Solvent Deuterium Isotope Effect on the Liver Alcohol Dehydrogenase Reaction

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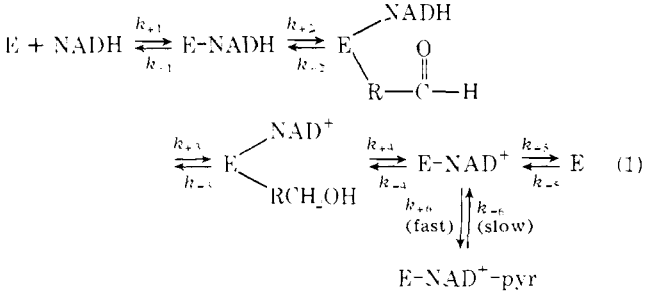
Abstract: Previous work on the kinetic mechanism of the liver alcohol dehydrogenase (LADH) catalyzed reduction of aromatic aldehydes by NADH has established that the transient kinetic reaction under conditions where enzyme is limited to a single reaction is biphasic and that the rapid transient shows a primary isotope effect when NADD is used. Furthermore, solution of the rate equations for reduction of aromatic aldehydes has established that this rapid transient is determined by a *single rate constant* for ternary complex interconversion (i.e., the chemical step). We have determined the deuterium solvent isotope effect on the rapid transient observed during aromatic aldehyde reduction. The isotope effect is 1.0 ± 0.1, indicating that the movement of proton is uncoupled from the addition of "hydride" to aldehyde carbonyl. Furthermore, we observe inverse isotope effects (k_{D_2O}/k_{H_2O} = 2–3) on the slow transient observed during aldehyde reduction; the rate-limiting step for this reaction has been established to be alcohol dissociation from the ternary complex. In addition, the solvent isotope effect on the transient kinetic rate of alcohol oxidation is also inverse as is the isotope effect on the rate of phenanthroline binding to active-site zinc. The pH dependence of these solvent isotope effects is in accord with proton transfer uncoupled from the rate-limiting process. The pK_a of the enzyme base catalytic group is perturbed by ~0.3–0.5 pK_a unit in D_2O and there is no solvent isotope effect at equivalent pH(D) values on the pH(D) dependence curves. These data and most of the other data collected on the chemical steps for LADH reaction are consistent with a modification of a recent proposal for LADH catalysis in which aldehyde binds to the active-site zinc and the zinc-bound water molecule serves as an acid–base catalyst.[1] The constraints suggested by the solvent isotope effect data are that transfer of proton to the departing alcohol molecule is not concerted with hydride transfer and indeed that proton transfer is uncoupled from either hydride transfer or alcohol desorption.

Introduction

Previous studies in our laboratory have shown that aromatic aldehyde reduction by NADH catalyzed by liver alcohol dehydrogenase can be limited to a single enzyme reaction by the addition of pyrazole.[2] Since the reaction is a bimolecular ordered process as shown in eq 1,[3] production of NAD^+ followed



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by pyrazole (pyr) addition to oxidized coenzyme can be employed to remove enzyme from the reactive pathway. Equation 1 shows the course of reaction under conditions of pyrazole addition. When k_{+6} is fast with respect to k_{+4}, enzyme is removed from further reaction with substrate, since the equilibrium constant for enzyme–NAD^+–pyrazole complex formation is very large and the rate constant for dissociation of pyrazole to regenerate the reactive enzyme–NAD^+ complex (k_{-6}) is on the order of <1 min^{-1}. Use of pyrazole at 0.01 M concentration gives $k_{+6} > 200$ s^{-1}, and at this concentration the absorbance change at 300 nm characteristic of the enzyme–NAD^+–pyrazole complex is associated with a rate constant equivalent to the turnover number (from steady-state kinetics) and the rate constant for the slow transient ($r_{1,\infty}$). (We use the rate expressions and nomenclature for the transient processes of Pettersson;[4] the ∞ subscript refers to the fact that the transient is saturated with respect to substrate. The parameters labeled are first-order rate constants derived from transient kinetics.) This equivalence has consequences on the identity of the rate-limiting step for the two kinetic transients as explained later in the Introduction.

Equations have been developed for the transient kinetic course under the reaction conditions specified.[4] Two transients are observed during aromatic aldehyde reduction; the rapid transient $r_{2,\infty} = k_{+3} + k_{-3} + k_{+4}$, while the slow transient $r_{1,\infty} = (k_{+3}k_{+4})/(k_{+3} + k_{-3} + k_{+4})$. Furthermore, at substrate concentrations greater than K_m, hyperbolic substrate dependence on the rapid transient is predicted and extrapolation yields $r_{2,\infty}$. For benzaldehyde three separate laboratories have shown that the rapid transient $r_{2,\infty}$ is ~300 s^{-1}.[5-7] Two other laboratories have observed rate constants of >500 s^{-1} at very high aldehyde concentrations.[8,9] We also have observed larger rates of aldehyde reduction with 0.01 M benzaldehyde than the extrapolated $r_{2,\infty}$. However, when this has occurred we have also observed deviation from exponential behavior. We believe this results from short-time mixing problems shown to occur for typical mixing devices employed in stopped-flow kinetic spectrometers.[10]

The slow transient r_1 for aromatic aldehyde reduction is experimentally equal to the rate constant for E–NAD^+–pyrazole complex formation and the steady-state turnover number. This result has been repeated by Shore and co-workers, who have reported results identical with ours.[8] Both Shore and co-workers and we interpret this observation as consistent with the approximation $r_{1,\infty} \simeq k_{+4}$, i.e., that the second transient is limited by the rate of alcohol dissociation from E–NAD^+–alcohol complex. Under this condition, the rate of enzyme–NAD^+–pyrazole complex formation can occur with a rate constant no larger than k_{+4}, i.e., since pyrazole and alcohol compete for the same enzyme site, complex formation with pyrazole can be no faster than alcohol dissociation. Referring back to the theoretical equation, $r_{1,\infty} = k_{+4}$ only if $k_{+3} \gg k_{-3} + k_{+4}$. It follows then that $r_{2,\infty} = k_{+3}$, i.e., the rapid transient from suicide transient kinetics is the uncontaminated "hydride transfer" rate constant. Characterization of substituent effects, isotope effects, and pH effects on the rapid transient should lead directly to mechanistic interpretation without the disadvantage of mixing of kinetic rate constants. For example, the rapid transient is associated with a primary isotope effect when NADD (4-deuterio-NADH) is used as a

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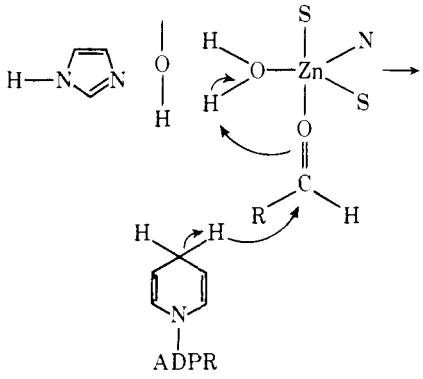
coenzyme[2]; the isotope effect is $k_H/k_D \simeq 3.0$. Furthermore, since there is no substrate concentration dependence on the primary isotope effect on r_2,[5] it should not even be necessary to study each parameter at saturation in order to reach mechanistic conclusions.

