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KINETICS OF ENZYME-COENZYME INTERACTIONS BY NMR SPECTROSCOPY

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Summary

The kinetics for the binding of coenzymes to H_4 and M_4 lactate dehydrogenase from chicken were investigated by nuclear magnetic resonance spectroscopy. With detailed computer analysis, some kinetic parameters were extracted from the chemical shifts and the linewidth of the observed coenzyme resonances at various enzyme/coenzyme ratios and temperatures. The results of the analysis indicated that the dissociation rates of coenzymes from the enzyme/coenzyme complexes are slower with the H_4 isozyme than those involving the M_4 isozyme. The lifetimes for the NAD^+-enzyme complexes are on the order of 1 msec while those for the NADH-enzyme complexes are on the order of 10 ms (at room temperature). Much shorter transverse relaxation times of the coenzyme resonances were observed in NADH-enzyme complexes than those in the NAD^+-enzyme complexes. The calculated kinetic constants are in good agreement with the previous studies by stopped-flow and temperature jump methods. A generalized NMR kinetic treatment for the binding of small molecules to a macromolecule is presented.

Introduction

Nuclear magnetic resonance spectroscopy (NMR) has been extensively applied to the kinetic studies of inhibitor or substrate binding to enzymes [1—6]. Most of these studies have been concerned with reversible small molecule-macromolecule interactions, and the effects that the macromolecule has on the chemical shifts, linewidths, and relaxation times of specific resonances in the ligand. Among these studies, the method of Swift and Connick [7] was most commonly employed for obtaining the kinetic parameters. However, this

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Abbreviations used: H_4 lactate dehydrogenase, heart type isozyme of lactate dehydrogenase; M_4 lactate dehydrogenase, Muscle type isozyme of lactate dehydrogenase.

treatment is limited to the cases where the relative population of the small molecule in the free state is much greater than that in the macromolecule-bound state. In view of this weakness, the original theory [8] from which the method of Swift and Connick was derived should be considered further by employing computer analyses to generalize its applications. It was found that, similar to the cases for chemical exchange of small molecules, the method of total line-shape analysis [9] could also be applied to the kinetic studies of small molecule-macromolecule interactions after some modifications.

The kinetics of the binding of coenzymes to lactate dehydrogenases have been studied by stopped-flow and temperature jump methods [10—13]. However, NMR spectroscopy can provide detailed information regarding the nature of coenzymes in the active sites of dehydrogenases [14], relaxation and correlation times of the coenzymes in the enzyme-coenzyme complex, and the dissociation rates of the coenzyme from the complex. In this communication, a generalized NMR treatment was employed for the kinetic studies of the binding of coenzymes to H_4 and M_4 lactate dehydrogenases in an attempt to elucidate the dfferences between these two isoenzymes.

Theory

In the binding of small molecules to macromolecules, the appearance of NMR spectra for the small molecules in both free and bound environments is sensitive to the dissociation rate occurring on the NMR time scale of observation. This 'critical rate' can be thought of as the inverse of the characteristic time with which the transverse components of the magnetization of a given resonance dephase after exchange from one site to another. Usually, the critical rate of a given resonance is defined relative to two factors: (1) the chemical shift difference between free and the bound state. Δ: (2) the inverse of the transverse relaxation time in the bound state, $1/T_{2b}$ [4—8]. If the exchange rate between the free and the bound state is faster than this critical rate, weight-averaged spectra will appear; the chemical shift, the linewidth, and the relaxation times of the observed resonances are the weight-average of these spectra in the free and the bound states. On the other hand, if the rate is slow compared to this critical rate, the resonances arising from the molecules in these two states are separated. Under this condition, only the resonances of the molecules in the free state can be observed. In both fast and slow exchange limits, simplified equations have been derived to obtain dissociation rate constants and relaxation times in the bound states [8]. Beyond these exchange limits, it is necessary to employ the modified Bloch equations to deduce these kinetic parameters. In order to simulate our experimental data for the coenzyme binding to lactate dehydrogenases, a generalized computer program was written according to the modified Bloch equations for a two-site exchange. Chemical shifts and line-width for the uncoupled, two-site case were simulated with the expression for the total magnetic moment $M(\omega)$ developed by Gutowsky and Holm [8,15]:

