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Thermodynamics and Molecular Kinetics of Liver Alcohol Dehydrogenase

KEITH DALZIEL*

Department of Biochemistry, University of Sheffield, Sheffield, 10, Great Britain

The effect of temperature on the initial rate of the liver alcohol dehydrogenase reaction in the range 8–36°C has been studied at pH 7.1. Heats, entropies and free energies of activation have been calculated from the variation with temperature of the parameters in the initial rate equation for the reaction in both directions. Thermodynamic data have been interpreted in terms of the Theorell-Chance mechanism. A satisfactory thermodynamic balance is demonstrated by comparison with independent data for the overall equilibrium, and the reactions of the enzyme with the coenzymes are discussed in terms of the absolute reaction rate theory.

The foundations of modern kinetic studies of coenzyme-substrate reactions were largely laid in 1951 in the papers by Theorell and Bonnichsen[1] and Theorell and Chance[2] on liver alcohol dehydrogenase. After much uncertainty[3,4], due mainly to the unsuspected effects of impurities in coenzyme preparations[5,6], and on the basis of further theoretical work[7,8], there is now fairly convincing evidence that, provided pure coenzymes are used, initial rate data for liver alcohol dehydrogenase at pH 6.0–9.0 are consistent with the mechanism proposed by Theorell and Chance. This is the limiting case of a compulsory order mechanism in which ternary enzyme-coenzyme-substrate complexes do not limit the maximum rate[2,8]. The chief evidence in its support is as follows: the rate of combination of reduced coenzyme and enzyme measured directly by rapid reaction technique agrees reasonably well with the value calculated from initial rate measurements on the overall reaction[2]; the maximum rate in each direction is equal to the rate of dissociation of the product coenzyme complex calculated from initial rate parameters for the reverse reaction[9]; the same values for maximum rates and for Michaelis constants for coenzymes are obtained with different substrates, which give quite different overall rates in rate-limiting concentrations[4]; values for the dissociation constants of the enzyme-coenzyme complexes calcu-

*Sorby Research Fellow of the Royal Society.

lated from initial rate data are in satisfactory agreement with those obtained by direct equilibrium measurements in absence of substrate[9,10].

In this paper, the variations of initial rate parameters for liver alcohol dehydrogenase with temperature at pH 7.1 are reported, and interpreted on the basis of the Theorell-Chance mechanism.

EXPERIMENTAL PROCEDURE

Crystalline alcohol dehydrogenase was prepared from horse liver and assayed as described previously[11]. Nicotinamide adenine dinucleotide (NAD) was purchased from Sigma Chemical Company ("β-DPN, 98 %"), and purified by ion-exchange chromatography[12]. The reduced coenzyme ($NADH_2$) was freshly prepared as described elsewhere[5].

Initial rate measurements were made with a recording fluorometer, and detailed descriptions have been given[4] of the apparatus, technique and method of estimation of the kinetic coefficients in the initial rate equation[8]

$$E/v_0 = \Phi_0 + \Phi_1/S_1 + \Phi_2/S_2 + \Phi_{12}/S_1S_2 \quad (1)$$

for the reduction of acetaldehyde (S_2) by $NADH_2$ (S_1), and the coefficients Φ_0' etc. in the analogous initial rate equation for the oxidation of ethanol by NAD.

The fluorescence intensities of both the Perspex fluorescent standard used in the fluorometer and $NADH_2$ in solution vary considerably with temperature, and calibration over the temperature range studied was necessary. The method was described previously[11], and the empirical relation

$$F_T/F_S = F_R = Ac - 0.0055Ac^2$$

was established between the concentration, c, and the fluorescence F_T of an $NADH_2$ solution relative to that of the standard, F_S. The calibration factor dF_R/dc, required to convert the measured initial rate of change of fluorescence dF_R/dt, into rate of change of $NADH_2$ concentration dc/dt, is

$$dF_R/dc = A - 0.011Ac$$

Calibration at temperatures from 7 to 37°C showed that A varies linearly with temperature, to within 2 % (Fig. 1). The calibration factor was calculated for each temperature and initial $NADH_2$ concentration from the appropriate value of A.

The highest and lowest reactant concentrations used were: $NADH_2$, 21 and 1 μM; acetaldehyde, 2600 and 13 μM; NAD, 330 and 2 μM; ethanol, 8000 and 200 μM. Each experiment consisted of triplicate series of measurements with from 3 to 5 different concentrations of both coenzyme and substrate, making from 9 to 25 different measurements in each series.

