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Temperature Dependence of the Primary Kinetic Isotope Effect in Hydride Transfer Reactions with NAD^+ and NADH Models

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N_1-(2,6-dichlorobenzyl)-1,4-($^1H_2$)dihydronicotinamide and N_1-(2,6-dichlorobenzyl)-1,4-(^1H,^2H)dihydronicotinamide were oxidized with nitrosobenzene (NOB), 2,6-dichloroindophenol (DCPIP), 2,4,6-trinitrobenzenesulfonic acid (TNBS), 10-methyl-acridinium ion (MAI), and 10-methyl-9-phenyl-acridinium ion (MPAI). In addition, 2-propanol-h_8 and 2-propanol-d_8 were oxidized by NAD^+ in the presence of yeast alcohol dehydrogenase. Investigation of the temperature dependence of the kinetic isotope effect indicated that the redox reactions with MAI and MPAI have very similar thermodynamic characteristics and proceed via a linear transition state. Redox reactions with NOB, DCPIP, and TNBS have entirely different thermodynamic characteristics, and probably proceed via a different transition state. Thermodynamic characteristics of enzymatic reaction are very similar to model redox reactions with MAI and MPAI.

INTRODUCTION

Transfer of a hydride equivalent from reduced pyridine-nucleotide coenzymes to a variety of substrates is catalyzed by enzymes of the dehydrogenase family (*1*). Since it was found that NAD(P)H as well as many simple dihydropyridine compounds will transfer hydride anion to various substrates, also in the absence of enzyme, the latter type of reactions has been studied extensively as a model for enzymatic processes (*2*).

For more than a decade, the main mechanistic question under discussion seemed to be whether the hydride equivalent is transferred in a single step, or via a single electron transfer pathway (*3*). This discussion unjustly overshadowed equally important questions about the structure of the transition state and the charge distribution in the same. Some time ago, it has been proposed by Harold Kwart, mainly on semiempirical grounds, that the study of the temperature dependence of the primary kinetic isotope effect can yield valuable information about the geometry of transition state (*4*). The applicability of these semiempirical criteria as a tool to probe the transition state structure met initially with severe criticism (*5, 6*).

A very limited amount of experimental data has been published in the literature, concerning the TDKIE of hydride transfer reactions mediated by NADH-models (*7–9*). In this work, we have expanded this experimental basis by performing TDKIE

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measurements with novel substrates, in order to better validate the TDKIE as a practical method to probe transition state structure of hydride transfer reactions. For comparison, a study of a TDKIE of an enzymatic hydride transfer from 2-propanol-h_8 or 2-propanol-d_8 to NAD^+, catalyzed by yeast alcohol dehydrogenase, has been included.

MATERIALS AND METHODS

The synthesis of N_1-(2,6-dichlorobenzyl)nicotinamide bromide was performed according to Krohnke and Ellegast (*10*). N_1-(2,6-dichlorobenzyl)1,4-($^1H_2$) dihydronicotinamide (DBND-4H_2)[1] was synthesized according to the method of Wallenfels *et al.* (*11*). DBND-4H,4D was synthesized in an analogous manner in D_2O (99.8%); care was taken to recrystallize the product of reaction several times to maximize its purity.

Absorption spectra and kinetic measurements were taken in a selfrecording spectrophotometer SPECORD UV VIS, Carl Zeiss, Jena (Germany), in thermostated cuvette holders at 25°C. Anaerobic conditions were obtained essentially as described by Wallenfels and Gerlach (*12*). The concentration of reactants was calculated from their absorption spectra, as previously described (*9, 13–15*). The TDKIE measurements were performed with extreme care, as previously described (*9*). In all calculations, the secondary KIE was assumed to be equal to unity (*7, 9*). Aqueous solutions and water : methanol mixtures were buffered with 0.1 M sodium phosphate buffer, pH 7.5.

The estimation of ΔE_a and A_{HH}/A_{DD} is sensitive to the secondary KIE and to the isotopic impurity of DBDN-4D,4D (*7, 15, 16*); the estimation of the primary KIE at different temperatures helps to minimize the random errors, but does little to eliminate the systematic errors. Therefore, the sources of inaccuracy in the estimation of the primary KIE remain the secondary KIE and the isotopic impurity of DBDN-4H,4D.

Thermodynamic parameters in Table 2 were calculated from relationships:

$$\Delta S^+ = R\,(\ln A - \ln RT + \ln Nh) - R \quad [1]$$

$$\Delta H^+ = E_a - RT \quad [2]$$

$$\Delta G^+ = \Delta H^+ - T\Delta S^+ \quad [3]$$

Yeast alcohol dehydrogenase (EC 1.1.1.1), NAD^+, and NADH were obtained from Sigma, and 2-propanol-d_8 was obtained from Aldrich. Initial rate data for the oxidation of 2-propanol-h_8 or 2-propanol-d_8 with NAD^+ in the presence of YADH, were collected at 4 or 5 different concentrations of alcohol at a constant NAD^+;