A recent study of modified enzyme in which amine groups have been modified yields enzyme in which hydride transfer may be rate limiting.[1] Isotope effects and pH dependencies are quite similar to those observed in transient kinetic experiments described previously. A mechanism was proposed in which hydride transfer is concerted with protonation of alcohol oxygen as follows:



(2)

Another feature of this mechanism is aldehyde coordination at the fifth coordination site and acid–base catalysis by zinc-bound water.

In order to study the concertedness of proton movement with hydride transfer, we have carried out aldehyde reduction in D_2O solvent. Transfer of proton shown in eq 2 could either occur concurrently with hydride transfer (producing a primary isotope effect) or subsequent to hydride transfer. If the equilibrium constant for proton transfer subsequent to hydride transfer is unfavorable, but the rate of hydride transfer is rapid, we would predict a preequilibrium effect on $r_{1,\infty} = k_{+4}$. This preequilibrium effect would be inverse since more of the catalytically active B–D would be present in D_2O than B–H in H_2O. We report below the results of D_2O solvent isotope effect studies on the LADH reaction.

Experimental Section

The enzyme preparation has been described previously.[11] Enzyme in D_2O was prepared by decanting the buffer in which enzyme crystals were suspended; centrifugation of this suspension was carried out followed by decantation of the supernatant. This process was repeated, after which dithiothreitol (DTT) in D_2O was added; the enzyme was incubated with DTT and then column chromatographed on a Bio-Gel P-30 column equilibrated with pD 8.75 pyrophosphate buffer 0.05 M in D_2O. After chromatography, Raman spectra of the enzyme solution were recorded in order to determine the amount of contaminating H_2O in our solutions; in no case was any H_2O band observed. However, small HOD bands were observed. Our estimates of H_2O contamination are $H_2O < 2\%$. The usual correction of pD = pH meter reading + 0.4 was applied in all of the following experiments.[12]

Aldehyde reduction was studied under conditions of suicide transient kinetics (pyrazole added) as described previously.[2] Azoaldehyde reduction was followed by changes in 410-nm absorption during reduction of the chromophoric aldehyde to alcohol. At very high concentrations of azoaldehyde (>100 μM), reduction was recorded at 430 nm in order to obtain smaller absorbance values then those observed at 410 nm. All transient kinetic studies were carried out on a Durrum stopped-flow spectrometer fitted with a 2-cm cuvette and capability for both absorbance and fluorescence experiments. β-Naphthaldehyde and benzaldehyde reductions were studied by observing changes in NADH concentrations at 340 nm. Experiments were carried out with NADD to slow the reaction rate in order to minimize the effect of the 2.5 ms dead time of our stopped-flow instrument.

Benzyl alcohol oxidation was also investigated under suicide transient kinetic conditions, in which isobutyramide was employed to remove the E-NADH complex from the reactive pathway after alcohol oxidation by NAD^+.[13] Production of NADH in this single turnover experiment was observed at 340 nm.

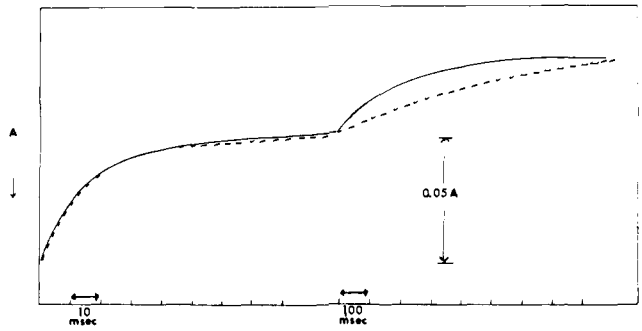


Figure 1. Time course of the transient kinetic reduction of azoaldehyde: (—) D_2O; (- - -) H_2O. [Azoaldehyde] = 2.7×10^{-5} M; [LADH] = 1.5×10^{-5} N; [NADH] = 7.4×10^{-5} M; [pyrazole] = 0.01 M; pH 8.75, pyrophosphate buffer 0.05 M. The time scale was changed by a factor of ten between the two halves of this kinetic trace as noted on the time axis.

Steady-state kinetic measurements were carried out using β-naphthaldehyde. Maximal velocities were recorded at 340 nm using a Cary 16 spectrometer. Data were treated by linear regression analysis to give a value of the maximal velocity. V_{max}/E_0 was then calculated from the concentration of enzyme sites measured by NAD^+–pyrazole titration.

The rate of phenanthroline binding to LADH was measured by observing the rate of quenching of enzyme fluorescence at varying concentrations of phenanthroline. These measurements were carried out on a Durrum stopped-flow spectrophotometer; fluorescence excitation was at 280 nm. Data were analyzed by plotting the observed rate of fluorescence quenching as a function of phenanthroline concentration; the slope of such a plot is proportional to the second-order rate constant for phenanthroline binding.[14]

NAD^+ dissociation from enzyme was followed by observing the increase in fluorescence from E-NADH–isobutyramide complex formed by mixing E-NAD^+ complex with NADH–isobutyramide. The fluorescence increase was observed on the fluorescence stopped-flow spectrophotometer employing an excitation wavelength of 340 nm and a cutoff filter to reduce scattering from the exciting line.[14]

NADH dissociation from enzyme was observed by following the decrease in E-NADH fluorescence as NAD^+–pyrazole bound to free enzyme. Conditions on the Gibson-Durrum stopped-flow spectrophotometer were identical with those reported in the NAD^+ dissociation experiment.[14]

The following buffers were used in the pH dependence studies: pH 6.0–7.5, 0.1 M phosphate buffer; pH 7.5–9.0, 0.05 M pyrophosphate buffer; pH 9.0–10.5, 0.1 M carbonate buffer.

