| \( K_{eq} \) (\( \mu M \)) | 12                  | 410                        |

* Concentration ranges were 0.2–50 \( \mu M \) for ethanol, 0.005–1 \( \mu M \) for NAD⁺, 0.2–2 \( \mu M \) for acetaldehyde, and 5–30 \( \mu M \) for NADH.
* Concentration ranges were 10–220 \( \mu M \) for cyclohexanol, 3–20 \( \mu M \) for NAD⁺, 0.5–10 \( \mu M \) for cyclohexanone, and 3–10 \( \mu M \) for NADH.
* Calculated from the Haldane relationship, \( K_{pq} = (V/K_{eq}K_{as}[H^+]^q)/(V/K_{eq}K_{as}) \).

aldehyde or ketone, and \( Q = \text{NADH} \) is the best explanation for the nonhyperbolic kinetics observed for the steady-state kinetics with ethanol, cyclohexanol, and 1-butanol.

Steps 1–5 represent the rate constants that describe the Ordered Bi Bi mechanism. For the class I alcohol dehydrogenases, the rate-limiting step for the reaction mechanism is often the release of NADH (\( k_7 \)) for the oxidation of alcohols or release of NAD⁺ (\( k_7 \)) for the reductive reaction. The abortive complex pathway bypasses step 5 and includes steps 6–8. With saturating concentrations of NAD⁺ and concentrations of alcohol sufficient to form the enzyme-alcohol-NAD⁺ complex (EBQ), release of NADH from this complex (\( k_7 \)) may become rate-limiting. The presence of such a pathway can lead to either substrate activation or inhibition (19, 22–24). Substrate activation would be observed when \( k_7 > k_6 \), and substrate inhibition would result when \( k_7 < k_6 \). Substrate inhibition could also occur with low concentrations of NAD⁺ and concentrations of alcohol sufficient to bind to free enzyme (Scheme 1, step 8; Refs. 19 and 21). The rate equation for the abortive complex pathway describing alcohol saturation kinetics under conditions of saturating NAD⁺ takes the form of the TWOONE equation (Equation 1) when \( k_{-6} \gg k_7 \) and \( k_6 \gg k_5 \) or \( k_7 \) is assumed (Equation 4) (23)

\[
\nu = \frac{V/K_{pq}B(V + B)}{K_{pq} + (V/K_{pq}B + B)} \tag{4}
\]

where \( K_0 \) is the Michaelis constant for the alcohol, \( K_{pq} \) is the dissociation constant of the alcohol from the enzyme-alcohol-NAD⁺ abortive complex, \( k_{-6}K_{pq} \), and \( V \) is \( k_7 \).

The oxidation of various alcohols may be expected to differ in the relative rate constants for the steps in the abortive complex pathway and produce either substrate activation or inhibition that would be diagnostic for the abortive complex pathway. The following experimental results provide evidence for the abortive complex pathway and estimates for the rate constants for the mechanisms of reactions with three alcohols.

### Substrate Inhibition and Abortive Complex Formation—The kinetics of inhibition by concentrations of cyclohexanone exceeding those required for maximum velocity was analyzed by an initial velocity study with varied concentrations of NAD⁺ at different fixed concentrations of cyclohexanol (Fig. 2A). The inhibition is described by both hyperbolic (1/V) and linear slope (K/V) effects (Fig. 2B). The intercept effect suggests that cyclohexanol is exerting its inhibitory effect by binding to a form of the enzyme distinct from that to which NAD⁺ binds, such as enzyme-NADH, to form an abortive complex. That the intercept effect is hyperbolic indicates that the inhibited rate reaches a lower limit, which in the context of the abortive complex pathway would be limited by \( k_7 \). The fit to the hyperbolic replot equation of the apparent \( V_{\text{max}} \) (from each NAD⁺ saturation curve) versus cyclohexanol concentration (Fig. 2B) gives values for \( V_{\text{max}}, V_{\text{min}}, \) and \( K_{pq} \), which can be assigned to \( k_7, k_6, \) and \( K_{pq} \) in Equation 4, respectively. The presence of a slope effect for the inhibition also indicates that cyclohexanol and NAD⁺ can compete for free enzyme. The enzyme-cyclohexanol complex apparently does not readily bind NAD⁺ to form the active ternary complex. Transient kinetic experiments also showed that 400 mM 2,2,2-trifluoroethanol, an analogue of ethanol that does not react with the NAD⁺, decreases the rate constant for the association of NAD⁺ to cyclohexanol; all other errors are ≤15% of the fitted values.

![Scheme 1](image-url)
against NAD\(^+\) for binding to free enzyme (Fig. 2D). The effects on intercepts (1/\(V\)) are relatively small and do not permit a good fit to the hyperbolic replot equation. Because 1-butanol saturation kinetics exhibit substrate activation with low concentrations of alcohol and inhibition by high concentrations, it appears that the activation arises from the abortive complex pathway, whereas the inhibition involves competition between NAD\(^+\) and 1-butanol for free enzyme.