[1] Abbreviations used: YADH, yeast alcohol dehydrogenase; TS, transition state; TDKIE, temperature dependence of the kinetic isotope effect; NOB, nitroso-benzene; DCPIP, 2,6-dichloroindophenol; TNBS, 2,4,6-trinitrobenzenesulfonic acid; MAI, 10-methyl-acridinium ion; MPAI, 10-methyl-9-phenyl-acridinium ion; BDN, N_1-(2,6-dichlorobenzyl)-1,4-dihydronicotinamide.

the same procedure was repeated at 4 or 5 different concentrations of NAD^+. Initial rate data were fitted to Eq. [4] with the Fortran programs of Cleland (*17*):

$$v_o/e_o = \frac{V_1[A][B]}{K_{ia}K_b + K_b[A] + K_a[B] + [A][B]} \quad [4]$$

where v_o is the initial rate (M.s^{-1}), e_o the concentration of enzyme active sites (M), V_1 the maximal catalytic constant (s^{-1}), K_a and K_b the Michaelis constants for NAD^+ and alcohol (M), K_{ia} the inhibitory constant for NAD^+ (M), and $[A]$ and $[B]$ the concentrations of NAD^+ and alcohol (M), respectively (*18*). Extreme care was taken to precisely measure the temperature in enzymatic reactions.

RESULTS

Stoichiometry and Product Identification

NOB, DCPIP, and TNBS were readily reduced by N_1-(2,6-dichlorbenzyl)-1,4-($^1H_2$)dihydronicotinamide, N_1-(2,6-dichlorobenzyl)-1,4-(^1H,^2H)dihydronicotinamide, or NADH in dry methanol, methanol : buffer mixtures, or neutral buffers. The three redox reactions strictly followed the first-order rate law under the pseudo-first order conditions, or the second-order rate law under the second order conditions, for at least 2 to 3 half-times, under a variety of conditions. It was previously shown that, in each case, a direct addition of hydride anion plus proton to NOB (*9*), DCPIP (*19*), or TNBS (*14, 15*) afforded the primary product of reduction; NOB afforded hydroxylamine, DCPIP the corresponding phenol, and TNBS the corresponding dihydro compound, respectively. In each case, the stoichiometry and the primary product of reaction were identified kinetically.

TDKIE in Model Reactions

NOB, DCPIP, and TNBS were readily reduced by DBDN-$4H_2$ or by DBDN-4H,4D in dry methanol or in methanol : buffer mixtures; anaerobic conditions were maintained in order to prevent the interference of radical oxygen species (*9, 13*). Table 1 shows the bimolecular rate constants (k) of each of the above redox reactions, measured at several temperatures between 14°C and 50°C.

The kinetic data from Table 1 were fitted to the following Arrhenius equations:

Nitrosobenzene:

$$\ln k_{HD} = (16.21 \pm 0.34) - (28.05 \pm 0.68\ \text{kJ/mol})/RT \quad [5]$$

$$r = 0.999$$

$$\ln k_{HH} = (16.78 \pm 0.40) - (28.02 \pm 0.67\ \text{kJ/mol})/RT \quad [6]$$

$$r = 0.997$$

TABLE 1
Temperature Dependence of Bimolecular Rate Constants (k) for the Reduction of Neutral Substrates with DBDN-4H$_2$ and DBDN-4H,4D, under Anaerobic Conditions

A. Reduction of nitrosobenzene by DBDN

temp, K	289.0	292.4	297.6	301.5	307.6	313.0	318.1
k_{HD}	93.7	111.0	131.6	148.4	188.7	232.7	275.9
temp, K	289.0	292.4	297.6	302.0	307.8	313.2	318.8
k_{HH}	165.0	184.9	239.8	287.1	347.2	391.5	497.7

B. Reduction of 2,6-dichloroindophenol by DBDN

temp, K	287.1	291.1	297.6	302.6	308.1	318.1	
k_{HD}	66.2	74.4	81.2	95.5	108.2	127.0	
temp, K	289.1	291.1	297.6	302.6	308.1	318.1	
k_{HH}	131.1	134.3	161.4	180.3	196.6	236.3	

C. Reduction of 2,4,6-trinitrobenzenesulfonic acid by DBDN

temp, K	—	292.1	296.7	302.1	307.1	313.1	322.6
k_{HD}	—	78.7	95.4	118.6	144.2	184.8	251.0
temp, K	287.4	292.1	296.7	302.1	307.1	313.1	322.6
k_{HH}	100.9	130.4	148.6	191.3	230.1	282.9	409.8

Note. Rate constants are given in $M^{-1}min^{-1}$. (A) [Nitrosobenzene] = $1{,}24.10^{-3}$ M; [DBDN-4H$_2$] and [DBDN-4H,4D] = $1{,}48.10^{-4}$ M in dry methanol. (B) [2,6-Dichloroindophenol] = $3{,}72.10^{-5}$ M; [DBDN-4H$_2$] = $6{,}48.10^{-4}$ M; [DBDN-4H,4D] = $5{,}98.10^{-4}$ M, in methanol/buffer mixture (1:1, v/v). (C) [2,4,6-Trinitrobenzenesulfonic acid] = $9{,}06.10^{-4}$ M; [DBDN-4H$_2$] = $1{,}60.10^{-4}$ M; [DBDN-4H,4D] = $1{,}66.10^{-4}$ M, in 0.1 M sodium phosphate buffer, pH 7.5.

