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Fluorescence Decay Studies of Reduced Nicotinamide Adenine Dinucleotide in Solution and Bound to Liver Alcohol Dehydrogenase†

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ABSTRACT: The monophoton counting technique was used to obtain the fluorescence decay kinetics of NADH (dihydronicotinamide adenine dinucleotide) bound to LADH (horse liver alcohol dehydrogenase). It was found that the fluorescence decay of the enzyme complex did not follow a single exponential decay law but that the data could be well described as a sum of two exponentials. The decay parameters of the enzyme complex do not depend on the degree of binding-site saturation. These results are interpreted in terms of a reversible excited-state reaction forming a nonfluorescent product. Fluorescence decay kinetics are also reported for NADH and related molecules in solution. The decay parameters, fluorescence emission maxima, and fluorescence intensities depend on solvent polarity and viscosity.

The nicotinamide-containing coenzymes play a major role in biological oxidation–reduction reactions and their chemical and spectroscopic properties have been extensively studied (Velick, 1961; Chaykin, 1967; Shifrin and Kaplan, 1961; Kosower, 1962). The emission quantum yield of NADH[1] is known to be solvent dependent and increases with decreasing polarity of the solvent (Scott et al., 1970). It has been shown, using several solvents, that the fluorescence yields and lifetimes follow the same pattern and that the quenching of the NADH fluorescence is, therefore, a dynamic process (Scott et al., 1970). Blomquist (1969) has shown that the fluorescence is enhanced somewhat in D_2O as compared to H_2O but that the fluorescence of NADH bound to LADH is not affected by D_2O. The bound NADH molecule thus seems to be shielded from solvent molecules.

The binding of NADH to LADH is accompanied by an increase in the fluorescence intensity, as well as by a shift of the absorption and emission spectra to shorter wavelengths (Sund and Theorell, 1963; Winer and Theorell, 1960). This effect is enhanced when ternary complexes involving substrate analogues (such as isobutyramide) are formed. One NADH molecule binds to each of the two catalytic sites of the enzyme (with increased fluorescence) and some more NADH molecules were found to bind, less tightly, to additional nonspecific sites (Weiner et al., 1972). The fluorescence of the latter is not enhanced.

Excitation spectra of the bound NADH lack the 260-nm band apparent in aqueous solution indicating that the coenzyme is bound in an unfolded conformation (while in aqueous solutions it assumes a folded conformation with the adenine and nicotinamide rings in close proximity (Velick, 1961; Weber, 1957)).

Due to the enhancement of NADH fluorescence upon binding to LADH, fluorescence has often been used to study the binding (Yonetani and Theorell, 1962) and it is believed that the two binding sites are equivalent and independent (Jörnvall and Harris, 1970; Theorell, 1964). On the other hand, some evidence has been presented that indicates that interaction between the two sites does exist (Lindman et al., 1972; Seydoux et al., 1974). Liver alcohol dehydrogenase was suspected to show "half of the sites reactivity" (Seydoux et al., 1974; Luisi and Bignetti, 1974) that requires a high degree of interaction between the two binding sites.

In the present study, the fluorescence of NADH in different solvents and when bound to LADH under various experimental conditions was studied by steady-state, as well as by nanosecond decay measurements. The decay mechanism in solution was found to be complex and several possible interpretations are discussed.

Experimental Section

β-NADH, α-NADH, AcPyNADH, and NMNH were obtained from Sigma. Reduced pyruvate adduct of NAD^+ was prepared as described in the literature (Everse et al., 1971). Reduced *N'*-methylnicotinamide was synthesized by the

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[1] Abbreviations used are: NADH, reduced β-nicotinamide adenine dinucleotide; NMNH, reduced nicotinamide mononucleotide; AcPyNADH, 3-acetylpyridine derivative of NADH; LADH, liver alcohol dehydrogenase; NAD^+, oxidized β-nicotinamide adenine dinucleotide; IBA, isobutyramide; OD, optical density.

