Circular Dichroism and Circular Polarization of Luminescence of Reduced Nicotinamide Adenine Dinucleotide in Solution and Bound to Dehydrogenases†

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ABSTRACT: The CD (circular dichroism) and CPL (circular polarization of luminescence) spectra of NADH in aqueous solution were studied and found to be markedly different. The spectra were not affected by cleavage of the coenzyme molecule with phosphodiesterase. The differences are thus not due to the existence of extended and folded conformations of NADH and it is concluded that they originate in excited state conformational changes of the nicotinamide-ribose fragment. Opposite signs of both the CD and CPL spectra were observed for NADH bound to horse liver alcohol dehydrogenase and to beef heart lactate dehydrogenase indicating structural differences between the nicotinamide binding sites. The binding of substrate analogues to enzyme-coenzyme complexes did not affect the CD spectra and hence no significant conformational changes are induced upon formation of the ternary complexes. No changes in the CPL spectrum of NADH bound to lactate dehydrogenase were observed upon adding oxalate to form the ternary complex. Marked differences were found between the CPL spectra of binary and ternary complexes with liver alcohol dehydrogenase, while the CD spectra of these complexes were identical. It is concluded that a conformational change of the excited NADH molecule occurs in the binary but not in the ternary complex involving LADH, thus indicating an increased rigidity of the latter complex.

A variety of spectroscopic techniques have shown that the binding of NADH[1] to dehydrogenases is accompanied by marked changes in the coenzyme conformation. While in neutral aqueous solution the NADH molecule is predominantly in the folded conformation (Oppenheimer et al., 1971), it fully assumes an extended conformation when bound to LDH (Holbrook et al., 1975), LADH (Bränden et al., 1975) as well as to other dehydrogenases (Harris & Waters, 1976). Binding of NADH to LDH or to LADH induces shifts in both absorption and emission spectra of the reduced nicotinamide ring to shorter wavelengths and a marked increase in its fluorescence quantum yield (Winer et al., 1959; Sund & Theorell, 1963). Further changes in the spectra are observed upon the formation of ternary complexes involving the enzyme, coenzyme, and certain substrate analogues (Sund & Theorell, 1963; Winer et al., 1959). These spectral changes have been used to monitor the binding of NADH to dehydrogenases, to follow the binding kinetics, and to gain insight into the nature of the binding sites.

Among the spectroscopic techniques used for structural investigations, CPL has been shown to be very useful and sensitive (Steinberg, 1978 (review)). This technique yields information about the conformations of chiral systems in their electronically excited states in the same way that CD reports on the ground state conformation (Steinberg, 1975, 1978). Since CPL combines the sensitivity of both optical activity and fluorescence techniques, it was found to be extremely sensitive to subtle conformational details of chiral systems (Schlessinger & Steinberg, 1972; Gafni et al., 1973; Gafni & Steinberg, 1974; Schlessinger & Levitzki, 1974; Steinberg, 1978). Of particular interest here is the study made of the CPL of ϵNAD$^+$ bound to several dehydrogenases. In this coenzyme analogue the modified adenine ring is fluorescent and its CPL spectra when bound to LDH and LADH were found to be very similar, indicating similar, if not identical, adenine binding sites in both enzymes (Schlessinger et al., 1975).

In the present study CD and CPL spectra of NADH were studied in aqueous solution and when bound to LADH and LDH in binary and ternary complexes involving substrate analogues. In contrast to the adenine binding sites, the nicotinamide binding sites in different dehydrogenases may be expected to differ from one another since these are close to the substrate binding sites which have to accommodate different substrates. A comparison of the CD and CPL spectra of a complex may reveal conformational changes which follow electronic excitation of the reduced nicotinamide ring, thus bearing on the rigidity of the enzyme-coenzyme complexes.

Materials and Methods

Horse liver alcohol dehydrogenase and beef heart lactate dehydrogenase were obtained from Boehringer Mannheim Co. as crystalline suspensions. The suspensions were centrifuged and the precipitated proteins dissolved in 0.1 M phosphate buffer of the desired pH and dialyzed against several changes of the same buffer for 48 h. Enzyme concentrations were determined from the optical density at 280 nm (Sund & Theorell, 1963; Velick, 1958). Phosphodiesterase (from *Crotalus terr. terr.*) was obtained from Boehringer Mannheim Co. as a solution in 50% glycerol. NADH was purchased from Sigma. Glass-distilled water was used to prepare all solutions.

