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Effect of Substrate Structure on the Rate of the Catalytic Step in the Liver Alcohol Dehydrogenase Mechanism*

Robert L. Brooks† and Joseph D. Shore

ABSTRACT: The catalytic step of the oxidation of alcohols by liver alcohol dehydrogenase has been resolved for several substrates by employing deuterated analogs using transient and steady-state kinetic techniques. With the two primary alcohols, ethanol and propanol, a presteady-state burst of liver alcohol dehydrogenase-bound NADH was observed. Substantial kinetic isotope effects on the rate of this burst were taken as evidence that this burst was the catalytic step. Methanol and 2-propanol did not produce a burst but only steady-state turnover which was, as had been previously reported, slower than the rate constant for dissociation of liver alcohol dehydrogenase bound NADH. Isotope effects on the turnover numbers at maximum velocity allowed the assignment of the catalytic step as rate limiting for these substrates. Large differences in catalytic rates were observed between the four substrates investigated. These differences are discussed with regard to the relative importance of steric effects, hydrophobic binding, and molecular orientation.

Several attempts have been made, using steady-state kinetics, to investigate the relationship between substrate structure and the rate of catalysis by liver alcohol dehydrogenase. These studies have been complicated due to the ordered reaction mechanism of the enzyme (Theorell and Chance, 1951) with the dissociation rate of binary enzyme–product coenzyme complex being rate limiting at maximum velocity (Dalziel, 1963). In an attempt to study the catalytic mechanism, Blomquist (1966) related the structure of substituted benzaldehydes to ϕ_2 values (Dalziel, 1957b). These values, however, are complex functions of the binding of substrate and release of product as well as the catalytic interconversion of ternary complexes. Dalziel and Dickinson (1966a, 1967) reported that several secondary alcohols exhibited maximum velocities considerably slower than primary alcohols, in which the dissociation rate of NADH[1] from binary enzyme–coenzyme complex is rate limiting. Despite the complex scheme required to explain results with secondary alcohols, they surmized that the maximum velocity for these substrates was limited by the rate of interconversion of ternary complexes.

Shore and Gutfreund (1970) recently reported the isolation of the hydride-transfer step in the LADH mechanism employing the use of deuterated ethanol and transient kinetic techniques. They reported the existence of a kinetic isotope effect of 6 on the rate constant for the initial formation of dehydrogenase-bound NADH. This substantial isotope effect was taken as evidence that the burst of bound NADH production was the hydride-transfer step or was controlled by the hydride-transfer rate. The primary objective of the present study is the determination of the effect of substrate structure on the rate of the catalytic step of the LADH mechanism. One secondary and three primary alcohols and their deuterated analogs were investigated, resulting in the discovery of large differences in rate constants for hydrogen transfer from these substrates to NAD^+.

Materials and Methods

Crystalline LADH was prepared from horse liver by the method of Theorell *et al.* (1966). The enzyme assay method of Dalziel (1957a) was used in conjunction with the NADH titration of enzyme in the presence of isobutyramide (Theorell and McKinley-McKee, 1961) to determine enzyme concentration. Coenzymes were purchased from either Boehringer Corp. or Sigma Chemical Corp., and NAD^+ was further purified by the procedure of Dalziel and Dickinson (1966b). Deuterioethanol and *d*-4-methanol were purchased from International Chemical and Nuclear Corp., Irvine, Calif.; deuterioisopropyl alcohol from Diaprep, Inc., Atlanta, Ga., and 1,1-dideuterio-*n*-propyl alcohol was synthesized using the method of Hill *et al.* (1952).

Initial rate studies for the determination of ϕ_0, the reciprocal of the turnover number, were made at saturating concentrations of NAD^+, greater than 1 mM, using an Eppendorf photometer adapted for fluorometry. Stopped-flow kinetic studies were performed on a Durrum-Gibson instrument which had a dead time of 2.5 msec. All kinetic studies were made at 25° in pH 7 phosphate buffer, ionic strength 0.1. First-order rate constants for the initial burst of bound NADH formation were calculated employing the Guggenheim method (Frost and Pearson, 1965) using only the initial exponential part of the rate curve or the method of Gutfreund and Sturtevant (1956) in which the steady-state portion was algebraically subtracted. Both methods of calculation resulted in the same numerical values for the rate constants.

* From the Edsel B. Ford Institute for Medical Research, Henry Ford Hospital, Detroit, Michigan 48202. *Received May 20, 1971.* This work was supported by a grant from the National Science Foundation (No. B-018027).

[1] Abbreviations used are: NAD^+ and NADH, oxidized and reduced nicotinamide-adenine dinucleotide; LADH, liver alcohol dehydrogenase.