$$M(\omega) = \frac{i\omega_1 M_0(k_{-1} + \alpha_b P_f^2 + \alpha_f P_b P_f)}{(P_b k_{-1} + P_f \alpha_b)(k_{-1} + \alpha_f) - P_b k_{-1}^2} \qquad (1)$$

where ω_1 is R.F. field frequency; M_0, the equilibrium magnetic moment; P_f and P_b are the relative populations of the free and bound states respectively;

$$\alpha_b = \frac{1}{T_{2b}} - i(\omega - \omega_b), \quad \alpha_f = \frac{1}{T_{2f}} - i(\omega - \omega_f),$$

ω_b and ω_f are the resonance frequencies in rad/s of the bound and free state resonances respectively. The imaginary part (absorption mode) of the total magnetic moment was generated as a function of frequency ω by computer under a given set of input parameters. The results were then compared with the observed coenzyme resonacnes under given enzyme/coenzyme concentrations ratios and temperatures.

Eqn. 1 has been widely employed in the total line-shape analysis of the chemical exchange between small molecules [8,9,15], such as rotational isomerization and keto-enol tautomerism. In the case of small molecules, the transverse relaxation times in the absence of exchange are usually long and the same in both states, However, in the case of enzyme-coenzyme interactions, T_{2b} values for coenzyme resonances are usually short compared to T_{2f} values.

From the described theory and kinetic treatment, one is able to obtain the dissociation rate constants (k_{-1}) as well as the bound relaxation times (T_{2b}) by different experimental conditions.

Materials and Methods

Chicken H_4 and M_4 lactate dehydrogenases were prepared in our laboratory by Mr. Francis Stolzenbach in a crystalline form [16]. SDS polyacrylamide and starch gel electrophoresis indicated that there was only one detectable band for each isoenzyme. Under the reported assay conditions, both isoenzymes exhibit the same specific activity of 500 units/mg [16]. In order to remove possible paramagentic contaminants, the enzymes were dialyzed extensively against 0.05 M Tris-HCl buffer solutions in ^2H_2O containing 3 mM EDTA at pH 7.6. The concentrations of the enzyme and coenzyme in the sample solution were determined before and after the NMR measurements.

NAD^+ (acid form) and NADH (sodium salt) were obtained from P.L. Biochemicals, Inc., Wisc. These chemicals were used without further purification. ^2H_2O (99.8% isotopic purity) was purchased from Mallinckrodt Chemical Works, Mo.

All the NMR spectra were recorded on a Varian HR 220 MHz spectrometer which was equipped with a Nicolet 1074 time average computer, in order to increase the signal-to-noise ratio. The ambient probe temperature of the spectrometer was $22 \pm 1°C$. The temperature study was conducted with a variable temperature controller on the spectrometer. Ethylene glycol was used to calibrate the probe temperature. The observed linewidth was measured as the width at the half heighth of a given coenzyme resonance with proper corrections for the spin-spin coupling. The accuracy of this measurement ranged from ±0.5 Hz to ±1 Hz, depending on the extent of broadening and the signal-to-noise ratio of the observed resonances. The chemical shift was measured from internal standard tetramethyl ammonium chloride with an accuracy of ±0.3 Hz.

The longitudinal relaxation times (T_1) of coenzyme resonances under various enzyme/coenzyme ratios were measured with the Varian HR 220 spectrometer according to the method of progressive saturation [17] as well as with a JEOL PS-100 spectrometer equipped with Fourier transform accessory. The transverse relaxation times (T_2) were obtained from measurements of the linewidth at observed resonances.

Results

In order to elucidate the kinetic parameters of binding as well as relaxation times of NAD^+ in the bound state (T_{2b}), the linewidth and the differential shifts of the coenzyme resonances were measured at different enzyme/coenzyme concentration ratios and temperatures (Figs. 1 and 2). The results are presented in Table I.