The sensitive fluorometric method, first developed by Theorell and Nygaard[13], is essential for reasonably accurate estimates of initial rate parameters for liver alcohol dehydrogenase, other than maximum rates. Since, for example, the Michaelis constants ($\Phi_1\Phi_0$) for $NADH_2$ and NAD are only 13 μM and 3 μM, respectively, at pH 7.1 spectrophotometry is hardly a

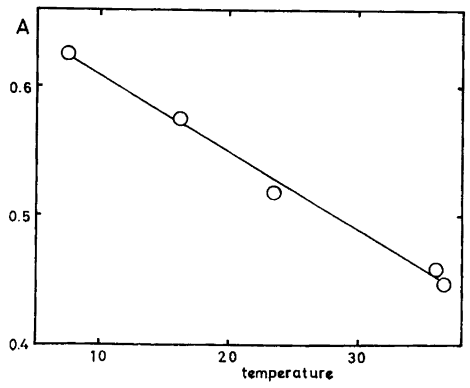

Fig. 1. Calibration of the fluorometer for $NADH_2$ measurements over a range of temperature. Variation with temperature of the constant A in the empirical equation $dF_R/dc = A - 0.011Ac$. (see text and reference 10).

feasible method. On the other hand, the highest concentration of $NADH_2$ that can be used in the fluorometer is limited by quenching, and the true maximum rate is significantly greater than the highest measured rate. For this reason, and as a check on the large temperature corrections for the fluorometer calibration, maximum rates of the $NADH_2$-acetaldehyde reaction at three temperatures were also determined by spectrophotometric initial rate measurements with 40–120 μM $NADH_2$.

The ionic strength of the reaction mixtures was 0.1 with respect to sodium phosphate buffer of pH 7.1 at 25°C. The change of pH with temperature[14] is −0.025 from 20 to 38°C.

Two distinct sets of experiments at temperatures from 8 to 36°C were carried out, separated by almost a year. Enzyme and $NADH_2$ were freshly prepared by the same methods for each set, but different NAD preparations were used. For the first set a commercial preparation was employed; for the second, following the discovery of a competitive inhibitor in commercial preparations[6], the NAD was purified by ion-exchange chromatography[12].

RESULTS

The kinetic coefficients in eqn. 1 have the dimensions of reciprocal velocity constants: $1/\Phi_0$ may be formally regarded as a first order rate constant (the maximum specific rate), $1/\Phi_1$ and $1/\Phi_2$ as second order rate constants, and $1/\Phi_{12}$ as a third order rate constant. The variation of these quantities with temperature is shown in the Arrhenius plots of Figs. 2 and 3 and the activation energies are given in Table 1.

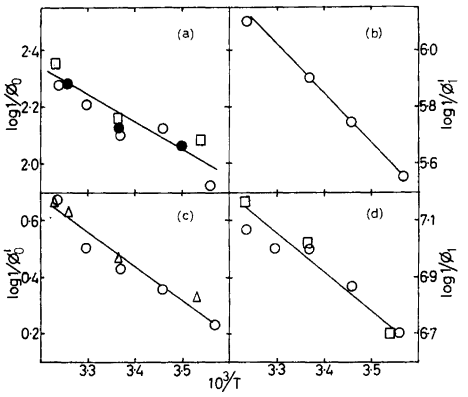

Fig. 2. Arrhenius plots showing the variation with temperature of the reciprocals of kinetic coefficients in Equation 1. (a) $1/\Phi_0$ (sec^{-1}), the maximum specific rate of the $NADH_2$-acetaldehyde reaction, measured in two series of experiments in the fluorometer (O, □) and by spectrophotometric initial rate measurements (●). (b) $1/\Phi_1'$ ($M^{-1}sec^{-1}$) from fluorometric initial rate measurements on the NAD-ethanol reaction with commercial NAD. (c) $1/\Phi_0'$ (sec^{-1}), the maximum specific rate of the NAD-ethanol reaction, from two series of initial rate measurements in the fluorometer with commercial (O) and chromatographically purified (△) NAD. (d) $1/\Phi_1$ ($M^{-1}sec^{-1}$) from two series of fluorometric initial rate measurements on the NADH-acetaldehyde reaction.