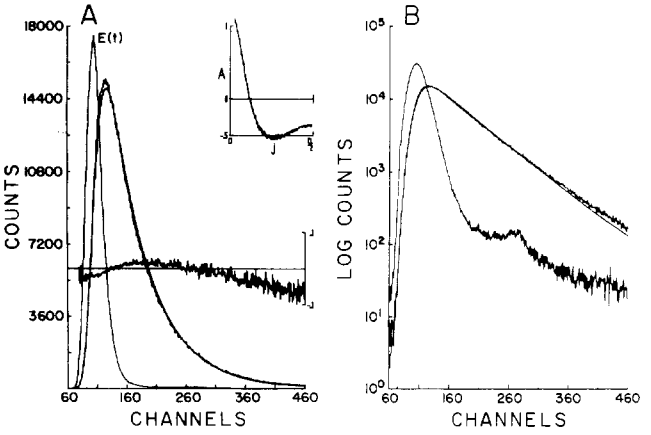


FIGURE 1: Experimental decay curves and the results of an analysis for a single exponential for the LADH-NADH-IBA ternary complex in 0.1 M phosphate buffer, pH 7. The excitation wavelength was 336 nm and the emission wavelength was 410 nm. The temperature was 1 °C. The timing calibration was 0.102 ns/channel. NADH/LADH binding sites = 0.5. (A) Shows the lamp flash, E(t), the decay curve, and the best theoretical decay curve based on a single exponential. τ = 6.3 ns, α = 0.22, and χ^2 = 7.31. The weighted normalized residuals and the autocorrelation function of the residuals are also shown. This representation of decay data is described in Gafni et al. (1975). (B) Shows the same data in logarithmic form together with the fit for a single exponential.

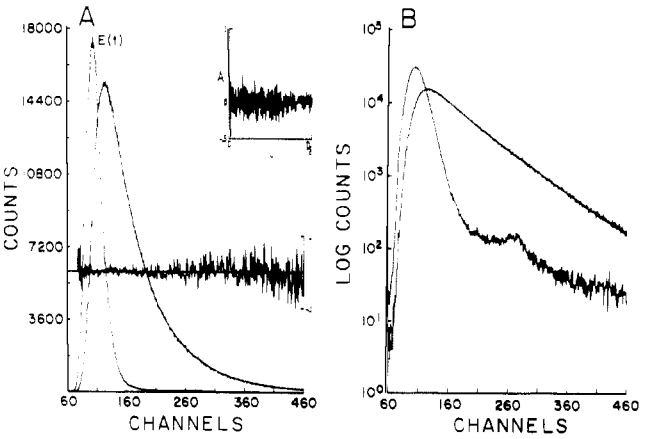


FIGURE 2: (A) Double-exponential analysis of the decay of LADH-NADH-IBA in 0.1 M phosphate, pH 7. Conditions as described for Figure 1. The theoretical curve is based on the following decay parameters: τ_1 = 2.85 ns, τ_2 = 6.92 ns, α_1 = 0.070, α_2 = 0.170. The pre-exponents normalized to one are α_1 = 0.29, α_2 = 0.71, χ^2 = 1.27. (B) Logarithmic representation of the lamp, the experimental decay, and the theoretical decay.

method of Karrer and Blumer (1947). Liver alcohol dehydrogenase was obtained from Boehringer Mannheim Co. as a crystalline suspension in 0.02 M phosphate (buffer pH = 7 containing 10% ethanol). The suspension was centrifuged and the precipitated protein was dissolved in 0.1 M phosphate buffer of the desired pH and dialyzed against several fresh additions of the same buffer for 48 h. The protein concentration was determined from the optical density at 280 nm (Sund and Theorell, 1963). The binding sites concentration was determined by fluorometric titration with NADH in the presence of 0.1 M isobutyramide (the binding capacity was usually found to be about 90%). Phosphodiesterase (from *Crotalus terr. terr.*) was obtained from Boehringer-Mannheim as a solution in 50% glycerol. Solvents: glass distilled water was used for all measurements. 2-Methoxyethanol, spectrophotometric grade, was obtained from Aldrich Chemical Co. Glycerol was spectral grade (for fluorescence microscopy; E. Merck, Darmstadt).

LADH-NADH binary complexes were prepared at different binding site to NADH ratios by adding the required volume of $\sim 10^{-4}$ M LADH solution to an NADH solution in phosphate buffer. The final concentration of NADH was 1.7×10^{-5} M (yielding an $OD_{336} < 0.1$ to eliminate inner filter effects). From the dissociation constant of the binary complex (Theorell and Winer, 1959) the binding of NADH was calculated to be ~90% for the complex prepared at a ratio of 1 between binding sites and coenzyme. The percent binding for the other complexes, where the above ratio was 2 or 3.3, was above 98%.

LADH-NADH-IBA ternary complexes were prepared in a similar way. Due to the much higher binding constant of NADH in the ternary complex (Yonetani and Theorell, 1962) practically all the NADH was bound to the enzyme.

Steady-state fluorescence and fluorescence excitation spectra were determined using a Perkin-Elmer MPF-4 spectrophotofluorometer using a half bandwidth of 5 nm in excitation and 3 nm in emission. These spectra are not corrected for instrumental response.

Nanosecond fluorescence decay measurements were done using an instrument constructed in this laboratory (Easter et al., 1976), operating under an HP 2100 computer control and collecting the decay curve and the lamp flash in a semi-simultaneous mode. All decay measurements were performed under single photon counting conditions. The 336- or 355-nm bands of an air flash lamp were isolated using the appropriate Baird Atomic band-pass filters and used for excitation. Emission was observed at a right angle through a Bausch and Lomb half-meter monochromator, the half-bandwidth for emission being 10–13 nm.