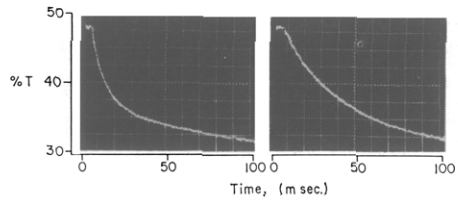

FIGURE 1: The rates of formation of enzyme-bound NADH for ethanol (left) and deuterioethanol on expanded time and amplitude scales. Syringe 1, 50 μN liver alcohol dehydrogenase; syringe 2, 3.0 mM NAD^+ and 14 mM substrate. Cuvet path length 2.0 cm; wavelength 325 mμ.

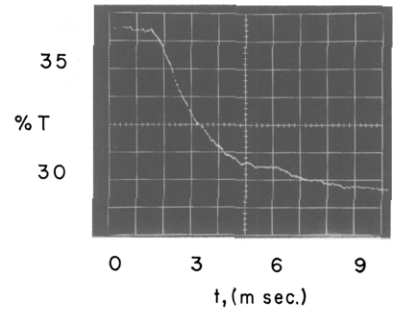

FIGURE 3: Oscilloscope trace of bound NADH production with 1-propanol. Syringe 1, 46.8 μN liver alcohol dehydrogenase; syringe 2, 3.1 mM NAD^+ and 3.0 mM 1-propanol. Cuvet path length 2 cm; wavelength 325 mμ.

Results

If saturating NAD^+ and high substrate concentrations are added to LADH in a stopped-flow spectrophotometer, two reactions can be seen at the isosbestic point for free and bound NADH: a rapid burst of bound NADH formation followed by the steady-state turnover of the enzyme. Figure 1 shows the burst reaction on an expanded time and amplitude scale, in which the rate difference between ethanol and deuterioethanol as substrates can be seen.

Using the method of Guggenheim it is possible to calculate the first-order rate constant for the initial burst of bound NADH formation. This calculation was performed for experiments at various concentrations of ethanol and deuterioethanol. The results were expressed as a double-reciprocal plot of first-order rate constant and substrate concentration, which can be seen in Figure 2. The true saturation concentration of ethanol for this reaction was approximately 50 mM, which could not ordinarily be observed in steady-state kinetics due to abortive enzyme–NADH–alcohol complex formation at concentrations higher than 8 mM. As reported previously (Shore and Gutfreund, 1970), a full burst of bound NADH, equal to the concentration of enzyme active sites, was obtained at 50 mM ethanol. The intercepts of the double-reciprocal plots for ethanol and deuterioethanol provide a basis for estimating the isotope effect on the burst reaction at saturating concentrations. It can be seen from the plots in Figure 2 that there is a sixfold isotope effect on the burst rate at saturating substrate concentrations.

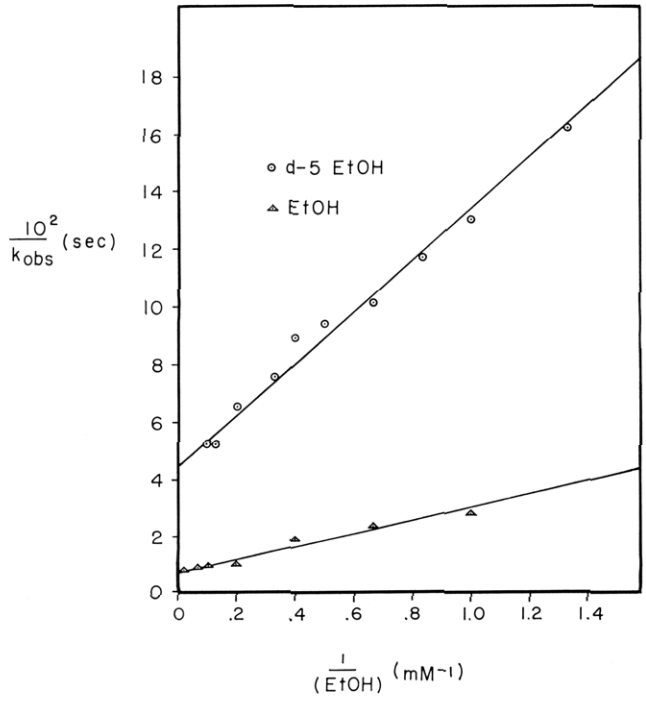

FIGURE 2: Double-reciprocal plot relating observed rate constant for bound NADH formation to substrate concentration. (Δ) Ethanol; (O) deuterioethanol; at 1.5 mM NAD^+.