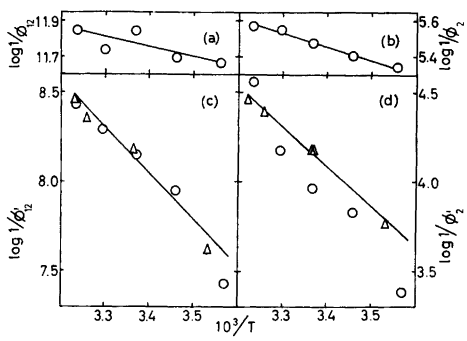

Fig. 3. Arrhenius plots showing the variation with temperature of initial rate parameters. In (a) and (b), values for $1/\Phi_{12}$ ($M^{-2}sec^{-1}$) and $1/\Phi_2$ ($M^{-1}sec^{-1}$) were obtained in a single set of experiments on the $NADH_2$-acetaldehyde reaction. In (c) and (d), measurements of the corresponding parameters for the NAD-ethanol reaction were made with commercial (O) and chromatographically purified (△) NAD.

Table 1. Apparent activation energies from the temperature variation of initial rate parameters at pH 7.1.

Parameter	Φ_0	Φ_1	Φ_2	Φ_{12}	Φ_0'	Φ_1'	Φ_2'	Φ_{12}'
E, kcal × mole^{-1}	4.4	6.3	3.5	~2.5	5.4	7.9	10.3	12.0

In Fig. 2(a), values for the maximum specific rate of the $NADH_2$ – acetaldehyde reaction, $1/\Phi_0$, obtained by spectrophotometric and fluorometric measurements are in satisfactory agreement. Figs. 2(c), 3(c) and 3(d) show parameters for the NAD-ethanol reaction obtained with commercial and chromatographically purified NAD. In accordance with earlier theory and experiment[9,12] only for the parameter Φ_2', (Fig. 3(d)) were significantly different values obtained with purified and commercial NAD. In this case, the activation energy was calculated from the measurements with purified NAD only, since the slope could be affected by the nucleotide impurity in commercial NAD if the heat of dissociation of its enzyme compound is different from that of the enzyme-NAD compound[12].

The precision of the activation energies in Table 2 is probably no better than ±1 kcal in general. The estimates of Φ_{12} are very approximate[4], but the difference between the apparent activation energies for $1/\Phi_{12}$ and $1/\Phi_{12}'$ is clearly significant, and there is an analogous difference between the temperature effects on $1/\Phi_2$ and $1/\Phi_2'$ (Fig. 3).

DISCUSSION

From evidence summarised in the introduction, the reciprocals of the kinetic coefficients Φ_0 and Φ_1 in equation (1) may be identified with the specific rate of dissociation of the product coenzyme-enzyme complex and the specific rate of formation of the reactant coenzyme-enzyme complex respectively. That is, $1/\Phi_1 = k_1$, $1/\Phi_0 = k_{-1}'$, $1/\Phi_1' = k_1'$, and $1/\Phi_0' = k_{-1}$, where the rate constants are defined by the following reaction sequence:

$$\mathrm{E} + \mathrm{NADH_2} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{E \cdot NADH_2}$$

$$\mathrm{E \cdot NADH_2} + \mathrm{CH_3CHO} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{E \cdot NADH_2 \cdot CH_3CHO} \underset{k'}{\overset{k}{\rightleftharpoons}}$$

$$\rightleftharpoons \mathrm{E \cdot NAD \cdot C_2H_5OH} \underset{k_2'}{\overset{k_{-2}'}{\rightleftharpoons}} \mathrm{E \cdot NAD} + \mathrm{C_2H_5OH}$$

$$\mathrm{E \cdot NAD} \underset{k_1'}{\overset{k_{-1}'}{\rightleftharpoons}} \mathrm{E} + \mathrm{NAD}$$

If, as seems certain, the reaction of substrate with bound coenzyme involves ternary complexes, even though these are not rate limiting under maximum rate conditions, the coef-

Table 2. Heats, entropies and free energies of activation at pH 7.1 and 23.5°C.