When fluorescence decays of viscous solutions, or those of complexes with LADH, were measured, care was taken to avoid interfering effects due to decay of the anisotropy (Shinitzky, 1972). This was done by exciting with an unpolarized beam of light (a depolarizing quartz wedge was used in front of the solution) and observing the fluorescence through a polarizer (polacoat) whose axis was at 54°44′ to the excitation observation plane. Under the experimental conditions used there was a significant shift correction (Gafni et al., 1975). This varied from 0.01 to 1 channels (0.001–0.1 ns). The shift was obtained from a separate experiment using anthracene as a single exponential standard. Scattered light was negligible in all the experiments. There was a small background fluorescence in glycerol. This was subtracted from decay curves prior to analysis. Under the experimental conditions used here, single lifetime standards, such as anthracene, gave good analyses for single-decay times.

The decay curves were analyzed using the methods of nonlinear least-squares (Grinvald and Steinberg, 1974) and Laplace transforms (Gafni et al., 1975). Both methods yielded very similar results. The parameters obtained by analysis were convolved with the experimental lamp flash and the fit between the calculated and experimental decay curves was evaluated from the weighted residuals, the autocorrelation function of the residuals and the reduced χ^2. The preexponential terms (the amplitudes) have been normalized to a sum of one for presentation of the data in the tables.

Results

The fluorescence decay data obtained with the ternary complex, LADH-NADH-isobutyramide, are shown in Figures 1A, B and 2A, B. Figure 1A shows a linear plot of the

TABLE I: Decay Parameters Obtained for Ternary Complexes of LADH-NADH-Isobutyramide in 0.1 M Phosphate Buffer at the Indicated pH.[a]

[LADH Binding Sites]/[NADH]	pH	Emission Wave length	τ_1	α_1	τ_2	α_2	$\bar{\tau}$
4	7	410	2.9	0.29	6.9	0.71	5.7
2	7	410	2.9	0.29	6.9	0.71	5.7
2	7	435	3.0	0.26	6.9	0.74	5.9
2	7	460	2.9	0.30	6.9	0.70	5.7
2	7	485	3.1	0.25	6.9	0.75	6.0
2	7	500	2.9	0.24	6.9	0.76	6.1
2	7	510	2.7	0.22	6.9	0.78	5.9
1.33	7	410	2.6	0.29	6.7	0.71	5.5
1.10	7	410	2.7	0.32	6.7	0.68	5.4
2	6.1	410	2.7	0.32	6.7	0.68	5.4
2	8.3	410	2.9	0.35	7.0	0.65	5.6
2	10.5	410	2.4	0.27	6.7	0.73	5.6
2	10.8	410	2.4	0.27	7.0	0.73	5.8
2[b]	7	410	2.4	0.45	5.8	0.55	4.3
2[c]	7	410	1.6	0.52	4.0	0.48	2.8

[a] The concentration of LADH was 5-10 μM. The concentration of isobutyramide was 0.1 M. The excitation wavelength was 336 nm and the emission wavelength is indicated for each case. The temperature was 1 °C except for "b" where the temperature was 12 °C and "c" where the temperature was 34 °C. $\bar{\tau}$, the mean lifetime = $\Sigma\alpha_i\tau_i/\Sigma\alpha_i$. χ^2 values for two component analyses ranged from 1.1 to 1.5. τ is given in nanoseconds; wavelength in nanometers.

lamp profile, the experimental decay curve, and the theoretical curve obtained by convolving the best parameters obtained by analysis for a *single* exponential decay (τ = 6.3 ns, α = 0.23) with the experimental lamp profile. The curve at the center of Figure 1A represents the residuals between the experimental and theoretical convolved decay curves. The inset at the upper right of Figure 1A shows the autocorrelation of the residuals. The results shown in Figure 1A together with the χ^2 of 7.61 clearly indicate that these experimental results are *not* in accord with a single exponential decay law (Gafni et al., 1975; Grinvald and Steinberg, 1974). At the request of the reviewer, a logarithmic representation of the same data is shown in Figure 1B. Our experience indicates that the linear representation shown in Figure 1A is preferred. The residuals and the autocorrelation of the residuals quickly indicate the deviation from a single exponential decay law. In contrast, the logarithmic representation shown on the right indicates the deviation only at the tail of the decay curve where the information content is minimal due to the small number of counts. In our view, the logarithmic plots are a holdover from the time when decay parameters were obtained graphically and it would be better not to use them at the present time.