In Table I, differential shifts of some NAD^+ resonances in the presence of H_4 and M_4 lactate dehydrogenases from chicken are presented under the conditions described. Since the pyridine H_2 and the adenine H_2 and H_8 resonances of NAD^+ appear as a broad singlet, the linewidth of these resonances can be accurately measured. In Table I, it can be shown that at a given enzyme/coenzyme ratio and temperature, the observed linewidth of NAD^+ resonances

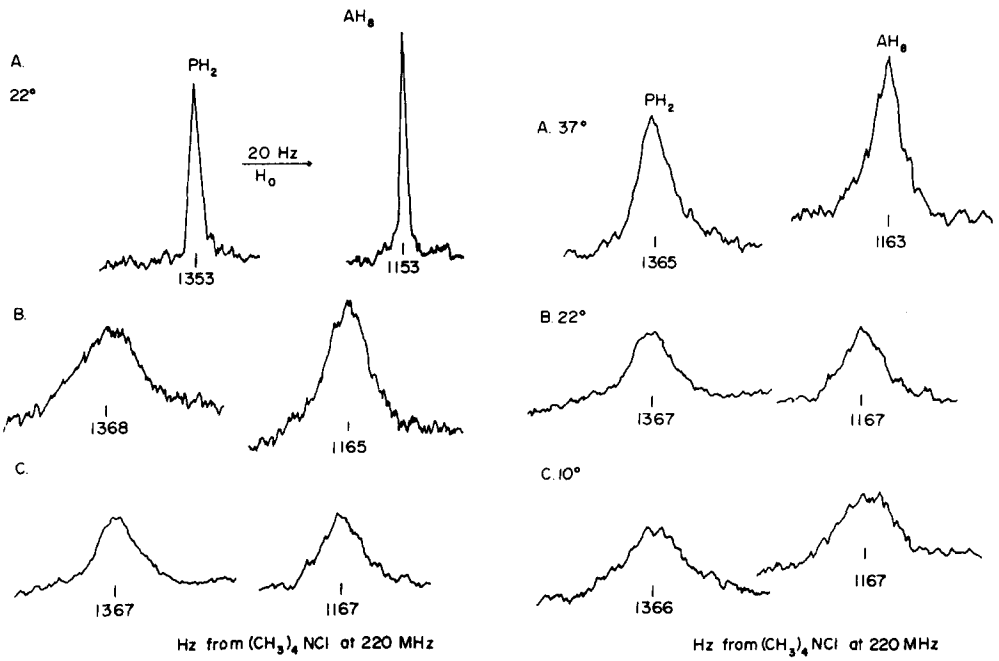

Fig. 1. Pyridine H_2 (PH_2) and adenine H_8 (AH_8) resonances of NAD^+ in the absence of (A) and in the presence of (B and C) lactate dehydrogenase at 22°C. In (A) 2 mM NAD^+; in (B) 3 mM NAD^+ and 1 mM H_4 lactate dehydrogenase; and in (C) 2 mM NAD^+ and 0.7 mM M_4 lactate dehydrogenase.

Fig. 2. Temperature dependence of pyridine H_2 (PH_2) and adenine H_8 (AH_8) resonances of NAD^+ (2 mM) in the presence of M_4 lactate dehydrogenase (0.7 mM). The temperatures are as shown in the figure.

TABLE I

OBSERVED CHANGES IN CHEMICAL SHIFT AND LINEWIDTH OF SOME NAD$^+$ RESONANCES AT VARIOUS TEMPERATURES AND COENZYME/ENZYME CONCENTRATION RATIOS

ν_{obs} and δ_{obs} are the observed linewidth and differential shift (in Hz) coenyzme resonances, respectively. + and — denote, respectively, the upfield and downfield shift relative to the free coenyzme resonances.

Isoenzyme	[Coenzyme]/ [Enzyme]	Temp. (°C)	Observed coenzyme resonances					
			P_{H2}		A_{H8}		A_{H2}	
			ν_{obs}	δ_{obs}	ν_{obs}	δ_{obs}	ν_{obs}	δ_{obs}
H_4 lactate dehydrogenase (1 mM)	3	10	24	—9	18	—10	+2	
	6		11	—4	12	—5	+1	
	3	22	19	—15	11	—12	+5	
	5		16	—9	9	—9	+3	
	6		14	—8	8	—6	+2	
	3	37	16	—15	9	—12	+4	
	5		12	—9	8	—8	+3	
	6		11	—8	7	—6	+2	
M_4 lactate dehydrogenase (0.7 mM)	3	10	13.5	—16	14	—15	—4	
	6		11	—9	9	—11	—2	
	9		9	—5	8	—6	—1	
	3	22	12	—14	10	—14	—4	
	6		0	—5	7	—6	—2	
	9		7	—3	6	—3	—1	
	3	37	9	—10	7	—10	—4	
	6		6;5	—4	6	—4	—2	