Error limits reported in the tables are the range of values from several experiments.

Results

Figure 1 shows the reaction rate profile for the reduction of 4,2′-imidazolylazobenzaldehyde (azoaldehyde) by NADH catalyzed by liver alcohol dehydrogenase in D_2O and H_2O. Note that under these concentration conditions the rapid transient $r_2 \simeq k_{+3}$ shows no solvent isotope effect, while the slow transient $r_1 \simeq k_{+4}$ exhibits a large "inverse" solvent isotope effect. Since the experiment could not be carried out under saturating conditions of aldehyde, we also investigated several concentrations for this reaction (Table I). At the highest concentrations of azoaldehyde, ~86% saturation was reached based on K_{app} for the first kinetic step. Benzaldehyde, which is soluble in sufficient amount to saturate the binary (E-NADH) complex, was also used as a substrate to investigate the hydride transfer reaction; the reaction was carried out at pH 10.0 in order to assure sufficient separation between r_2

Table I. Deuterium Solvent Isotope Effect for Aldehyde Reduction

pL	r_2			r_1			V/E			[S], μM	S	[R], μM	R[a]	K_{app},[f] μM
	D_2O	H_2O	k_D/k_H	D_2O	H_2O	k_D/k_H	D_2O	H_2O	k_D/k_H					
6.0				34 ± 4	39 ± 1	0.87				50	β-NAPA	25	H	9.4 ± 1[b]
6.0				27.4 ± 1.8	27.8 ± 0.8	0.99				15	β-NAPA	25	H	
6.0							18.7 ± 0.6	31.6 ± 0.6	0.59	50	β-NAPA	25	H	
6.5							19.8 ± 1.2	26.1 ± 0.7	0.76	50	β-NAPA	25	H	
7.0				33 ± 5	21 ± 1	1.57				50	β-NAPA	25	H	9.1 ± 6[b]
7.0				28.0 ± 0.2	18.4 ± 1.3	1.52				25	β-NAPA	25	H	
7.0							16.8 ± 0.2	17.2 ± 0.3	0.98	50	β-NAPA	25	H	
7.2	151 ± 33	154 ± 23	0.98	23 ± 3	13 ± 3	1.77				45	β-NAPA	25	D	
7.5							11.9 ± 0.6	8.8 ± 0.2	1.35	50	β-NAPA	25	H	
8.0							6.31 ± 0.03	3.86 ± 0.22	1.63	50	β-NAPA	100	H	
8.5							3.19 ± 0.05	1.67 ± 0.01	1.91	50	β-NAPA	100	H	
8.75	35.0 ± 0.5	35.5 ± 0.7	0.99	4.21 ± 0.04	1.49 ± 0.02	2.82				15	azoald	55.6	D	20[d]
8.75	38.8 ± 0.8	39.3 ± 0.3	0.99	3.19 ± 0.02	1.60 ± 0.01	1.99				17	azoald	75	D	53[c]
8.75	46.3 ± 0.8	47.3 ± 0.6	0.98	3.28 ± 0.03	1.66 ± 0.04	1.98				22	azoald	75	D	
8.75	65 ± 2	63.3 ± 0.9	1.02	3.85 ± 0.03	1.35 ± 0.01	2.85				27	azoald	74	D	
8.75	69.9 ± 0.8	63 ± 1	1.11	4.28 ± 0.09	1.33 ± 0.02	3.22				27	azoald	75	D	
8.75	73 ± 1	65.9 ± 0.9	1.11	3.54 ± 0.08	1.75 ± 0.04	2.02				32	azoald	75	D	
8.75	72 ± 2	74 ± 3	0.98	4.61 ± 0.07	1.58 ± 0.02	2.91				32	azoald	75	D	
8.75	137 ± 8	119 ± 8	1.15	3.4 ± 0.3	0.98 ± 0.05	3.47				88	azoald	25	D	
8.75	217 ± 31	179 ± 13	1.21							118	azoald	25	D	
8.75	280 ± 37	245 ± 38	1.14							119	azoald	25	D	
8.75	188 ± 5	169 ± 12	1.11	1.93 ± 0.10	0.74 ± 0.04	2.61				39	β-NAPA	25	D	33 ± 4[b]; 30 ± 2[e]
9.0							1.22 ± 0.04	0.62 ± 0.04	1.97	50	β-NAPA	100	H	
9.5							0.58 ± 0.03	0.33 ± 0.01	1.76	50	β-NAPA	100	H	
9.9	65.8 ± 4.2	70.0 ± 1.7	0.94	3.18 ± 0.05	1.11 ± 0.02	2.86				500	benzald	20	D	
10.2				0.19 ± 0.02	0.074 ± 0.005	2.57				20	β-NAPA	29	H	
10.0							0.27 ± 0.01	0.16 ± 0.02	1.67	50	β-NAPA	100	H	
10.5							0.10 ± 0.01	0.07 ± 0.01	1.43	50	β-NAPA	100	H	

[a] H = NADH, D = NADD. [b] Reference 13. [c] Reference 2. [d] Reference 25. [e] Reference 11. [f] For first kinetic step.

Table II. Deuterium Solvent Isotope Effect on Benzyl Alcohol Oxidation

pL	r_D'	r_H'	r_D'/r_H'	$10^4 \times$ [benzyl alcohol], M	$10^4 \times$ [NAD^+], M	[IBA], M	[E], μM
6.0[a]	0.19 ± 0.01	0.21 ± 0.01	0.94	1.0	1.0	0.1	3.8
	0.32 ± 0.02	0.32 ± 0.03	1.00	2.0			
	0.61 ± 0.04	0.77 ± 0.10	0.79	4.0			
	1.30 ± 0.07	1.57 ± 0.30	0.83	10.0			
	1.8 ± 0.1	2.4 ± 0.3	0.76	40.0			
7.0[a]	2.85 ± 0.14	3.85 ± 0.15	0.74	1.0	0.84	0.08	3.8
	3.12 ± 0.07	5.24 ± 0.37	0.60	1.5			
	6.5 ± 0.2	7.0 ± 0.4	0.93	4.0			
	9.8 ± 0.6	9.3 ± 0.7	1.05	30.0			
8.75[b]	6.6 ± 0.2	5.4 ± 0.2	1.23	0.5	1.0	0.10	3.8
	10.2 ± 0.5	8.9 ± 0.1	1.15	1.0			
	14.0 ± 0.6	11.9 ± 0.6	1.18	2.0			
	18.0 ± 0.5	14.6 ± 0.4	1.23	10.0			
8.75[b]	17.2 ± 0.2	14.0 ± 0.3	1.23	3.0	1.0	0.05	4.4
	16.7 ± 0.2	14.0 ± 0.1	1.19	4.0	1.0	0.05	10.3
	15.0 ± 0.4	12.3 ± 0.1	1.22	4.0	1.0	0.05	10.3