The decay data obtained with the ternary enzyme complex was analyzed for a double-exponential decay law and the results are shown in Figure 2A. The logarithmic representation of the fit to a double-exponential decay is shown in Figure 2B. The decay parameters obtained were τ_1 = 2.9, α_1 = 0.29, τ_2 = 6.9, α_2 = 0.71 and χ^2 = 1.27. The decay data is thus consistent with a double-exponential decay law within our present criteria. It must be emphasized that these are kinetic experiments and, thus, other more complex decay laws are not precluded.

The decay parameters obtained for NADH bound to LADH with isobutyramide under a variety of conditions are shown in Table I. In all cases the data was examined as illustrated in Figures 1A and 2A. The χ^2, the residuals and the autocorrelation of the residuals indicated that the data could not be explained in terms of a single-exponential decay law but that the results were consistent with a double exponential with the parameters shown in Table I. The χ^2 values obtained were in the range 1.1-1.5.

The accuracy expected from the analysis of fluorescence decay data depends on the degree of correlation between the decay parameters (Gafni et al., 1975; Isenberg, 1973). In order to evaluate the recovery of parameters to be expected from the results shown in Table I and the other results presented in this paper the following computational experiment was carried out. A decay curve was synthesized using the parameters obtained with the real data and to the same number of total counts obtained in typical experiments. The synthetic decay curve was convolved with an experimental lamp flash and photon counting noise was added. Five synthetic data sets were generated using different sets of random numbers to introduce the photon counting noise. The synthetic decay curves were analyzed by the method of nonlinear least-squares. Table II indicates the parameters used to initiate the experiment, the mean parameters recovered, and the standard deviation. There can be no doubt that even for the worst cases, the origin of any uncertainty in the results presented in this paper must be attributed to systematic errors in the instrument or impurities in the samples and not to errors introduced by the analysis methods.

At a given pH and temperature the decay parameters are not significantly dependent on the ratio of NADH to LADH binding sites. Under a given set of conditions there is no significant change in the decay parameters when the emission wavelength is varied between 410 and 510 nm, and the mean

TABLE II: Recovery of Parameters from Synthetic Decay Data with the Analysis Methods Employed in this Study.[a]

Theoretical Parameters				Mean Parameters Recovered			
τ_1	τ_2	α_1	α_2	τ_1	τ_2	α_1	α_2
2.9	6.9	0.29	0.71	2.87 ± 0.06	6.89 ± 0.02	0.287 ± 0.004	0.713 ± 0.007
2.4	5.8	0.45	0.55	2.39 ± 0.02	5.78 ± 0.01	0.446 ± 0.005	0.554 ± 0.004
1.6	4.0	0.52	0.48	1.58 ± 0.02	3.97 ± 0.02	0.511 ± 0.01	0.489 ± 0.009
0.8	3.1	0.94	0.06	0.79 ± 0.01	3.01 ± 0.07	0.936 ± 0.006	0.064 ± 0.004
1.4	2.7	0.6	0.4	1.37 ± 0.03	2.67 ± 0.03	0.583 ± 0.03	0.417 ± 0.02

[a] The procedure used is described in the text. τ is given in nanoseconds.

TABLE III: Decay Parameters Obtained for Binary Complexes of LADH-NADH in 0.1 M Phosphate Buffer at pH 7.[a]

[LADH Binding Sites]/ [NADH]	τ_1	α_1	τ_2	α_2	$\bar{\tau}$
3.3	1.8	0.58	4.2	0.42	2.81
2	1.7	0.54	4.0	0.46	2.76
1	1.6	0.53	4.0	0.47	2.73

[a] The temperature was 1 °C. The exciting wavelength was 336 nm and the emission wavelength was 410 nm. τ is given in nanoseconds.

lifetimes are practically constant across the emission band.

Although the fluorescence intensity of the NADH-LADH complex decreases at alkaline pH, the decay parameters do not change markedly in the pH region 6.1–10.8. The decay parameters do, however, depend on the temperature with both lifetimes decreasing in about equal proportion when the temperature is increased. It should be pointed out that the change in decay parameters with temperature is not the result of thermal decomposition of the ternary complex, since this has been shown to be stable under the conditions used (Yonetani and Theorell, 1962).

Decay parameters obtained with the binary LADH-NADH complexes at various degrees of saturation of the protein with coenzyme are presented in Table III. As with the ternary complexes, the decay parameters are not significantly dependent on the ratio of binding sites to NADH. The observed lifetimes are considerably shorter than those obtained for the ternary complex at the same temperature. The lifetimes for the binary complexes at 1 °C are similar to those obtained with the ternary complex at 34 °C.

Since one possible explanation for the double-exponential decay obtained with LADH-NADH complexes involves two different binding sites on the protein, it was of interest to study the decay of the coenzyme in pure solvents. The results of such experiments are summarized in Table IV together with decay parameters obtained for some other 1,4-dihydropyridine derivatives. It is evident that the decay times depend on both the solvent and the temperature and increase with decreasing temperature and polarity of the solvent. Multi exponential decay laws were observed with all solvents other than water. As indicated in Table II, some of the decay data in glycerol obeyed neither a single- nor a double-exponential decay law.