in the presence of H_4 lactate dehydrogenase is considerably broader than that observed in the presence of chicken M_4 lactate dehydrogenase. In chicken M_4 lactate dehydrogenase, the observed differential shifts of NAD$^+$ resonances decreased with increasing temperature for a given coenzyme/enzyme ratio, while those of NAD$^+$ resonances increased with increasing temperature in the presence of chicken H_4 lactate dehydrogenase. In contrast, as shown in Fig. 2 and Table I, the linewidth of all the NAD$^+$ resonances decreased with increasing temperatures in the presence of both lactate dehydrogenase isoenzymes.

As described in a previous paper [14] the spectral behavior of NADH upon binding to dehydrogenases is significantly different from that of NAD$^+$ because of the strong binding of the reduced coenzyme to enzymes. In order to determine the suitable kinetic parameters as well as the relaxation times in the bound state, the differential shifts as well as linewidth were measured at high coenzyme/enzyme ratios and at different temperatures (Figs. 3 and 4).

In Table II, the linewidth as well as differential shifts of selected NADH resonances are listed for NADH/H_4 lactate dehydrogenase concentration ratios of 10:1, 20:1, and 30:1 at three different temperatures. As shown in this Table, the observed linewidth and differential shifts increased significantly with decreasing coenzyme/enzyme ratios. For the coenzyme/enzyme ratios below 10:1, the NADH resonances become too broad to be observed.

A similar study with M_4 lactate dehydrogenase is presented in Table II and Fig. 4. At the same coenzyme/enzyme concentration ratio (10:1), significant differences were observed regarding the appearance of NADH resonances in the

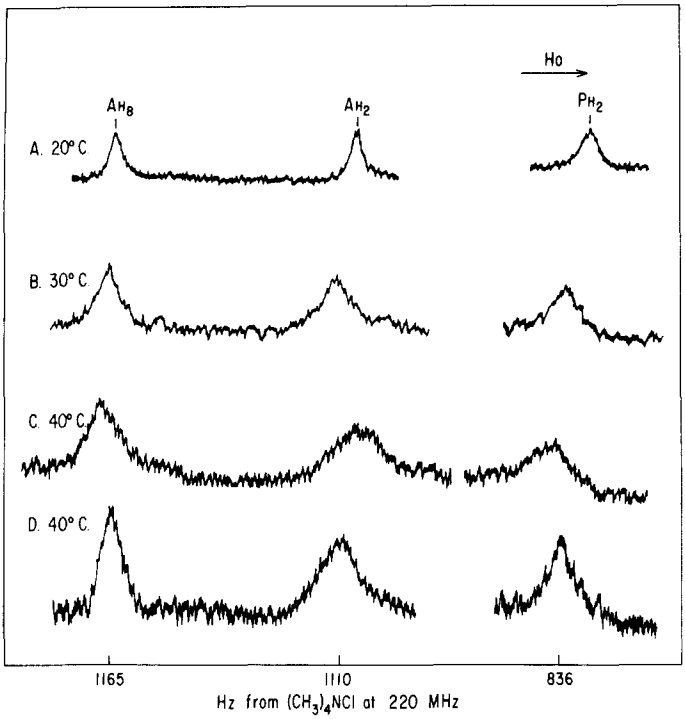

Fig. 3. Temperature dependence of adenine H_8 and H_2 resonances (AH_8 and AH_2) as well as pyridine H_2 resonances (PH_2) of NADH (6 mM in A, B, and C; 10 mM in D) in the presence of 0.6 mM H_4 lactate dehydrogenase from chicken. The temperatures are shown in the figure.