[a] 0.1 M phosphate buffer. [b] 0.05 M pyrophosphate buffer.

and r_1 (Table I). Note that this rate constant is smaller than that previously reported; at these high concentrations of benzaldehyde we observe substrate inhibition at basic pH. Table I also lists the apparent binding constants derived from the transient kinetics of aldehyde reduction. In all cases studied, the rate constant for the rapid transient, r_2, shows no D_2O solvent isotope effect within experimental error. On the other hand, r_1 shows a large inverse isotope effect (Figure 1). Since $r_1 \simeq k_{+4}$ shows a measurable D_2O solvent isotope effect, we have investigated the pH dependence of this rate process. The results of these studies are shown in Figure 2; the data are more difficult to obtain at acidic pH because at pH <7.5 r_2 and r_1 are difficult to separate; however, the qualitative trend of a pK_a shift of ~0.3 pK_a unit is clear.

A study of the solvent isotope effect on V_{max}/E is also reported (Figure 2); at pH >7.5, the inverse isotope effects on r_1 and V_{max}/E are identical in keeping with the postulate that k_{+4} is rate limiting for both processes. At more acidic pH values the isotope effect on V_{max}/E becomes "normal", indicating that E-NAD^+ dissociation becomes partially rate limiting. The "normal" kinetic isotope effect we observe on V_{max}/E for aldehyde reduction is consistent with a change in rate-limiting step at acidic pH. The solvent isotope effect for NAD^+ dissociation at acidic pH values is k_{D_2O}/k_{H_2O} = 36 $s^{-1}/60\ s^{-1}$ = 0.6. The kinetic expression for the turnover number is $V_{max}/E_0 = (k_{+4}k_{+5})/(k_{+4} + k_{+5})$. At basic pH values, $k_{+5} \gg k_{+4}$, so $V_{max}/E_0 = k_{+4}$; however, at acidic pH k_{+5} becomes partially rate limiting. Since k_{+4} shows no kinetic isotope effect at pH 6.0, the isotope effect on k_{+5} determines the overall isotope effect on V_{max}/E_0. Calculation using the formula $V_{max}/E_0 = (k_{+4}k_{+5})/(k_{+4} + k_{+5})$ [where k_{+4} is the second step rate constant and k_{+5} the NAD^+ off rate[13]] gave a predicted $V_{max}/E_0(H_2O)$ = 23 s^{-1} and $V_{max}/E_0(D_2O)$ = 17.5 s^{-1}. This agrees well with our measured values of $V_{max}/E_0(H_2O)$ = 31.6 s^{-1} and $V_{max}/E_0(D_2O)$ = 18.7 s^{-1}.

Results of oxidation of benzyl alcohol are shown in Figure

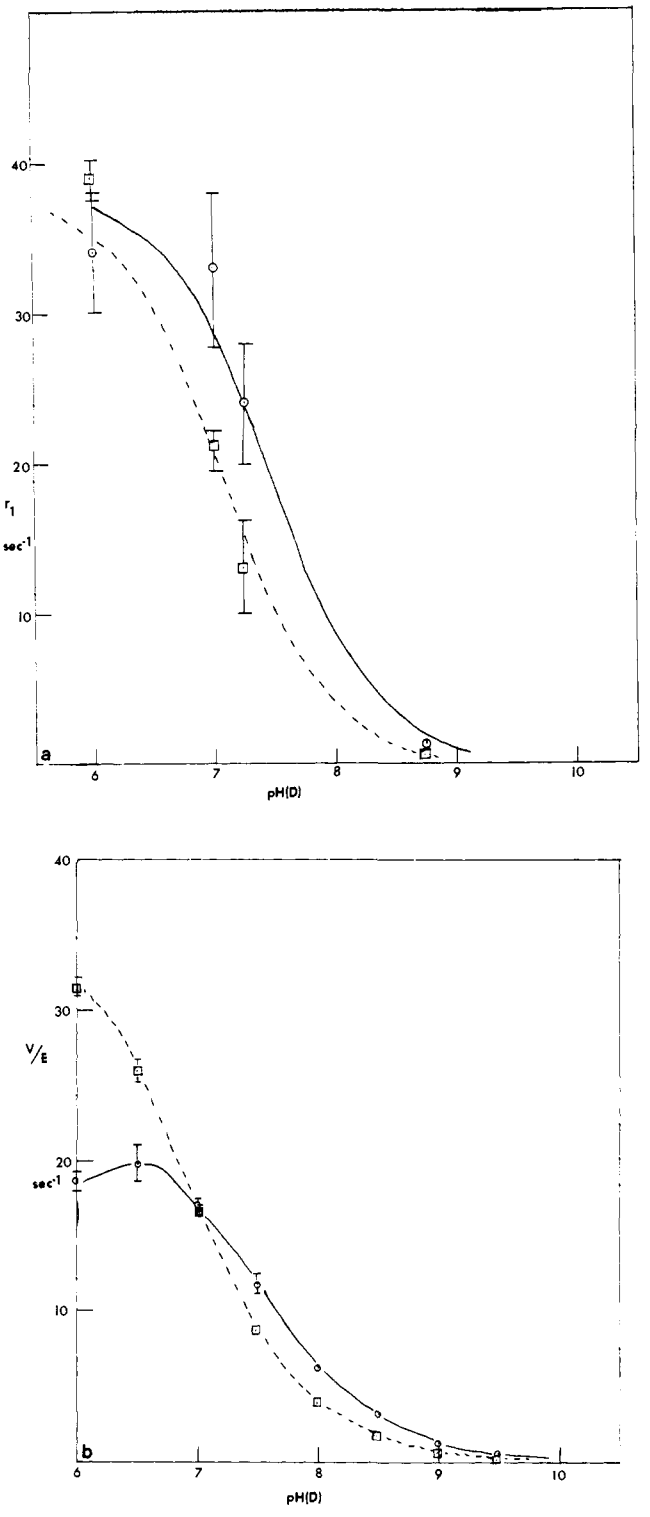


Figure 2. (a) pH dependence of the deuterium solvent isotope effect on r_1: (⊙, —) D_2O; (⊡, - - -) H_2O. Concentration conditions in Table I. The theoretical curves shown were derived using the following parameters: H_2O – pK_a = 7.1; D_2O – pK_a = 7.5 with k_{HA} = 39 s^{-1}; k_a = 0 s^{-1}. (b) pH dependence of the deuterium solvent isotope effect on V_{max}/E (⊙, —) D_2O; (⊡, - - -) H_2O. Concentration conditions are in Table I.