The effect of substrate structure on the initial burst rate for bound NADH production was investigated using 1-propanol since it has been reported that higher alcohols are better substrates for LADH (Dalziel and Dickinson, 1966a). The results of a typical experiment can be seen in Figure 3, in which 1.5 mM 1-propanol and saturating NAD^+ were added to LADH. Although the first two-thirds of the reaction could not be followed due to the instrument dead time, the last third of the reaction has a half-time of approximately 1 msec, resulting in a calculated first-order rate constant of 650 sec^{-1}. A similar reaction, using 1,1-dideuterio-1-propanol, resulted in a rate constant of 150 sec^{-1}. Furthermore, the rate constants for these reactions were the same regardless of whether 1.5 or 5.0 mM 1-propanol was used, indicating that the concentration required for saturation was much lower than for ethanol.

To further characterize the effect of substrate structure on the catalytic step of the LADH mechanism, methanol and isopropyl alcohol were investigated. Both substrates had previously been reported to have turnover numbers considerably slower (Mani *et al.*, 1970; Dalziel and Dickinson, 1966a) than the directly determined dissociation rate of NADH (Shore, 1969; Shore and Gutfreund, 1970), indicating that some other step is rate determining at maximum velocity. Stopped-flow experiments at high enzyme concentrations indicated no burst of bound NADH formation preceding the steady-state turnover of the enzyme with both substrates. Consequently, it seemed probable that the rate-determining step with these substrates was the transfer of hydrogen from the substrate to NAD^+. The steady-state kinetics with these substrates and their deuterated forms were investigated at saturating NAD^+ concentrations. Figure 4 shows the Lineweaver–Burk plots for methanol and *d*-4-methanol. It can be seen that there is an isotope effect of 4 on the maximum velocity, demonstrating that the hydride-transfer step is probably rate determining. The results with isopropyl alcohol and *d*-8-isopropyl alcohol can be seen in Figure 5. The isotope effect on the maximum velocity was 2.3, indicating that for this substrate the hydride-transfer step is at least partially rate determining at saturating concentrations. Thus, for ethanol and 1-propanol, in which the turnover number is

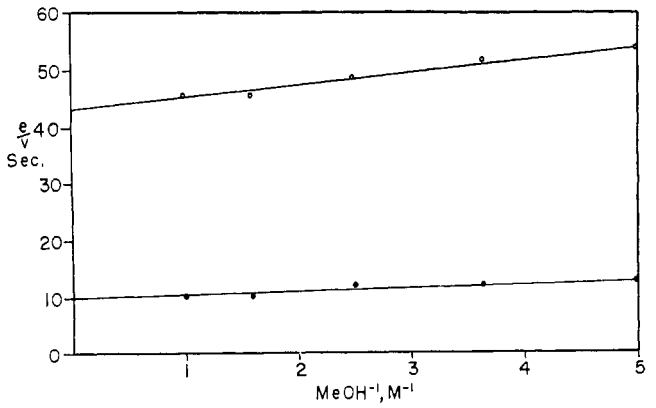

FIGURE 4: Double-reciprocal plots of initial velocity and substrate concentration at 1.8 mM NAD^+. Methanol (●) and deuteriomethanol (○).

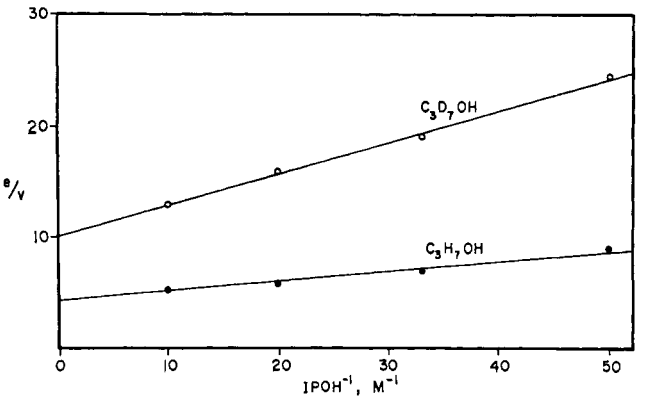

FIGURE 5: Double-reciprocal plots of initial velocity and substrate concentration at 1.0 mM NAD^+. Isopropyl alcohol (●) and deuterioisopropyl alcohol (○).

controlled by the dissociation rate of binary dehydrogenase–NADH complex, a substantial isotope effect was seen on the burst of bound NADH production. When methanol and isopropyl alcohol, which have turnover numbers considerably slower than the NADH dissociation rate, were used as substrates, a substantial isotope effect on the turnover number was observed.