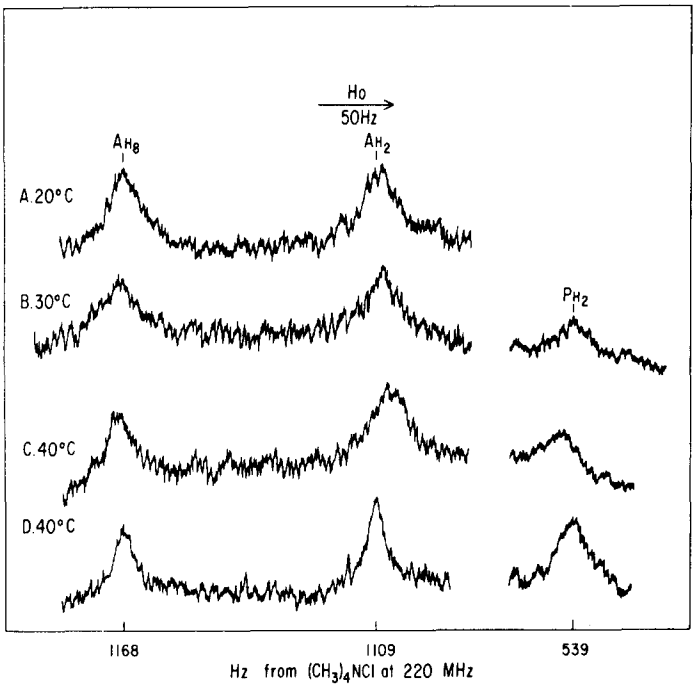

Fig. 4. Temperature dependence of adenine H_8 and H_2 resonances (AH_8 and AH_2) and Pyridine H_2 resonance (PH_2) of NADH (5 mM in A, B, and C; 10 mM in D) in the presence of 0.5 mM M_4 lactate dehydrogenase from chicken. The temperatures are shown.

TABLE II

OBSERVED CHANGES IN CHEMICAL SHIFT AND LINEWIDTH OF SOME NADH RESONANCES AT VARIOUS TEMPERATURES AND COENZYME/ENZYME CONCENTRATION RATIOS

Isoenzyme	[Coenzyme]/ [Enzyme]	Temp. (°C)	Observed coenzyme resonances					
			P_{H2}		A_{H8}		A_{H2}	
			ν_{obs}	δ_{obs}	ν_{obs}	δ_{obs}	ν_{obs}	δ_{obs}
H_4 lactate dehydrogenase	10	20	3.0	−0.5	3.5	+1.0	4.5	−0.5
(0.6 mM)	10	30	12	−2	13	+3	14	−3
	20		7	−1	10	+2	12	−2
	30		3.5	0	4	+1	7	−1
	10	40	10	−5	13	+6	15	−7
	20		7	−3	9	+3	10	−4
	30		3.5	−1	4	+1	7.5	−2
M_4 lactate dehydrogenase	10	20	10	−3	11	+3		
(0.5 mM)	20		8	−2	8	+2	11.5	−3
	10	30	11	−4	11	+5	16	−7
	20		8	−2	8	+2	10	−4
	30		4	−1	4.5	+1	7	−2
	10	40	10	−4.5	11	+8	15	−9
	20		6.5	−2	6	+4	9	−5
	30		3.5	−1	4.5	+2	7.5	−4

presence of the two lactate dehydrogenase isozymes. In the presence of H_4 lactate dehydrogenase, little shift and significant broadening of NADH resonances were observed at 20°C. However, both the differential shift as well as linewidth of NADH increased significantly with increasing temperature. In contrast, in the presence of M_4 lactate dehydrogenase, there were only slight changes in both linewidth and differential shifts of NADH resonances with increasing temperature. As shown in Figs. 3 and 4, the differential shift and linewidth of NADH resonances became comparable in the presence of either isozyme at 40°C.

Discussion

In the binding of coenzymes to dehydrogenases, the appearance of coenzyme resonances is very sensitive to the dissociation rates of coenzyme molecules from the complex, if the rates are on the order of NMR time scale of observations. The NMR time scale is determined by (1) the bound shift of a given coenzyme resonances (Δ) and (2) the inverse of its transverse relaxation time in the bound state ($1/T_{2b}$). The principal goal of this communication is to provide a generalized NMR kinetic treatment to resolve these two parameters for the enzyme-coenzyme interactions.