Reaction	Specific rate	ΔH^* kcal × mole^{-1}	ΔS^* e. u.	ΔF^* kcal × mole^{-1}
$E + NAD \rightarrow E \cdot NAD$	$1/\Phi_1' = 9 \times 10^5 M^{-1}sec^{-1}$	7.3	− 9	10.0
$E \cdot NAD + C_2H_5OH \rightarrow E \cdot NADH_2 + CH_3CHO$	$1/\Phi_2' = 1.5 \times 10^4 M^{-1}sec^{-1}$	9.7	− 9	12.3
$E \cdot NADH_2 \rightarrow E + NADH_2$	$1/\Phi_0' = 2.7$ sec^{-1}	4.8	−42	17.2
$NAD + C_2H_5OH \rightarrow NADH_2 + CH_3CHO$	Sum	21.8	−60	39.5
$E + NADH_2 \rightarrow E \cdot NADH_2$	$1\Phi_1 = 1 \times 10^7 M^{-1}sec^{-1}$	5.7	− 9	8.4
$E \cdot NADH_2 + CH_3CHO \rightarrow E \cdot NAD + C_2H_5OH$	$1/\Phi_2 = 3 \times 10^5 M^{-1}sec^{-1}$	2.9	−26	10.6
$E \cdot NAD \rightarrow E + NAD$	$1/\Phi_0 = 125$ sec^{-1}	3.8	−38	15.0
$NADH_2 + CH_3CHO \rightarrow NAD + C_2H_5OH$	Sum	12.4	−73	34.0

ficients Φ_2 and Φ_2' will be functions of several rate constants[8], *e. g.* $\Phi_2 = (k_{-2}k_{-2}' + k_{-2}k' + k_{-2}'k)/k_2k_{-2}'k$. No information is available about the relative values of these rate constants. The activation energy derived from $1/\Phi_2$ will be a function of those for the several forward and reverse steps involved and will correspond to the height of the highest energy barrier above the energy level of the reacting bound coenzyme and substrate.

Heats, entropies and free-energies of activation in Table 2 were calculated from the activation energies of Table 1 and the best estimates[9] of the rate constants at 23.5°C by the relations of the absolute reaction rate theory[15,16]:

$$\Delta H^* = E - RT = E - 0.6 \text{ (kcal} \times \text{mole}^{-1}) \quad (2)$$

$$\Delta S^* = 2.3R \text{ (log } k - \log k_B T/h) + \Delta H^*/T \quad (3)$$
$$= \Delta H^*/T - 4.57 \text{ (13.22} - \log k)$$

$$\Delta F^* = \Delta H^* - T\Delta S^* \quad (4)$$

Thermodynamic data for the overall oxidation of ethanol by NAD may be calculated as the differences between the sums of these pseudo-thermodynamic quantites for the forward and reverse steps in Table 2, and are $\Delta H = 21.8–12.4 = 9.4$ kcal, $\Delta S' = -60 + 73 = 13$ e. u. and $\Delta F' = 39.5 - 34.0 = 5.5$ kcal. These quantities relate to an arbitrary standard state of the hydrogen ion of pH 7.1 and to the apparent equilibrium constant at pH 7.1, $K_0/(H^+) = (NADH)(CH_3CHO)/(NAD^+)(C_2H_5OH)$. The standard data for pH 0, or 1 molal activity of the hydrogen ion, are

$$\Delta H^\circ = \Delta H = 9.4 \text{ kcal}$$
$$\Delta S^\circ = \Delta S' - 2.3R(\text{pH}) = 13–33 = -20 \text{ e. u.}$$
$$\Delta F^\circ = \Delta F' + 2.3RT(\text{pH}) = 5.5 + 9.6 = 15.1 \text{ kcal} \times \text{mole}^{-1} \text{ at } 23.5°C$$

and relate to the pH-independent equilibrium constant $K_0 = (NADH)(H^+)(CH_3CHO)/(NAD^+)(C_2H_5OH)$. From direct studies of the variation of K_0 with temperature, Bäcklin[17] found $\Delta H^\circ = 7.13$, $\Delta S^\circ = -26.5$ and $\Delta F^\circ = 15.0$. The values obtained from the six kinetic parameters agree as well as could be

Table 3. Thermodynamic data for the oxidation of ethanol by NAD at pH 7.1 and. 23.5°C, calculated from activation data of Table 2.