Discussion

The absence or presence of a primary deuterium isotope effect presents a convenient method for determining whether a carbon–hydrogen bond is being broken in the rate-determining step of a reaction (Richards, 1970; Jencks, 1969). It is also a convenient method for determining which step in a multistep sequence is involved in hydrogen transfer. The magnitude of these isotope effects is quite variable, and Richards (1970) has suggested a range of 2–15 as being quite normal. Jencks (1969), in a simplified calculation involving only zero-point energies, reported that at room temperature a theoretical isotope effect of seven could be expected when a hydrogen atom is transferred from a carbon atom to an acceptor. Therefore, the range of isotope effects which has been observed with the various substrates used in this work is well within the realm of normal primary isotope effects involving the cleavage of a carbon–hydrogen bond. Since the deuterated substrates studied contained deuterium in positions other than the sterospecific position on the α-carbon involved in catalytic transfer, it is possible that part of the observed isotope effects are due to secondary effects. The magnitude of the isotope effects obtained with these substrates, however, are considerably larger than would be expected for a secondary isotope effect. It is probable that secondary isotope effects are only minor contributors to the observed rate differences between deuterated and nondeuterated substrates.

The results obtained with the four alcohols studied are presented in Table I. In this table, k is used to represent the rate constant for the step in which the hydride-transfer reaction takes place, as evidenced by a substantial deuterium isotope effect. For ethanol and 1-propanol the rate constants for the burst of bound NADH production are listed while for methanol and isopropyl alcohol, in which the dissociation of NADH is not rate limiting at maximum velocity, the turnover numbers are reported. The wide range of values for the deuterium isotope effects may be explained in several ways. Either some other step is partially rate determining for the reactions in which a lower isotope effect was observed or there is a unique difference in the mechanism of transfer of hydrogen with different substrates. Despite these variations in magnitude of isotope effects, the existence of effects greater than two indicates that the rate constants observed for each substrate are at least partially limited by the hydride-transfer rate. It is therefore possible to compare these rates for the various substrates tested.

In the series of primary alcohols methanol, ethanol, and 1-propanol, the relative rates are 1:1400:6500. The very large difference existing between methanol and ethanol cannot easily be explained by simple chemical reactivities. An explanation based on steric or electronic effects is not tenable since these effects do not normally exhibit such a discontinuity. However, an explanation based on rate differences due to molecular orientation could account for this discontinuity. The proper molecular orientation for undergoing hydride transfer at an optimum rate would be attained by binding to the two binding sites which have been proposed for alcoholic substrates, a hydroxyl binding site and a hydrophobic binding site (Dalziel and Dickinson, 1967). In the case of LADH, the distance between these two binding sites might be so large that only an alcohol having a chain length of at least two carbon atoms can effectively bridge the gap between the binding sites and attain the proper orientation. Thus, methanol, having a chain length of only one carbon atom, may bind only to the hydroxyl binding site and would avail itself

TABLE I: Comparison of Catalytic Rates for Alcohol Substrates.[a]

Alcohol	k (sec^{-1})	Isotope Effect
Methanol	0.1	4.3
Ethanol	140	6.2
1-Propanol	650	4.3
Isopropyl alcohol	0.23	2.3

[a] The first-order rate of catalytic hydrogen transfer is represented by k. For ethanol and 1-propanol, k is the transient rate of bound NADH formation while for methanol and isopropyl alcohol it is the turnover number.

of the proper orientation for only a small amount of the time during which it is bound. This small residence time in the proper molecular orientation would effectively reduce the catalytic rate but not the kinetic isotope effect. The fact that methanol is bound to one site only as compared with other primary alcohols which can bind to two sites should be reflected in the affinity of the enzyme–coenzyme binary complex for substrate. This is substantiated by the observation that 50 mM ethanol is required for reaction saturation as compared with 1 M for methanol.

Little difference would be expected for the relative catalytic rate constants with ethanol and 1-propanol as substrates, based on steric and electronic properties. The increased alkyl chain length of 1-propanol results in increased affinity since only 1.5 mM was required for saturation as compared with 50 mM for ethanol. The almost fivefold enhancement in rate constant for hydride transfer with 1-propanol can probably also be attributed to an orientation effect due to greater hydrophobic interaction.

The extremely slow rate of hydride transfer for isopropyl alcohol, as compared with ethanol and 1-propanol, indicates that proper orientation of the substrate is not due to a nonspecific hydrophobic interaction. Since secondary alcohols have two alkyl groups available for binding the orientation requirements may be more stringent than for primary alcohols. Butan-2-ol has been reported to have a turnover number twice as fast as isopropyl alcohol (Dalziel and Dickinson, 1966a) indicating that hydrophobic interactions are still important for secondary alcohols. The poor efficiency of these substrates demonstrates than an additional factor must be responsible, since even butan-2-ol is considerably slower than ethanol.

The primary purpose of this study has been to demonstrate that the rate of the catalytic ternary complex isomerization of the LADH reaction mechanism is extremely dependent on substrate structure. Further studies should help to characterize the details of the mechanism and explain the large orientation effect.

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