NMNH was prepared by cleavage of NADH with phosphodiesterase. Twenty micrograms of phosphodiesterase was added to 2 mL of 2×10^{-4} M NADH solution in 0.1 M phosphate buffer (pH 7.4) and the mixture was kept at room temperature (~23 °C) for 2 h. Complete cleavage of the NADH was evidenced by the disappearance of the 260-nm band from the fluorescence excitation spectrum (Weber,

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[1] Abbreviations used: NADH, reduced β-nicotinamide adenine dinucleotide; NMNH, reduced nicotinamide mononucleotide; ϵNAD$^+$, nicotinamide 1,N^6-ethenoadenine dinucleotide; LDH, beef heart lactate dehydrogenase; LADH, horse liver alcohol dehydrogenase; GPDH, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase; CD, circular dichroism; CPL, circular polarization of luminescence; IBA, isobutyramide.

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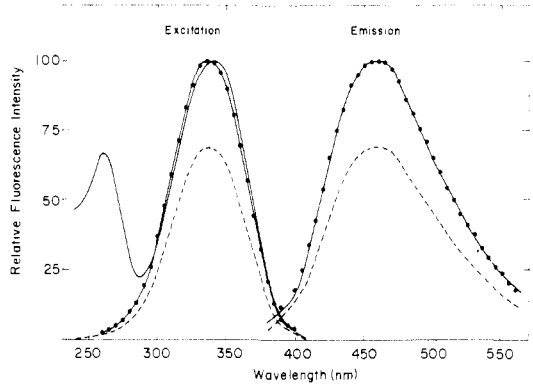

FIGURE 1: Excitation and emission spectra of NADH in 0.1 M phosphate buffer (pH 7.4) before (solid line) and after (dashed line) cleavage with phosphodiesterase (see text for experimental details). The solid lines with circles are the spectra of cleaved NADH normalized at the maxima to the corresponding spectra of the intact coenzyme. The fluorescence was excited at 340 nm, and the excitation spectra were studied for the emission at 460 nm. NADH concentration was 2×10^{-5} M.

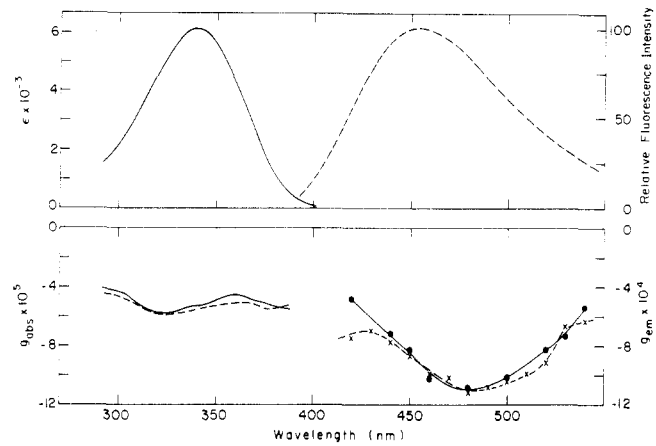

FIGURE 2: Spectroscopic results obtained for NADH and for phosphodiesterase treated NADH (NMNH) in 0.1 M phosphate buffer (pH 7.4). Upper part: absorption and emission spectra of NADH; excitation wavelength was 340 nm. Lower part: anisotropy factors in absorption and emission of NADH (dashed lines) and of NMNH (solid lines). Data points are indicated on the CPL curves. NADH concentrations were 2×10^{-4} M in absorption, CD and CPL studies and 2×10^{-5} M in fluorescence studies.

1957). Binary complexes of NADH with LDH or LADH were prepared by adding the required volume of $\sim 10^{-3}$ M NADH solution to the enzyme solution in buffer. The final concentration of NADH was 2×10^{-4} M. The complexes were prepared with binding sites to NADH ratios of 1 or 2. At the concentrations used, the coenzyme was practically fully bound in all the complexes. LADH:NADH:IBA and LDH:NADH:oxalate ternary complexes were prepared in a similar way, the concentrations of substrate analogues being 0.1 M. The fluorescence, fluorescence excitation, and the linear polarization of fluorescence were measured using 8–10-fold diluted solutions of the binary and ternary complexes.