Because the relative population of the coenzyme in the free and the bound states are comparable in the case of the binding of NAD^+ to lactate dehydrogenases the simplified equations derived by Swift and Connick [7] cannot be applied to extract the kinetic parameters. Moreover, the range of the dissociation rates for enzyme-coenzyme binding vary from slow, intermediate, to fast exchanges and Δ and T_{2b} are not easily obtained directly from the observed

coenzyme resonances. Therefore a computer program was devised on the basis of the modified Bloch equations, Eqn. 1, to evaluate the parameters. The computerized calculation is based on the assumption that the coenzyme molecules exchange between the free and the enzyme-bound states as described by the equation:

$$E + C \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EC$$

where E, C, and EC represent free enzyme, free coenzyme, and enzyme-coenzyme complex respectively, k_{+2} and k_{-1} are the respective association and dissociation rate constants. The linewidth and the differential shift of the coenzyme resonances under a given experimental condition can be simulated by the computer program only with a proper and unique choice of T_{2b}, (Δ), and k_{-1} *. The computerized calculations according to Eqn. 1 require the precise knowledge of the relative populations of the coenzyme in free and the complexed state. It has been shown by fluorescence studies that the dissociation constants (k_d values) for the binary complex between NADH and lactate dehydrogenases from chicken are of the order of 10^{-7}—10^{-6} M [18]. Under the experimental conditions used, the concentrations of NADH employed in our kinetic study are usually of the order of 10 mM which is several orders of magnitude higher than k_d values of the enzyme-coenzyme complex. We can therefore assume that more than 99% of the enzyme is in the complexed state.

In the case of the binding of NAD^+ to lactate dehydrogenases from chicken, no information is available regarding the k_d values of NAD^+-lactate dehydrogenase complex, since NAD^+ does not cause quenching of protein fluorescence of these enzymes. However, the steady state kinetic studies indicate that at room temperatures, K_m values for NAD^+ are of the order of 10^{-4} M which is at least one order of magnitude lower than the concentrations of the coenzymes employed in the binding study [19]. Previous studies with lactate dehydrogenase from beef heart, chicken muscle, and lobster tail also indicated that K_m or k_d values for NAD^+-lactate dehydrogenase complex are not sensitive to temperature changes from 10 to 37°C [14]. Therefore, one can assume that under these experimental conditions, the fraction of the coenzymes in the bound state can be approximately given by:

$$\frac{[EC]}{[C]_0} \simeq \frac{[E]_0}{[C]_0}$$

where $[E]_0$ is the initial enzyme concentration, $[C]_0$ the initial coenzyme concentration, and [EC] is the concentration of enzyme-coenzyme complex.

In the case of the binding of NAD^+ to H_4 lactate dehydrogenase, the exchange rates fall in the region of intermediate to fast exchange. However, in order to fit the experimental data given in Table I the estimated bound shifts for the pyridine H_2 and the adenine H_8 resonances were found to decrease with increasing temperature. For example, the calculated bound shifts for the

* In the computer analysis, the known parameters for a given observed coenzyme resonance are (1) differential chemical shift (δ_{obs}), (2) observed linewidth (ν_{obs}) and (3) relative population of the coenzyme molecule in the free and the bound state (P_f and P_b). According to Eqn. 1, T_{2b}, Δ, and k_{-1} can be uniquely obtained by computer simulations.

pyridine H_2 resonance of NAD^+ vary from −65 Hz at 10°C to −50 Hz at 37°C. Similar results were also obtained for the binding of NAD^+ to M_4 lactate dehydrogenase where the exchange rates are relatively fast in this temperature range. The estimated bound shifts of NAD^+ resonances in the case of M_4 lactate dehydrogenase are smaller than those for H_4 lactate dehydrogenase. The fact that the downfield bound shifts of NAD^+ resonances decrease with increasing temperature has been interpreted as the result of the unfolding of coenzyme molecules in the active site of dehydrogenases [14]. The best fit bound shifts (Δ) and bound relaxation times (T_{2b}) of the pyridine H_2 and the adenine H_8 NAD^+ resonances upon the binding to H_4 and M_4 lactate dehydrogenases are presented in Table III.