2,6-Dichloroindophenol:

$$\ln k_{HD} = (10.96 \pm 0.24) - (16.16 \pm 0.36\ \text{kJ/mol})/RT \qquad [7]$$

$$r = 0.995$$

$$\ln k_{HH} = (11.47 \pm 0.23) - (15.86 \pm 0.32\ \text{kJ/mol})/RT \qquad [8]$$

$$r = 0.997$$

2,4,6-Trinitrobenzenesulfonic acid:

$$\ln k_{HD} = (16.74 \pm 0.43) - (30.05 \pm 0.77\ \text{kJ/mol})/RT \qquad [9]$$

$$r = 0.999$$

$$\ln k_{HH} = (17.21 \pm 0.48) - (30.06 \pm 0.84\ \text{kJ/mol})/RT \qquad [10]$$

$$r = 0.999$$

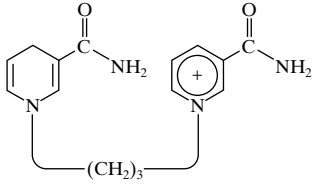
From Eqs. [5]–[10], the difference in activation energies ($E^{DD} - E^{HH}$) and, after correction for the isotopic impurity of DBDN-4H,4D (*20*), the ratio of frequency factors (A_{HH}/A_{DD}) for hydride vs deuterium transfer were calculated and presented

TABLE 2
TDKIE Criteria and Thermodynamic Parameters for Hydride Transfer Reactions to Neutral and to Positively Charged Substrates, at 25°C

Redox pair		$E_a^{DD}-E_a^{HH}$ (kJ/mol)	A_{HH}/A_{DD}	k_{HH}/k_{DD}	ΔG^+ (kJ/mol)	ΔH^+ (kJ/mol)	$-\Delta S^+$ (e.u.)	Reference
Nitrosobenzene	DBDN-4H$_2$	0.2	7.6	8.3	59.4	25.5	113.7	Table 1
	DBDN-4D$_2$				64.7	25.8	130.6	
2,6-Dichloro-indophenol	DBDN-4H$_2$	0.8	5.1	3.9	60.9	13.8	157.8	Table 1
	DBDN-4D$_2$				65.7	14.6	171.4	
2,4,6-Trinitroben-zenesulfonic acid	DBDN-4H$_2$	0.1	4.0	3.9	60.4	27.6	110.1	Table 1
	DBDN-4D$_2$				63.8	27.5	121.7	
Compound-1[a]	(4H$_2$,4′H)	0.0	2.7	2.7	77.0	34.7	141.8	*8, 16*
	(4D$_2$,4′D)				79.5	34.7	150.1	
10-Methyl-acridinium ion[b]	MPDN-4H$_2$	7.7	0.2	5.2	67.6	31.9	119.6	*7*
	MPDN-4D$_2$				71.1	39.5	106.0	
10-Methyl-9-phenyl-acridinium ion[b]	BDN-4H$_2$	4.3	0.7	4.2	71.0	23.1	161.0	*8, 16*
	BDN-4D$_2$				74.1	27.4	158.4	

Note. MPDN-4H$_2$, N_1-methylphenyl-1,4-(^{1}H$_2$)dihydronicotinamide; MPDN-4D$_2$, N_1-methylphenyl-1,4-(^{2}H$_2$)dihydronicotinamide; BDN-4H$_2$, N_1-benzyl-1,4-(^{1}H$_2$)dihydronicotinamide; BDN-4D$_2$, N_1-benzyl-1,4-(^{2}H$_2$)dihydronicotinamide.

Compound-1



[a] In dimethylsulfoxide-D$_6$.
[b] In acetonitrile.

in Table 2. In Table 2, also, thermodynamic parameters ΔG^+, ΔH^+ and ΔS^+, plus kinetic isotope effects (k_{HH}/k_{DD}) for all three redox reactions were calculated at 25°C. For comparison, all above kinetic and thermodynamic parameters for another three substrates, 10-methyl-acridinium ion (*7*), 10-methyl-9-phenyl-acridinium ion (*8*), and compound-1 (*8*), were included in Table 2.

Solvent Effects in Hydride Transfer Reactions

The presence of water, a polar protic solvent of high dielectric constant, has a very strong influence on reaction rate of redox reactions of NOB, DCPIP, and TNBS with DBDN-4H$_2$. Figure 1 shows the relationship between the natural logarithm of bimolecular rate constants for above reactions (ln k) and the molar concentration of water in buffer : methanol mixtures, determined at pH 7.5. The kinetic data in Fig. 1 were treated assuming a linear relationship between ln k and the molar concentration of water in buffer : methanol mixtures, according to Eq. [11]:

$$\ln k = \ln k_o - A\,[H_2O] \qquad [11]$$