As the temperature, in glycerol, is decreased from 34 to −20 °C the lifetimes associated with NADH become longer and the relative amplitudes change. The result of these two effects is an increase of the mean lifetimes by a factor of 5.5 over this temperature range, as is shown in Figure 4. At 34 °C the decay parameters in glycerol are very similar to the ones observed in ethanol at 1 °C, while at −20 °C they approach those obtained for the ternary complexes with LADH at 1 °C. The decay parameters of NADH in glycerol at 34 °C depend to some extent on the emission wavelength and the mean lifetime increases by about 13% between 410 and 510 nm. In glycerol the other 1,4-dihydropyridine derivatives listed in Table IV also decay according to a multi-exponential decay law. The parameters depend on the particular compound.

In water, NADH and the other compounds decay with practically a single short decay constant. A long lifetime component associated with a very small amplitude is attributed to instrumental error. The parameters obtained for NADH in buffer are in good agreement with those previously reported by Schuyler et al. (1972).

The steady-state fluorescence of NADH in glycerol increases in intensity as the temperature is decreased. This is

TABLE IV: Decay Parameters Obtained with NADH and Related Molecules in the Solvents Indicated.

Compound	Solvent	T (°C)	Emission Wavelength	τ_1	α_1	τ_2	α_2	$\bar{\tau}$
NADH	0.1 M Phosphate, pH 7	1	450	0.54	1.0	14.3	5×10^{-4}	0.54
	0.1 M Phosphate, pH 7	3	450	0.52	1.0	2.4	5×10^{-3}	0.52
	0.1 M Phosphate, pH 7	~21	450	0.44	1.0	12.6	5×10^{-4}	0.44
	Ethanol	1	450	0.8	0.94	3.1	0.06	0.94
	Ethanol, 1 mM $MgCl_2$	3	450	1.5	0.59	3.5	0.41	2.3
	2-methoxyethanol	−2	420	0.9	0.95	3.0	0.05	1.0
	Glycerol	34[a]	410	0.9	0.94	2.7	0.06	0.98
	Glycerol	34[a]	460	0.96	0.92	2.3	0.08	1.07
	Glycerol	34	510	1.0	0.92	2.4	0.08	1.11
	Glycerol	12	410	1.5	0.77	4.0	0.23	2.1
	Glycerol	−2	410	2.7	0.67	6.0	0.33	3.8
	Glycerol	−20	410	3.1	0.38	7.1	0.62	5.3
	Glycerol	−20	460	3.0	0.36	7.0	0.64	5.5
NMNH[b]	0.1 M Phosphate, pH 7	0	430	0.36	1.0	8.9	2×10^{-4}	0.36
NMNH[b]	0.1 M Phosphate, pH 7	2	450	0.29	1.0	5.5	2×10^{-3}	0.30
NMNH[b]	Glycerol	−1	410	1.7	0.57	4.4	0.43	2.9
Reduced *N'*-methylnicotinamide	0.1 M Phosphate, pH 7	0	460	0.36	0.98	6.3	0.02	0.48
Reduced *N'*-methylnicotinamide	Glycerol	0	460	1.4	0.60	2.7	0.40	1.9
α-NADH	Glycerol	2	460	2.2	0.62	5.2	0.38	3.4
3-acetyl derivative of NADH[c]	0.1 M Phosphate, pH 7	2	480	0.12	1.0	1.8	4×10^{-3}	0.12
3-acetyl derivative of NADH[c]	Glycerol	1	460	1.0	0.75	2.9	0.25	1.5

[a] Two component analysis was not adequate (χ^2 values obtained were above 1.8). Three component analysis gave good fits to the experimental data (for the 410-nm emission the parameters were: $\tau_1 = 0.7$, $\alpha_1 = 0.77$, $\tau_2 = 1.6$, $\alpha_2 = 0.23$, $\tau_3 = 6.3$, $\alpha_3 = 5.10^{-3}$. [b] Made by reacting NADH with phosphodiesterase in buffer for 30 min at room temperature. [c] Excitation wavelength 355 nm. τ in nanoseconds. Wavelength in nanometers.