Evidence that the enzyme-NADH-alcohol abortive complex can form was obtained by measuring the quenching of NADH fluorescence during titration of the enzyme-NADH complex with cyclohexanol and 2,2,2-trifluoroethanol (Fig. 3). The dissociation constants of the alcohols from the abortive complexes were 11 and 12 mM for cyclohexanol and 2,2,2-trifluoroethanol, respectively.

The effects of the abortive complex on the kinetics were studied further by a quantitative analysis of the inhibition by cyclohexanol. The cyclohexanol saturation data (Fig. 1A) were fit with the hyperbolic replot equation (Equation 3; solid line). The values for \(V_{\text{max}}\), \(V_{\text{min}}\), and \(K_i\) from the fit to the hyperbolic replot equation of the data for cyclohexanol in Fig. 1B were 0.45 ± 0.03 s\(^{-1}\), 0.10 ± 0.05 s\(^{-1}\), and 34 ± 20 mM, respectively. The 1/\(V\) data in Fig. 1D were fit as well by a line as by the hyperbolic replot equation.

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and the presence of saturating concentrations of cyclohexanol. Data were combined for a quantitative analysis using KINSIM.

complete description of the substrate activation and inhibition value measured for $k_7$.

from stopped-flow experiments as described under "Experimental Procedures." Value from measurement with ethanol was used.

as an alternative to the classical Ordered Bi Bi pathway, as would 1-butanol. 1-Butanol and cyclohexanol, at high concentrations, can also bind appreciably to free enzyme and prevent NAD$^+$ binding, as $k_8$ (Scheme 1) is relatively small. This kind of inhibitory effect is also observed in the slope (KJV) effects observed for both 1-butanol and cyclohexanol inhibition against NAD$^+$ (Figs. 2 and D).

Sensitivity analysis was used to determine which rate constants in the simulation made major contributions to the overall velocity at different concentrations of alcohol (Table IV). At higher concentrations of alcohol, $k_5$ and/or $k_7$, the NADH release steps are important in controlling the rates. Simulations of the data using only the Ordered Bi Bi mechanism (without the abortive complex pathway) did not adequately describe the data (dashed lines in Fig. 1, A, B, and D).

**DISCUSSION**

The Abortive Complex Pathway—The nonhyperbolic kinetics of HsADH$_2$ (both substrate activation and inhibition) appear to be attributable to the presence of an abortive complex pathway as an alternative to the classical Ordered Bi Bi pathway (Scheme 1). This pathway involves the formation of the ternary enzyme-NADH-alcohol abortive complex from which the rate constant for dissociation of NADH ($k_{6b}$) is different from that for the binary enzyme-NADH complex ($k_6$). When $k_{6b} > k_6$, substrate activation is observed, and when $k_{6b} < k_6$, substrate inhibition is observed. Several lines of direct evidence support this explanation. It was shown that cyclohexanol and 2,2,2-trifluoroethanol could bind to the enzyme-NADH complex and that cyclohexanol decreased the rate of NADH dissociation to a level that is consistent with the level of inhibition observed in the steady-state kinetics. Effects of ethanol on NADH release were not detectable, but simulation of the steady-state kinetics

| TABLE III | Rate constants used to simulate the steady-state saturation kinetics |
|-----------|-------------------------|
| Rate constant | Ethanol | Cyclohexanol | 1-Butanol |
| $k_1$ (M$^{-1}$s$^{-1}$) | $6 \times 10^3$ | $6 \times 10^3$ | $6 \times 10^3$ |
| $k_2$ (s$^{-1}$) | 40 | 40 | 40 |
| $k_3$ (M$^{-1}$s$^{-1}$) | $2.4 \times 10^6$ | $2.2 \times 10^6$ | $1.2 \times 10^6$ |
| $k_4$ (s$^{-1}$) | 1200 | 750 | 300 |
| $k_5$ (s$^{-1}$) | 500 | 190 | 380 |
| $k_6$ (s$^{-1}$) | 600 | 170 | 600 |
| $k_7$ (s$^{-1}$) | 50 | 50 | 17 |
| $k_8$ (M$^{-1}$s$^{-1}$) | $9.2 \times 10^2$ | $5.6 \times 10^2$ | $2.8 \times 10^2$ |

$^a$ Rates obtained for EqADH (35).

$^b$ Rate constants used to simulate the steady-state saturation kinetics (Table III). The ratio of $k_{6b}/k_6$ is consistent with the measured values (Figs. 2B, 3, and 4, A and B).

within the experimental error.