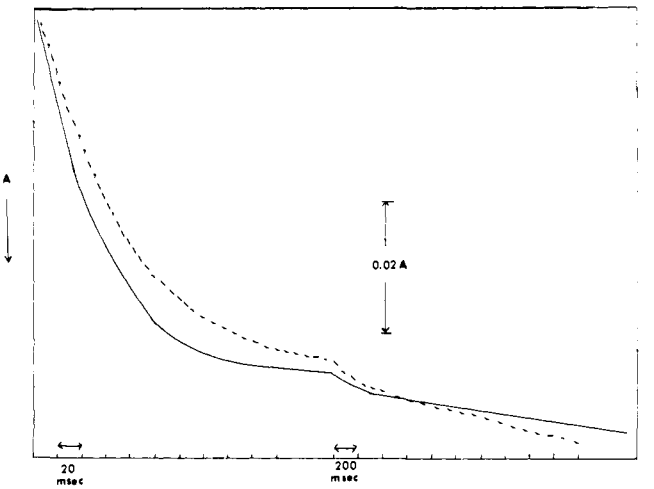


Figure 3. Time course of the transient kinetic oxidation of benzyl alcohol: (—) D_2O; (- - -) H_2O. [Benzyl alcohol] = 4 × 10^{-4} M; [LADH] = 1.0 × 10^{-5} N; [NAD^+] = 2.0 × 10^{-4} M; [isobutyramide] = 0.05 M. pH 8.75, pyrophosphate buffer 0.05 M.

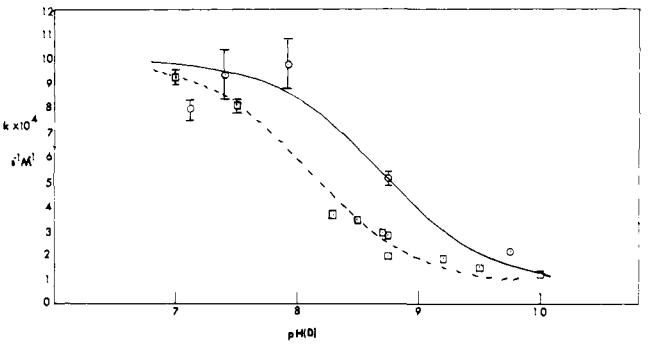


Figure 4. pH dependence of the deuterium solvent isotope effect on the rate constant for phenanthroline binding to LADH: (⊙, —) D_2O; (⊡, - - -) H_2O. The theoretical curves shown were derived using the following parameters: H_2O – pK_a = 8.1; D_2O – pK_a = 8.7 with k_{HA} = 0.10 $\mu M\ s^{-1}$; k_a = 0.012 $\mu M\ s^{-1}$.

3. There is a small inverse isotope effect on the single observed transient, r', for alcohol oxidation; this inverse isotope effect persists at acid pH as well as at pH 8.75 (Table II). Table III shows extrapolated values of the transient r_∞' and apparent binding constant for benzyl alcohol. The small inverse isotope effect on r_∞' is seen at all pH values; as seen in Table III, the isotope effect on the apparent binding constant is larger. Both oxidation and reduction reactions were carried out under suicide transient kinetic conditions where enzyme is removed from the reaction pathway by formation of a tight ternary complex.[2]

In addition to oxidation and reduction reactions, we have also measured the solvent isotope effect on the rate of phenanthroline binding to LADH, having previously reported the pH dependence of this rate in H_2O.[14] Phenanthroline has been shown to bind to the active-site zinc atom at two coordination positions by displacement of the water molecule of the fourth coordination site in native LADH. Figure 4 shows the inverse

Table III. Benzyl Alcohol Oxidation Parameters[a]

pL	10^4K_{app}, M		ratio	r', s^{-1}		ratio
	D_2O	H_2O	D_2O/H_2O	D_2O	H_2O	D_2O/H_2O
6.0	12.0	5.70	2.10	2.40	1.64	1.47
7.00	2.86	1.50	1.91	10.33	9.87	1.05
8.75	1.07	1.12	0.955	20.82	17.81	1.17

[a] Extrapolated from data shown in Table II.

Table IV. Deuterium Isotope Effects on Coenzyme Dissociation

NAD+ dissociation						
reactants						
[enzyme], μN	[NAD+], μM	[NADH], μM	[IBA], mM	k_{H_2O}, s^{-1}	k_{D_2O}, s^{-1}	k_{D_2O}/k_{H_2O}
7.90[a]	63	140	1.25	11.60 ± 0.2	14 ± 1	1.25
7.77[b]	123	180	1.24	7.80 ± 0.2	9.9 ± 0.5	1.27
NADH dissociation						
reactants						
[enzyme], μN	[NAD+], mM	[NADH], μM	[pyr], mM	k_{H_2O}, s^{-1}	k_{D_2O}, s^{-1}	k_{H_2O}/k_{D_2O}
8.35[b]	0.181	21	10	2.72 ± 0.04	2.15 ± 0.03	0.79
3.60[a]	8.2	33	8.2	4.65 ± 0.06	3.42 ± 0.05	0.74

[a] Experiments carried out at pH 8.69, T = 25 °C. [b] Experiments carried out at pH 8.75, T = 25 °C.

isotope effect observed for the process; there is a pK_a shift of ~0.5 pK_a unit associated with phenanthroline binding in D_2O.