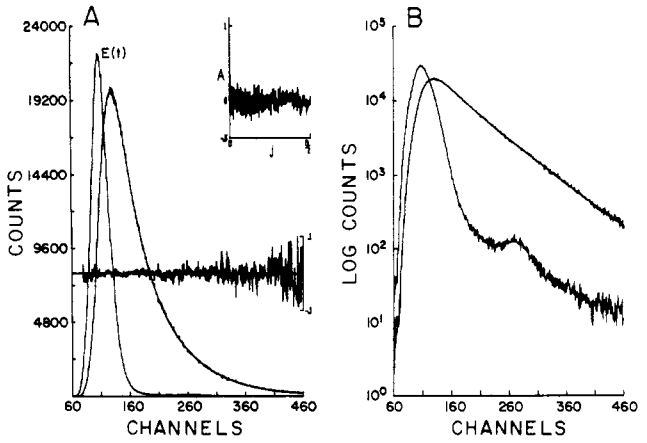


FIGURE 3: (A) Fluorescence decay of NADH in glycerol. The exciting wavelength was 336 nm and the emission wavelength was 410 nm. The temperature was −20 °C and the timing calibration was 0.102 ns/channel. A small background fluorescence (due to impurities in the glycerol) was subtracted from the decay curve. Shown are the lamp flash E(t), the decay curve, and the theoretical curve based on an analysis for a double exponential with $\tau_1 = 3.12$, $\tau_2 = 7.12$, $\alpha_1 = 0.118$, $\alpha_2 = 0.192$. The pre-exponential terms normalized to one are $\alpha_1 = 0.38$ and $\alpha_2 = 0.62$. $\chi^2 = 1.42$. The normalized residuals between the experimental and theoretical decay curves are also shown, as well as the autocorrelation function of the residuals. (B) A logarithmic representation of the data. The lamp, the experimental, and the theoretical decay are shown.

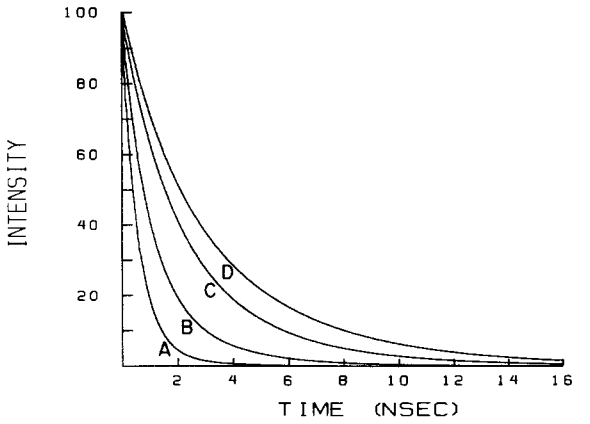


FIGURE 4: The fluorescence decay (impulse response) of NADH in glycerol. The emission wavelength was 410 nm. The temperatures were: A, 34 °C; B, 12 °C; C, −2 °C; and D, −20 °C.

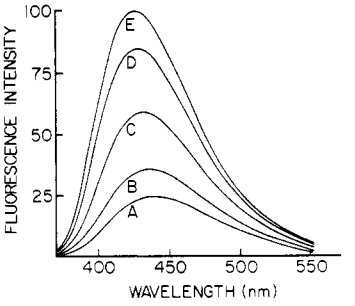


FIGURE 5: Fluorescence emission spectra of NADH in glycerol at different temperatures. The same coenzyme solution and the same instrumental sensitivity were used in all the experiments. Excitation wavelength 336 nm. The temperatures used were: A, 34 °C; B, 24 °C; C, 12 °C; D, 2 °C; E, −3 °C.

accompanied by a shift of the emission band to shorter wavelength as shown in Figure 5. At the same time the fluorescence quantum yield (as estimated from the relative intensities of the fluorescence peaks) is enhanced by a factor of 4 between 34 and −3 °C. This agrees with the increase in the mean lifetime of 3.8 for the same temperature range and with the finding of Scott et al. (1970) that the quantum yields and decay times increase simultaneously. The shape of the long wavelength fluorescence excitation bands of NADH in glycerol are independent of temperature, indicating that the changes in the chromophore take place in the excited state.

Discussion

The data presented here indicate that neither the binary complexes of NADH and LADH nor the ternary complexes of enzyme, coenzyme, and isobutyramide show mono-exponential fluorescence decay. The experimental data is in good agreement with a double-exponential decay. As is the case with any kinetic experiment, this does not rule out other decay laws that might also be found to agree with the data.

In a situation where there are two binding sites for a fluorophore on a macromolecule and where a double-exponential decay is observed it is always tempting to relate the two decay times to different micro-environments at the two sites. It is, however, important to keep in mind that the dynamic photophysics of an excited-state system can easily lead to multi-exponential decay kinetics. For example, 2-naphthol dissolved in slightly acid pH in water shows double-exponential decay kinetics (Loken et al., 1972). We suggest that the results presented here are in accord with the latter interpretation.

If we focus our attention on excited-state reactions, the double-exponential decay of the coenzyme–enzyme complexes might be attributed to two emitting species. This is unlikely, however, since the decay parameters do not show a significant dependence on the emission wavelength. In addition, if a large proportion of the emission originated from a species formed in the excited state, an exponential term with a negative amplitude would be expected and this has not been found.