Since the first order dissociation rate constant, k_{-1}, does not depend on the enzyme concentration or coenzyme/enzyme concentration ratio at a given temperature, the Δ and T_{2b} which characterize a given coenzyme resonance of the system must be those yielding the observed linewidth and the observed chemical shift for the same value of k_{-1}. The fact that the computer simulation of both adenine and pyridine resonances of the coenzyme in a given system gave the same k_{-1} suggests that both moieties of the coenzyme molecule exhibit the same time scale of dissociation from the enzyme surface. The agreement between the observed and the calculated differential shifts as well as linewidth at various coenzyme/enzyme concentration ratios was within ±10%.

From the results of kinetic analysis, it was found that both NAD^+ and NADH exhibit longer T_{2b} times and greater k_{-1} values for complexes with M_4 isozyme than with H_4 isozyme (under the same experimental conditions). The calculated k_{-1} values for the enzyme-coenzyme complexes are close to those obtained by temperature jump study of Heck in which $TNAD^+$ and NADH

TABLE III

CALCULATED RELAXATION TIMES OF COENZYME RESONANCES IN THE BOUND STATES AND BOUND SHIFTS

Enzyme	Co-enzyme	Temp. (°C)	Relaxation times in the bound states and bound shifts								
			P_{H2}			A_{H8}			A_{H2}		
			T_{1b}	T_{2b}	Δ	T_{1b}	T_{2b}	Δ	T_{1b}	T_{2b}	Δ
H_4 lactate dehydrogenase	NAD^+	10		6.7	−65		11.0	−50			
		22	80	8.3	−55	110	13.3	−45	100		
		37		10.0	−50		15.5	−40			
M_4 lactate dehydrogenase	NAD^+	10		13.0	−55		32.0	−60			
		22	60	16.0	−45	110	32.0	−45	130		
		37		22.0	−40		30.0	−30			
H_4 lactate dehydrogenase	NADH	40	120	5.0	−120	110	6.0	−90	200	8.0	+110
M_4 lactate dehydrogenase	NADH	40	180	5.0	−120	190	7.0	−80	120	8.0	+120

T_{1b} denotes longitudinal relaxation time in the bound state in ms. The accuracy of this measurement is ±30%. T_{2b} denotes transverse relaxation time in the bound state in ms. The accuracy of the estimations from computer calculation is ±10%. Δ denotes the bound shift in Hz. The accuracy of estimations from computer simulations is ±10%.

were employed for binding with porcine heart lactate dehydrogenase [11]. The k_{-1} values for NADH-enzyme complexes were also in good agreement with those of the steady-state kinetic analysis [24] and stopped-flow measurements [13]. One also finds that k_{-1} values for NAD^+ in enzyme-coenzyme complex are several times greater than that of the turnover number of chicken lactate dehydrogenase measured by steady state rate of NADH oxidation (300 s^{-1}/subunit) [16].

This comparison suggestst that the dissociation of NAD^+ from the enzyme surface during the enzyme catalyzed reaction is not a rate-determining step. There appear to be intermediate steps which are rate-determining during the catalytic reaction. This is consistent with the previous observations by stopped-flow study [13].

The activation energy for the dissociation process can be determined from the temperature dependence of the dissociation rate constants of a given system. Examination of the dissociation rate constants over the temperature range of 10—40°C yields sets of activation energies of these two systems as given in Table IV.

A similar kinetic analysis can be applied to the binding of NADH to dehydrogenases. In most cases, however, the exchange rates fall in the region of slow to intermediate exchanges. Thus T_{2b}, and k_{-1} should be obtained from the computer simulations by following slowly the changes of NADH resonances with increasing temperature as well as with different enzyme/coenzyme ratios. The best fit Δ and T_{2b} values of some coenzyme resonances and their calculated kinetic constants are presented in Tables III and IV.

The results of this study show that the dissociation of NADH from its complex with H_4 lactate dehydrogenase is considerably slower than that from its complex with M_4 lactate dehydrogenase. Also, the dissociation rates of NADH from the complexes are slower than those of NAD^+. T_{2b} values estimated from

TABLE IV

ESTIMATED KINETIC CONSTANTS FOR THE DISSOCIATION OF COENZYMES FROM ENZYME-COENZYME COMPLEX

Accuracy of the rate constant estimation is ±10 to ±20%.