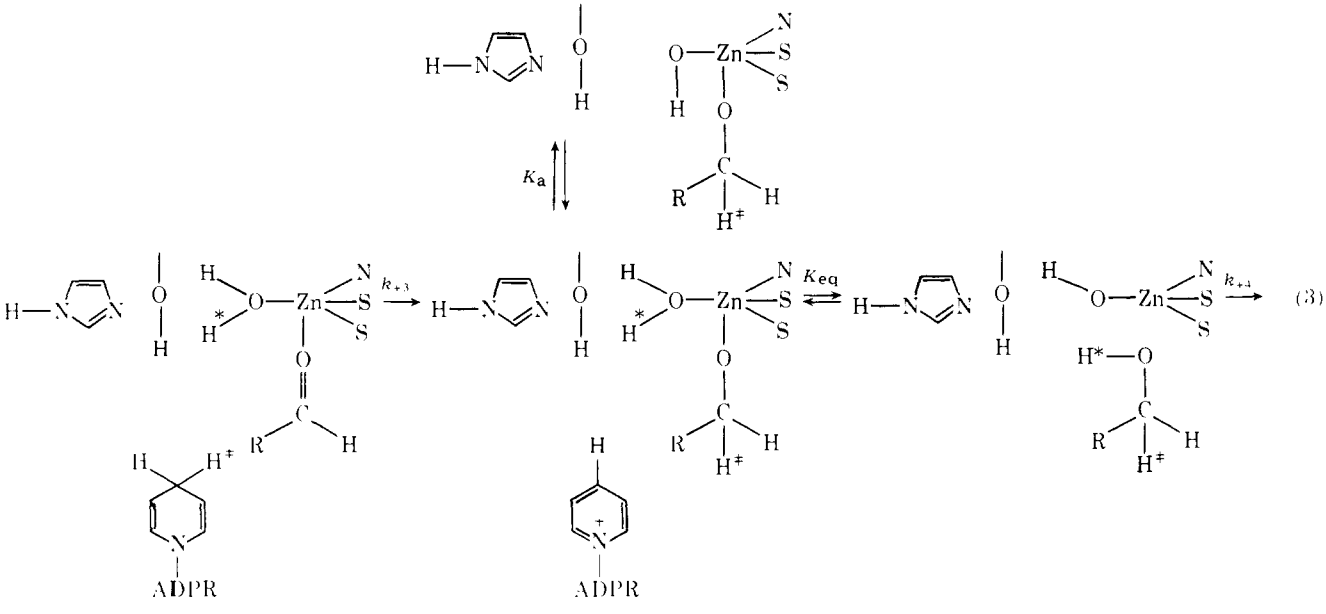
We have also measured the D_2O isotope effect on two reactions of binary complexes in which protonic equilibria have been postulated to occur.

Table IV reports the deuterium solvent isotope effect on the rate of dissociation of E-NAD+ complex. This rate was measured by displacing NAD+ from the binary complex with NADH and isobutyramide. Dissociation of NAD+ can then be measured by observing the fluorescence increase on forming E-NADH-isobutyramide ternary complex; a small inverse isotope effect is observed. Likewise, the dissociation of E-NADH was observed by displacing coenzyme with NAD+-pyrazole; in this case, the decrease of fluorescence of E-NADH is followed. Table IV indicates the small normal deuterium solvent isotope effect on E-NADH dissociation.

Discussion

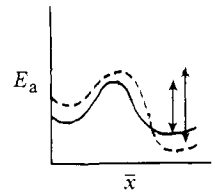
Deuterium solvent isotope effects have proven useful for studying events leading to the transition state in chemical reactions involving exchangeable protons. In enzyme chemistry, acid-base catalysis is often involved with bond making and bond breaking at the reacting chemical moiety, and solvent deuterium isotope effects have been employed to indicate if movement of proton is concerted with the chemical bond formation or cleavage step. For example, a primary deuterium solvent isotope effect on the hydrolysis of an acyl-enzyme intermediate of chymotrypsin has been interpreted in terms of synchronous movement of proton with nucleophilic attack on acyl-enzyme intermediate.[15] Conversely, the inverse "solvent" isotope effect during association of subunits of formyltetrahydrofolate synthetase indicates that proton movement is not concerted with subunit association.[16] A deuterium solvent isotope effect study in a dehydrogenase enzyme reaction (glutamate dehydrogenase) has been reported.[17] There is a pK_a equilibrium solvent effect on the first of three transient kinetic phases; however, a kinetic isotope effect of ~2.0 is observed for the third transient. The deuterium solvent isotope effect has recently been reported for benzaldehyde reduction and benzyl alcohol oxidation catalyzed by yeast alcohol dehydrogenase.[18a] An unusually small solvent isotope effect resulting from a pK_a change of <0.1 pK_a unit is observed for both processes.

A recent mechanistic proposal for the action of liver alcohol dehydrogenase is shown in eq 2.[1] The authors proposed a concerted movement of proton and hydride, thereby reducing the amount of charge separation built up in the transition state. However, the present data seem to suggest that there is not concerted movement of hydride and proton and indeed that movement of the proton occurs subsequent to hydride transfer ($r_{2,\infty} \simeq k_{+3}$) and prior to or following alcohol dissociation ($r_{1,\infty} \simeq k_{+4}$). We therefore propose a modification of the mechanism shown in eq 3.[18b] This is in keeping with the pH independence of k_{+3},[13] the absence of a primary deuterium solvent isotope effect on k_{+3} (Table I, Figure 1), the pH dependence of k_{+4}[13] reflecting the unfavorable preequilibrium for transfer of proton from solvent to the zinc alcoholate oxygen (K_A, eq



(3)

Chart I[a]



[a] (- - -) acidic form; (—) basic form.