The decay parameters of NADH are seen to be quite sensitive to the solvent environment. A conformational change at the coenzyme binding sites on LADH might be expected to influence the decay parameters of the binary or ternary complexes. There is considerable evidence that the two coenzyme binding sites of LADH are identical (Jörnvall et al., 1970; Theorell, 1964). Since the fraction of single-bound enzyme species will change as a function of saturation, the finding that the degree of saturation does not affect the decay parameters is in accord with previous evidence for the identity of the sites.

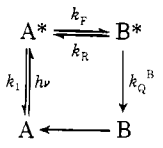
Lindman et al. (1972) studied changes in ^{35}Cl NMR as a function of the degree of saturation of LADH with coenzyme. They found that, while for the binary complex 1 mol of NADH/mol of enzyme affects chloride ion binding on *both* subunits, for the ternary complexes 2 mol of NADH were required for a full effect to be observed. In these experiments a relatively high concentration (~1 M) of chloride ion was used. This is known to affect both the enzyme conformation and the binding of coenzyme (Coleman and Weiner, 1973). The results presented here indicate that, in the absence of chloride ions, binding of a second NADH to the enzyme does not influence the conformation of the already occupied subunit in a way detectable by decay measurements of NADH. The decay parameters of NADH in the binary, as well as in ternary complexes, remain unaltered. Thus, there is either no interaction between the two subunits or a full conformational change takes place in both, upon binding NADH to one subunit. The latter conclusion is supported by the observation (Gafni and Brand, unpublished) that an NADH bound to one subunit in a ternary complex protects a whole LADH molecule against heat denaturation.

The association constant of the LADH–NADH complex is pH dependent and the complex dissociates at a pH near 10 (Li et al., 1962; Yonetani and Theorell, 1962) with a decrease in the NADH steady-state fluorescence intensity. The observation that the decay parameters do not depend on pH is, therefore, of interest and leads to the conclusion that the decomposition of the complex is not due to a gradual conformational change, but rather reflects a two-state dissociation process. This is in accord with the observation (Li et al., 1962) that the activity and intrinsic optical rotatory dispersion of LADH are unchanged by exposure of the enzyme to pH 10.5 for 2–3 h.

There is a significant difference between the decay parameters obtained with the binary and ternary complexes. This is in agreement with previously reported quantum-yield differences (Sund and Theorell, 1963; Winer and Theorell, 1960) and indicates that the microenvironment about the coenzyme is altered in the presence of isobutyramide.

Since the biexponential decay does not appear to reflect two different binding sites nor two distinct spectrally emitting species, an alternative explanation for this finding must be sought. It is proposed that the decay behavior has its origin in a reversible excited-state reaction that transforms the fluorescent reduced-nicotinamide chromophore to a nonfluorescent product. A reversible excited-state process of this type is described in Scheme I, where $k_1 = k_{F1}^{A} + k_Q^{A}$ is the sum of the

Scheme I



rate constants for fluorescence and quenching of A*. The concentration of species A* as a function of time, following excitation, is described by eq 1 (Loken 1973)

$$[A^*] = \frac{[A_0^*]}{\frac{1}{\tau_2} - \frac{1}{\tau_1}}\left[\left(\frac{1}{\tau_2} - X\right)e^{-t/\tau_1} + \left(X - \frac{1}{\tau_1}\right)e^{-t/\tau_2}\right] \quad (1)$$

where $X = k_{F1}^{A} + k_Q^{A} + k_F$, $Y = k_Q^{B} + k_R$, and $1/\tau_{1,2} = \frac{1}{2}\{(X + Y) \pm [(X + Y)^2 - 4(XY - k_R k_F)]^{1/2}\}$. Hence the fluorescence will follow a double-exponential decay law. Since the rate constants of Scheme I change with temperature and solvent, both of these might be expected to affect the rates of the excited-state reaction and thus influence the decay parameters. This is what has been observed.

Several specific excited-state processes may be considered in order to explain the experimental observations described here. Folding and unfolding of the NADH may have a role but is not sufficient to explain the lack of monoexponential decay behavior found with NMNH and reduced *N'*-methylnicotinamide both of which lack the adenine moiety.

Syn and anti configurations of nucleotides are known to exist (Egan et al., 1975; Rhodes and Schimmel, 1971) and have been shown to interconvert in aqueous solution on the nanosecond time scale. However, these two configurations do not exist in *N'*-methylnicotinamide and, thus, cannot account for the lack of monoexponential behavior at least with this derivative.

If the kinetic scheme proposed here is correct, the nature of the nonfluorescent excited-state species and the process by which it is formed remain to be determined. Electron ejection or hydride-ion transfer in the excited state may well be involved. Frisell and Mackenzie (1959) have shown that 1,4-dihydronicotinamide derivatives are active as photochemical reducing agents. The excited-state mechanism may be ionic or free radical depending on the experimental conditions (Kurz et al., 1961).