NAD$^+$ association was measured with varied concentrations of NAD$^+$ and pyrazole, and the observed rate constants were fitted to the sequential Bi equation with SEQUEN to yield values for a limiting rate constant of 300 s$^{-1}$ and a bimolecular association rate constant for NAD$^+$ of 600 mM$^{-1}$ s$^{-1}$. The limiting unimolecular step is likely to be due to an isomerization in the NAD$^+$ association step, such as a conformational change upon coenzyme binding as is observed for EqADH (31). The rate constant for NADH association to enzyme, as measured by protein fluorescence quenching, is 2 μM$^{-1}$ s$^{-1}$. The same value was obtained for NADH association in the presence of ethanol, but a value of 1 μM$^{-1}$ s$^{-1}$ was obtained in the presence of saturating concentrations of cyclohexanol.

**Simulation of the Abortive Complex Pathway**—Because a complete description of the substrate activation and inhibition by the various alcohols requires assignment of rate constants for each step in the mechanism, the steady-state and transient data were combined for a quantitative analysis using KINSIM. The steady-state kinetics for the oxidation of ethanol, cyclohexanol, and 1-butanol at a fixed concentration of NAD$^+$ (Fig. 1) were simulated using determined or estimated values for the rate constants (Table III) for each of the abortive complex pathway in Scheme 1. The values for constants $k_2$, $k_3$, $k_{-3}$, $k_4$, and $k_{-4}$ were taken from studies with horse liver enzyme for each of the respective alcohols (35, 36). Rate constants $k_1$, $k_5$, $k_{-5}$, $k_{-d}/k_d$ (for reactions with ethanol and cyclohexanol), and $k_{-7}$ were measured by the experiments described above, and an estimate for $k_{-1}$ was calculated from $K_{	ext{app}}$ (Table II) and the value measured for $k_1$. The initial estimate for $k_{-4}$ for cyclohexanol was in the range of the measured rate constants in Figs. 2B, 4A, and 4B (0.08 to 0.17 s$^{-1}$) but was refined to produce the best simulation of the data. The value for $k_{-4}$ required by the simulation was 0.23 s$^{-1}$, which is in good agreement with the measured constants. The values for the remaining steps were adjusted to make the simulations agree with the data.

As would be predicted for activation by ethanol and 1-buta-
of ethanol revealed that NADH release from the abortive complex would only have to be 10% faster than that from the enzyme-NADH complex to account for the observed substrate activation. Further simulations also demonstrated that the pathway outlined in Scheme 1 could also explain the observed kinetics for cyclohexanol and 1-butanol. The results also indicate that binding of alcohol to free enzyme can inhibit the binding of NAD+ and decrease velocities of alcohol oxidation. The abortive complex pathway is the simplest mechanism that explains all of the results.

Previous studies on horse liver alcohol dehydrogenase also invoked the abortive complex pathway mechanism to account for substrate activation and inhibition and included a random pathway for binding of NAD+ and alcohol (19, 22–24). In contrast to the kinetics observed with HsADH1, the saturation kinetics for EqADHE showed substrate inhibition by ethanol and substrate activation by cyclohexanol. Ethanol inhibits EqADHE by decreasing the rate constant for NADH dissociation by 4-fold (24).

An abortive complex pathway has also been proposed to account for cooperativity in other enzymes. Sheep liver cytosolic aldehyde dehydrogenase forms complexes with NADH and aldehyde that either lead to substrate activation or inhibition because of rate-limiting NADH release that is either faster or slower (depending on the aldehyde) from the abortive complex than from the binary enzyme-NADH complex (37). The negative cooperativity for glucose saturation observed for monocromic wheat germ hexokinase type L1 and the substrate inhibition by glucose seen for rat liver glucokinase have been attributed to the presence of an abortive complex pathway that involves the formation of an enzyme-MgADP-substrate complex (38). Dihydrofurate reductase from Escherichia coli also shows substrate activation with NADPH as the varied substrate and rate constants determined for each step in the mechanism under physiological conditions are not in the substrate binding site. Microscopic rate constants need to be determined in order to accurately simulate ethanol concentration ranges where the other human liver enzymes appear to have hyperbolic saturation kinetics (6). In addition to the ready formation of the enzyme-NADH-alcohol complex, HsADH1 is unusual among the class I human liver alcohol dehydrogenases in that it has good activity on 3-β-hydroxy-5-β-steroids (44) and can bind with high affinity to some bulky inhibitors, such as N-1-methylheptylformamide and N-cyclopentyl-N-cyclopropylformamide (45). HsADH1 is also the best of the human class I enzymes at catalyzing aldehyde dismutation (46). Perhaps this property is related to an increased ability to form the enzyme-NAD+-aldehyde ternary complex, which is analogous to the enzyme-NADH-alcohol abortive complex. All of these properties suggest that the active site of HsADH1 differs critically from the other class I enzymes. HsADH1 has Val-141 and Val-143, whereas the other enzymes have Leu, Phe, or Met at position 141 and Thr or Ile at 143 (47). Modeling of the HsADH1 structure from the known horse liver enzyme suggests that small adjustments to the Cα backbone may be required to accommodate the valine residues. Perhaps the differences at positions 141 and 143 can account for the properties of HsADH1, but other residues, distant from the active site, may also play a role.

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