3), and the observed inverse solvent D_2O isotope effect on k_{+4} (Table I, Figure 1). This latter inverse solvent isotope effect reflects the rapid preequilibrium protonation of zinc alcoholate, since Zn–OD_2 in D_2O is a poorer acid than Zn–OH_2 in H_2O. One would expect a shift in pH dependence equivalent to ~+0.5 pK_a unit in D_2O[19] (see Appendix); this shift is observed both in the pH dependence of the rate constant for association of phenanthroline (Figure 3) and enzyme and in k_{+4} and V_{max}/E_0 (Figure 2) (at basic pH where k_{+4} limits turnover). Furthermore, the proposed mechanism in which Zn^{2+} acts as an acid catalyst is consistent with the smaller substituent effects for k_{+3} than those observed for $NaBH_4$ reduction of para-substituted aldehydes. It should be mentioned at this point that the pH dependence cited above for parameters associated with aldehyde reduction is also observed in a modified enzyme where hydride transfer (k_{+3}) may be rate limiting.[1] However, in this system a larger substituent effect is observed than that observed by transient kinetic studies on k_{+3}, so that unlike the pH dependencies, the substituent effects mentioned above have not been confirmed by two independent studies.[1]

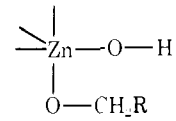
The absence of a preequilibrium solvent isotope effect on aldehyde reduction indicates that unlike phenanthroline binding (which shows such an effect), there is no displacement of water bound to zinc during aldehyde binding. The mechanism shown in eq 2 and 3 is consistent with zinc catalysis without involving displacement of water. This is accomplished by binding zinc to the fifth coordination site. Such fifth site coordination has been established for the binding of the inhibitor imidazole to carbonic anhydrase involving a Zn–N distance of 2.8 Å for the inner sphere fifth coordination bonding as opposed to 2.0 Å for Zn–N distance for the four histidine ligands.[20]

Tables II and III show the inverse isotope effect for the transient kinetic time course of alcohol oxidation at several pH values. Both the apparent binding constants and the apparent rate constants show inverse solvent isotope effects. Recent theoretical treatments of the single exponential transient for alcohol oxidation indicate that $r' \simeq k_{-3}$; that is, the rate constant for the transient in alcohol oxidation is also associated with the chemical step.[21]

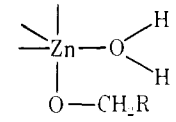
The mechanism shown in eq 3 seems inconsistent with two observations made on the transient kinetics of alcohol oxidation, namely the pH dependence and the small substituent effect. The substituent effect during aldehyde reduction (k_{+3}) is much smaller than observed for $NaBH_4$ reduction of the same series of aldehydes; this fact has been interpreted in terms of an early transition state. Conversely, one would predict that such an early transition state would lead to a large substituent effect for alcohol oxidation and that such substituent effect would be in a direction opposite to that for aldehyde reduction. In fact, the substituent effects are in opposite directions, but the ρ value for alcohol oxidation is rather small.[22] One way to rationalize such a small value is to argue that movements of proton and hydride are concerted, resulting in little overall change in charge distribution on proceeding to the transition state. This point of view would be supported by the pH dependence observed for alcohol oxidation, in which the transient kinetic rate for alcohol oxidation becomes smaller at acidic pH values.[13,23]

However, we would like to suggest that this pH dependence for alcohol oxidation can be interpreted in terms of the proposed mechanism of eq 3. Since both aldehyde and water remain bound to the Zn^{2+} atom simultaneously and since the pK_a of Zn–OH_2 is ~8.0,[14] then ionization of water should lead to an electronic effect on the reaction rate constant. Furthermore, if there is an early transition state leading to aldehyde reduction, this electronic effect would be negligible in the aldehyde reduction direction and large for alcohol oxidation. This is a consequence of the transition state consideration shown in Chart I.

The observed pH dependence is quite consistent with this picture in which ionization of Zn–OH_2 in the zinc alcoholate complex would lead to larger electron density in the C–H bond undergoing cleavage and therefore to faster rate of alcohol oxidation for:



than for:



However, as shown in Chart I the early transition state could lead to a negligible rate effect of ionization on aldehyde reduction.

In summary, the mechanism shown in eq 3 is consistent with the transient kinetic data reported in this paper as well as previous transient kinetic studies of substituent effects, pH dependence, and isotope effects. Furthermore, the mechanism shown is consistent with previous spectroscopic data which indicate direct liganding between a chromophoric aldehyde and zinc at the active site of liver alcohol dehydrogenase in an inactive ternary complex.[24]

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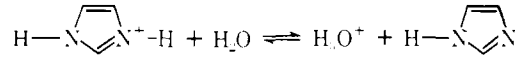
Appendix

The "normal" solvent isotope effect mentioned for ionization of an amino acid side chain such as the imidazole of histidine results from the solvent isotope effect on the following equilibrium:

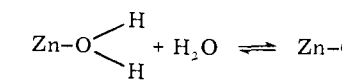


$$K_{H_2O}/K_{D_2O} = (\phi_{H_2O})^2\phi_{IM^+}/\phi_{IM}(\phi_{H_3O^+})^3$$

This corresponds to a $\Delta pK_a \simeq 0.5$.

The solvent isotope effect can be calculated as shown by considering that the fractionation factors for imidazolium cation, imidazole, and H_2O are all $\phi \simeq 1.0$; however, the fractionation factor for H_3O^+ is $\phi = 0.69$ (see ref 26 for a discussion of these calculations).

For the mechanism of eq 3, the solvent isotope effect (SIE) on k_{+4} would be a function of the SIE on the ionization of Zn–OH_2:



$$K_{H_2O}/K_{D_2O} = (\phi_{Zn-OH_2})^2(\phi_{H_2O})^2/(\phi_{H_3O^+})^3(\phi_{Zn-O-H})$$
$$= (\phi_{Zn-OH_2})^2/(0.69)^3$$

This assumes that Zn–OH has a fractionation factor of ϕ = 1.0. Now if we estimate the $Zn–OH_2$ fractionation factor by linear interpolation between $\phi_{H_3O^+}$ (pK_a = 1.74) and ϕ_{H_2O} (pK_a = 15.74), then the fractionation factor for $Zn–OH_2$ ($pK_a \simeq 8–9$) would be predicted to be ϕ = 0.85. For this equilibrium then $K_{H_2O}/K_{D_2O} = (0.85)^2/(0.69)^3 = 2.2$, and $\Delta pK_a = 0.3$. It can be seen that the estimated SIE for ionization of $Zn–OH_2$ is smaller than for imidazole. However, because of error in this estimate and in the measured SIE for k_{+4}, we are not prepared to state that either imidazole or $Zn–OH_2$ is acting as the ionizable acid–base catalyst in the second rate process observed during aldehyde reduction. In eq 3 we have shown only one of many possibilities for the ultimate fate of proton removed from alcohol during alcohol oxidation.