There is a pronounced dependence of the mean lifetime (and quantum yield) of NADH and its derivatives on solvent polarity and viscosity. The steady-state emission maximum is shifted to the blue as solvent polarity decreases and as the viscosity (in glycerol) is increased, while there is only a small effect on the absorption spectrum. In the ternary complexes with LADH negligible changes of the mean lifetime with emission wavelength were observed. However, in glycerol the mean lifetime does show a small increase toward the red edge of the emission spectrum. This was also the only case where a two-component analysis did not give an adequate fit to the data. It appears that the excited nicotinamide ring may undergo interactions with the solvent environment. These interactions may influence and be related to the reversible two-state excited-state reaction postulated above.

It is expected that further studies of the excited-state interactions of NADH and its enzyme complexes will add new insight regarding its role in biochemical catalytic reactions.

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Molecular Mechanism of Cardiotoxin Action on Axonal Membranes†

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ABSTRACT: Cardiotoxin isolated from *Naja mossambica mossambica* selectively deactivates the sodium–potassium activated adenosine triphosphatase of axonal membranes. Tetrodotoxin binding and acetylcholinesterase activities are unaffected by cardiotoxin treatment. The details of association of cardiotoxin with the axonal membrane were studied by following the deactivation of the sodium–potassium activated adenosine triphosphatase and by direct binding measurements with a tritiated derivative of the native cardiotoxin. The maximal binding capacity of the membrane is 42–50 nmol of cardiotoxin/mg of membrane protein. This high amount of binding suggests association of the toxin with the lipid phase of the membrane. It has been shown that cardiotoxin first associates rapidly and reversibly to membrane lipids, then, in a second step, it induces a rearrangement of the membrane structure which produces an irreversible deactivation of the sodium–potassium activated adenosine triphosphatase. Solubilization of the membrane-bound ATPase with Lubrol WX gives an active enzyme species that is resistant to cardiotoxin-induced deactivation. Cardiotoxin binding to the membrane is prevented by high concentrations of Ca^{2+} and dibucaine. Although cardiotoxins and neurotoxins of cobra venom have large sequence homologies, their mode of action on membranes is very different. The cardiotoxin seems to bind to the lipid phase of the axonal membrane and inhibits the sodium–potassium activated adenosine triphosphatase, whereas the neurotoxin associates with a protein receptor in the post-synaptic membrane and blocks acetylcholine transmission.

Cardiotoxin is the most abundent constituent of cobra venoms (Lee et al., 1968; Larsen and Wolff, 1968; Slotta and Vick, 1969; Lo et al., 1966; Takechi et al., 1971). Its lethal potency in mice is about one-twentieth of that of neurotoxins. Toxins of the cardiotoxin group are composed of 60 residues in a single polypeptide chain cross-linked by four disulfide bridges (Lee, 1972; Yang, 1974). Cardiotoxins affect various kinds of cells, both excitable and nonexcitable, causing irreversible depolarization of the cell membrane and consequently impairing both the function and the structure of cells (Lee, 1971; Lee et al., 1968; Patel et al., 1969). Comparative pharmacological and sequence studies have indicated that other toxic proteins, found in snake venom, such as the direct lytic factor (DLF) (Condrea et al., 1964; Aloof-Hirsch et al., 1968), cobramines (Larsen and Wolff, 1968) or cytotoxins (Braganca et al., 1967; Patel et al., 1969) belong to the cardiotoxin family.

Excitable cells are very vulnerable to cardiotoxin action: (1) Cardiotoxin causes contracture followed by paralysis of the skeletal muscle (Lee et al., 1968; Tazieff-Depierre et al., 1969). (2) In isolated heart preparations, cardiotoxins at low concentrations cause an augmentation of contraction; systolic arrest occurs at high concentrations of the toxin (Lee et al., 1968). (3) Cardiotoxins block axonal conduction in peripherical nerves (Condrea et al., 1967).

Snake neurotoxins and cardiotoxins have different pharmacological effects but they are proteins originating from a common ancestor gene (Lee, 1972; Yang, 1974). Whereas the molecular mechanism of the association of snake neurotoxins with the membrane-bound acetylcholine receptor is now well understood (for a review, see Rang, 1975), very little is known about the molecular basis of cardiotoxin action. To study this problem, we have chosen to analyze the interaction of *Naja mossambica mossambica* cardiotoxin with crab axonal membranes that have been obtained in a very purified form and that have been chemically and functionally well characterized (Balerna et al., 1975).

Materials and Methods

Cardiotoxins and Chemicals. Cardiotoxins were purified from *Naja mossambica mossambica* venom following the method described by Louw (1974). A slight modification was introduced by replacing the CM-cellulose chromatography

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