Enzyme	Coenzyme	Temp. (°C)	Rate constant (s^{-1})	ΔE^* (kcal/mol)
H_4 lactate dehydrogenase	NAD^+	10	300	8.0
		22	600	
		37	1100	
M_4 lactate dehydrogenase	NAD^+	10	600	6.9
		22	1200	
		37	1800	
H_4 lactate dehydrogenase	NADH	10	30	19.3
		20	100	
		32	300	
		40	600	
M_4 lactate dehydrogenase	NADH	22	350	10.6
		32	600	
		40	1000	

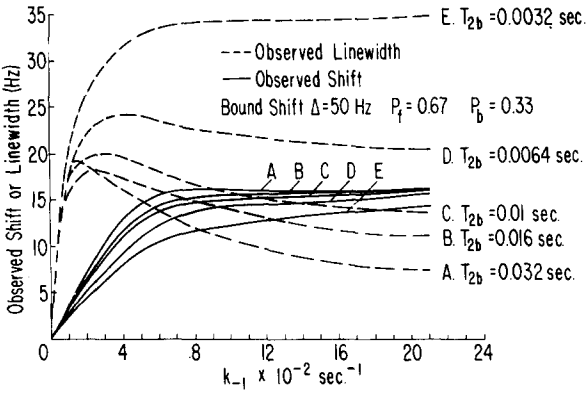

Fig. 5. Computer calculations showing the effect of T_{2b} and k_{-1} on the observed shift as well as linewidth of the coenzyme resonances, assuming that the bound shift Δ = 50 Hz, the relative population of the free and the bound state, P_f and P_b are 0.67 and 0.33 respectively.

various NADH resonances are also much shorter than those estimated for the corresponding NAD^+ resonances. NADH exhibits higher activation energies of dissociation from the complex than those of NAD^+. All of these findings were consistent with the strong binding of NADH to dehydrogenase as relative to NAD^+.

In the course of computer simulation, it is worthwhile to mention that the linewidth and the differential shift of the coenzyme resonances vary differently with respect to the changes in T_{2b} and k_{-1}. This can be shown by a typical example given in Fig. 5, where Δ = 50 Hz and the relative population in the bound state, P_b is 0.33. Generally speaking, in the region of intermediate to fast exchanges ($k_{-1} \geqslant 100$ s^{-1}), the observed linewidth is more sensitive to changes in k_{-1}, when T_{2b} is long. The differential shift is sensitive to the rate changes only when T_{2b} is short. From the variations of the observed linewidth and differential shift with temperature, T_{2b} and k_{-1} can be obtained from such a computer analysis.

Conclusions

The principle and the method described in this paper can be generally applied to other kinetic studies of small molecule-macromolecule interactions with rates of dissociation occurring within the NMR time scale. In contrast to the limitation imposed by other kinetic equations for NMR treatment, the computer analysis can be applied to cases with a wide range of exchange rates and relative populations in the free and bound states. Compared to the total lineshape analysis for the chemical exchange between the small molecules, T_{2b} is the only additional parameter introduced to the computer's kinetic analysis of small molecule-macromolecule interactions.

This method can also be extended to the simulation of the multiple spin systems where the spin-spin couplings are involved. Studies with multiple spin systems using the modified dynamic NMR computer program by G. Binsch [22] are now in progress. Compared to the other methods for fast reaction kinetics

such as stopped-flow and temperature jump techniques [11—12,23], a comparable accuracy and sensitivity of the rate meausrements can be obtained with this method of analysis. However, NMR kinetic treatments can also provide information regarding to relaxation and correlation times in the bound environments. In the case NAD^+ binding to chicken lactate dehydrogenases NMR treatments seem to be unique in that there is no direct apparent optical or fluorescence change which can be monitored for enzyme/coenzyme interactions.

The results of this study suggest that M_4 and H_4 isoenzymes of chicken lactate dehydrogenase exhibit significantly different T_{2b} times and relative rates of coenzyme dissociation from enzyme/coenzyme complexes. These kinetic differences could account for the differences in the catalytic mechanisms between the two isoenzymes under physiological conditions [19].

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