Reaction	ΔH kcal×mole^{-1}	$\Delta S'$ e. u.	$\Delta F'$ kcal×mole^{-1}
$E + NAD \rightleftharpoons E \cdot NAD$	3.5	29	−5.0 (−5.2[a])
$E \cdot NAD + C_2H_5OH \rightleftharpoons E \cdot NADH_2 + CH_3CHO$	6.8	17	1.7
$E \cdot NADH_2 \rightleftharpoons E + NADH_2$	−0.9	−33	8.8 (8.8[a])
$NAD + C_2H_5OH \rightleftharpoons NADH_2 + CH_3CHO$	9.4 (7.1[b])	13 (6.5[b])	5.5 (5.4[b])

[a] Calculated from the data of Theorell and McKee[10].
[b] Calculated from the data of Bäcklin[17].

expected from the combined experimental errors. This is a further test of the Haldane relation $K_0/(H^+) = \Phi_0\Phi_1\Phi_2/\Phi_0'\Phi_1'\Phi_2'$, which is characteristic of the Theorell-Chance mechanism, and depends upon the identification of $1/\Phi_0$ and $1/\Phi_0'$ with the rates of dissociation of the enzyme-coenzyme compounds[8].

Thermodynamic data in Table 3 for the three successive reactions in the oxidation of ethanol by NAD, according to the Theorell-Chance mechanism, were similarly calculated as the differences between the activation data (Table 2) for the forward and reverse steps, and again refer to the arbitary standard state of pH 7.1. The parameters Φ_2 and Φ_2', although functions of several rate constants, are related[9] to the apparant equilibrium constant for the overall reaction of bound coenzymes and substrates at fixed pH by $K = \Phi_2/\Phi_2' = (E \cdot NADH_2)(CH_3CHO)/(E \cdot NAD)(C_2H_5OH)$, and thus thermodynamic data for this reaction can be calculated as the differences between activation data derived from these two parameters (Table 2). The largest free energy change accompanies the dissociation of the stable compound $E \cdot NADH_2$. Oxidation of ethanol by $E \cdot NAD$ involves little free energy change. This important fact and its possible physiological significance was pointed out long ago by Theorell and Bonnichsen[1] in other terms: the binding of the coenzymes by the enzyme favours substrate oxidation thermodynamically because the dissociation constant of $E \cdot NADH_2$ is smaller than that of $E \cdot NAD$. Judged by initial rate data[12], this is true for a number of dehydrogenases, and may be a general phenomenon of importance in metabolism. From the high yields of alcohol dehydrogenase and other dehydrogenases from liver, it seems likely that in the tissue the coenzymes will be largely present in the bound forms[1].

Although the free energy change accompanying ethanol oxidation by $E \cdot NAD$ is small, there is a large increase of heat content compensated by an increase of entropy. This step is mainly responsible for the heat content change in the overall reaction. The oxidation of ethanol by $E \cdot NAD$ is favoured by increase of temperature, K at pH 7.1 increasing from 0.05 at 23.5°C to 0.08 at 38°C. No significant enthalpy change accompanies the combination of enzyme and $NADH_2$, but $E \cdot NAD$ appears to be slightly endothermic. The value $\Delta H = 3.5$ kcal in Table 3 was obtained from activation energies derived from $1/\Phi_0$ and

$1/\Phi_1'$. The dissociation constant for E · NAD is also related[8] to the kinetic parameters by $K_1' = \Phi_{12}'/\Phi_2'$, and the activation energies from these two parameters (Table 1) give $\Delta H = 1.7$ kcal. However, no account has been taken of pH effects on the rate constants and dissociation constants, which for E · NAD, in contrast to E · $NADH_2$, are considerable[9] near pH 7.1. There is evidence that combination of NAD (but not $NADH_2$) requires ionisation of an acidic group in the enzyme with p$K \sim 6.4$, and that the pK values of this and another weaker group are shifted to lower levels in the compound[9,10]. The small ΔH value at pH 7.1 may represent the heats of ionisation of these groups, and activation data from true, pH-independent rate constants would be expected to give a smaller value of ΔH.

ΔS for the formation of both enzyme-coenzyme compounds are large positive values (Table 3). Also, whilst the entropy of activation for the combination of the enzyme with both coenzymes is the expected value for a bimolecular reaction, the reverse, dissociation reactions have large negative ΔS^* values (Table 2). These findings are consistent with neutralisation of the two negative charges on the phosphate groups of the coenzymes by positively charged binding sites in the enzyme, and with considerable charge separation on formation of the activated complexes in the dissociation reactions. The latter is largely responsible for the stability of the compounds. The possibility of a structural contribution to the large entropy changes cannot, of course, be dismissed[18].

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