References and Notes

(1) R. T. Dworschack and B. V. Plapp, *Biochemistry*, **16**, 2716 (1977).
(2) J. T. McFarland and S. A. Bernhard, *Biochemistry*, **11**, 1486 (1972).
(3) K. Dalziel, *J. Biol. Chem.*, **239**, 2850 (1963).
(4) G. Pettersson, *Eur. J. Biochem.*, **69**, 273 (1976).
(5) J. Kvassman and G. Pettersson, *Eur. J. Biochem.*, **69**, 279 (1976).
(6) J. W. Jacobs, J. T. McFarland, I. Wainer, D. Jeanmaier, C. Ham, K. Hamm, M. Wnuk, and M. Lam, *Biochemistry*, **13**, 60 (1974).
(7) M. F. Dunn and S. A. Bernhard, unpublished results.
(8) C. F. Weldig, H. R. Halvorson, and J. D. Shore, *Biochemistry*, **16**, 2916 (1977).
(9) M. Hadorn, V. A. John, F. K. Meier, and H. Dutler, *Eur. J. Biochem.*, **54**, 65 (1975).
(10) R. E. Hansen, in "Rapid Mixing Notebook", Vol. I, Note 5, Update Instruments, Inc., Madison, Wis., 1978, p 3.
(11) S. A. Bernhard, M. F. Dunn, P. L. Luisi, and P. Shack, *Biochemistry*, **9**, 185 (1970).
(12) P. K. Glasoe and F. A. Long, *J. Phys. Chem.*, **64**, 188 (1960).
(13) J. T. McFarland and Y. H. Chu, *Biochemistry*, **14**, 1140 (1975).
(14) M. C. DeTraglia, J. Schmidt, M. F. Dunn, and J. T. McFarland, *J. Biol. Chem.*, **252**, 3493 (1977).
(15) E. Pollock, J. L. Hogg, and R. L. Schowen, *J. Am. Chem. Soc.*, **95**, 2279 (1973).
(16) J. A. L. Harmony, R. H. Hines, and R. L. Schowen, *Biochemistry*, **14**, 5379 (1975).
(17) A. H. Cohen, R. R. Wilkinson, and H. E. Fisher, *J. Biol. Chem.*, **250**, 5243 (1975).
(18) (a) J. P. Klinman, in "Isotope Effects on Enzyme Catalyzed Reactions", W. W. Cleland, M. H. O'Leary, and D. B. Worthrup, Eds., University Park Press, Baltimore, 1976. (b) We have not shown canonical structures for transfer of proton through the proton relay system. Our proposal deals only with the *change* in the state of protonation of this system during catalysis. We have also shown proton transfer preceding break up of zinc alcoholate complex; however, the data are equally consistent with unfavorable equilibrium dissociation to Zn and alcoholate anion followed by rapid proton transfer. We prefer the mechanism of eq 3 on the basis of our preference for S_N1 decomposition of the *protonated* Zn alcohol complex.
(19) J. F. Coetzee and C. D. Ritchie, in "Solute-Solvent Interactions," Marcel Dekker, New York, 1969, pp 499–538.
(20) K. K. Kannan, M. Petef, K. Fridborg, H. Cid-Dresdner, and S. Lövgren, *FEBS Lett.*, **73(1)**, 115 (1977).
(21) J. Kvassman and G. Pettersson, *Eur. J. Biochem.*, **87**, 417–427 (1978).
(22) J. J. Hardman, L. F. Blackwell, C. R. Boswell, and P. D. Buckley, *Eur. J. Biochem.*, **50**, 113 (1974).
(23) R. L. Brooks, J. D. Shore, and H. Gutfreund, *J. Biol. Chem.*, **247**, 2382 (1972).
(24) M. F. Dunn and J. S. Hutchison, *Biochemistry*, **12**, 4882 (1973).
(25) J. T. McFarland, Y. H. Chu, and J. W. Jacobs, *Biochemistry*, **13**, 65 (1974).
(26) K. B. Showen, in "Transition States of Biochemical Processes", R. D. Gandorn and R. L. Schowen, Eds., Plenum Press, New York, 1978.

Kinetic and Thermodynamic Study of the Specificity in the Elementary Steps of α-Chymotrypsin-Catalyzed Hydrolysis Reaction

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Abstract: Pre-steady-state kinetic processes of α-chymotrypsin-catalyzed hydrolysis were studied at several temperatures for *N*-(2-furyl)acryloyl derivatives of Tyr, Phe, and Leu methyl esters, and the kinetic and thermodynamic parameters of the individual elementary steps were determined. For a wide range of pH, the rate profile of the hydrolyses of these esters, under the condition of enzyme in excess of the substrate, confirmed the generally accepted three-step mechanism and did not require the postulation of the existence of additional intermediates. The spectra of the two intermediates (enzyme–substrate complex and acyl-enzyme) expected for the three-step mechanism were quantitatively measured. From the pH dependences of three kinetic parameters (enzyme–substrate dissociation constant, acylation rate constant, and deacylation rate constant), their pH-independent limiting values were obtained, showing varying specificity depending on the particular elementary step. The temperature dependences of the reaction parameters indicated that the specificity in the deacylation step is controlled by the entropic term as far as the specific substrates are concerned. This was not so in the case of nonspecific substrates. The acylation step for specific substrates exhibited less negative or positive $\Delta S^{\ddagger}$ values, and the enzyme–substrate dissociation constant was rather insensitive to the change in temperature.

α-Chymotrypsin is a well-known endoproteinase which hydrolyzes the peptide bonds at the C end of hydrophobic amino acid residues. A number of studies on its specificity have been performed on the overall reaction rates (k_{cat}/K_m) or on the steady-state kinetic parameters (k_{cat} and K_m).[1,2] However, these parameters do not always represent the microscopic rate or equilibrium constant for the elementary reactions of this enzyme. In order to obtain an insight into the mechanism of the α-chymotrypsin catalysis, studies on the specificity[3,4] and thermodynamic parameters[5] of the individual rate processes of the reaction, as shown by eq 1, are necessary, especially by means of pre-steady-state kinetic measurements:

$$E + S \overset{K_s}{\rightleftarrows} E \cdot S \overset{k_2}{\rightarrow} ES' \overset{k_3}{\rightarrow} E + P_2 \quad (1)$$
$$+ P_1$$

(E, S, E·S, ES′, P_1, and P_2 denote enzyme, substrate, enzyme–substrate complex, acyl-enzyme, first leaving group, and second leaving group (acid), respectively, and K_s, k_2, and k_3 are the dissociation constant of E·S complex, acylation rate constant, and deacylation rate constant, respectively.) However, detailed kinetic and thermodynamic studies of the individual steps have been restricted to the deacylation step of the acyl-enzyme[6-10] or to the binding of substrate analogues.[11-14]

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