Absorption spectra were measured with a Zeiss Model PMQ II spectrophotometer. Fluorescence spectra and fluorescence excitation spectra were determined with a Perkin-Elmer MPF-3 spectrophotofluorometer using a half bandwidth of 5 nm in excitation and emission. The spectra were not corrected for instrumental response. Linear polarization of fluorescence was measured with an instrument constructed in this laboratory and described elsewhere (Ehrenberg, 1976). The excitation light was polarized at 90° to the excitation emission plane. The fluorescence polarization is defined as $P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$ where $I_{\parallel}$ and $I_{\perp}$ are the intensities of fluorescence light polarized parallel and perpendicular, respectively, to the polarization of the excitation light. CD spectra were obtained using a Cary 60 spectropolarimeter equipped with a 6002 CD accessory. CPL was measured using an instrument constructed in this laboratory and described elsewhere (Steinberg & Gafni, 1972). The samples were placed in a 2-mm optical path cuvette. A high-pressure mercury lamp (HBO 100 W/2, Osram) was used for excitation of the luminescence. The excitation light was monochromated with a Bausch and Lomb high intensity monochromator set to 365 nm and filtered with a Schott UG-1 filter to remove stray light. The luminescence light was monochromated using a Jarrell-Ash double monochromator (Model 82-410) with a spectral resolution of 16 nm. A 2 M sodium nitrite solution in water (1-cm optical length) was used in conjunction with the emission monochromator to remove stray light due to the excitation beam. CD spectra are presented in terms of the absorption anisotropy factor, g_{abs}, defined as $(\epsilon_l - \epsilon_r)/\epsilon$ where ϵ_l and ϵ_r are the extinction coefficients for left- and right-handed circularly polarized light, respectively, and ϵ is the extinction coefficient for unpolarized light. CPL spectra are presented in terms of the emission anisotropy factor, g_{em}, which is equal to $\Delta f/(\frac{1}{2}f)$ where $\Delta f/f$ is the fraction of circularly polarized light in the emission and is defined to be positive for left-handed circularly polarized light (Gafni & Steinberg, 1972).

All the measurements were done at room temperature (~23 °C).

Results

Figures 1 and 2 present the spectroscopic results obtained for NADH and phosphodiesterase-treated NADH in neutral aqueous solution. The cleavage of coenzyme introduces small shifts (~2–3 nm) to shorter wavelengths in the fluorescence excitation spectrum as well as in the long wavelength absorption band of the reduced nicotinamide ring. The fluorescence excitation spectrum of the cleaved coenzyme lacks the 260-nm band present in the corresponding spectrum of intact NADH (Weber, 1957). The fluorescence quantum yield (excitation at 340 nm) is reduced by 30% upon cleavage without any change in the shape or wavelength of the emission. This quenching is accompanied by a shortening of the fluorescence decay time by 33%, and is, therefore, dynamic in nature (Gafni & Brand, 1976). The anisotropy factors of NADH and NMNH are identical both in absorption and in emission. However, the values of g_{em} in both cases are more than an order of magnitude larger than those of g_{abs}. The absorption anisotropy factors do not vary significantly across the 340-nm absorption band, while appreciable wavelength dependence is observed in the values of g_{em}.

The absorption and emission spectra as well as spectra of the linear polarization of the fluorescence of the LADH:NADH:IBA ternary complex are shown in the upper part of Figure 3. The polarization of the fluorescence excited at 335 nm is seen to be constant across the emission band and to have a value which is very close to the theoretical upper limit of 0.5. The polarization of the fluorescence at 440 nm does not depend on the excitation wavelength above 320 nm, while below this wavelength the polarization drops sharply. A similar high degree of linear polarization has been reported for the fluorescence of the LDH:NADH complex when excited in the 320–370-nm range (Velick, 1961). The lower part of Figure 3 presents the CD and CPL spectra of both the LADH:NADH and LADH:NADH:IBA complexes. The CD spectra of these two complexes are identical, in agreement with previous ob-

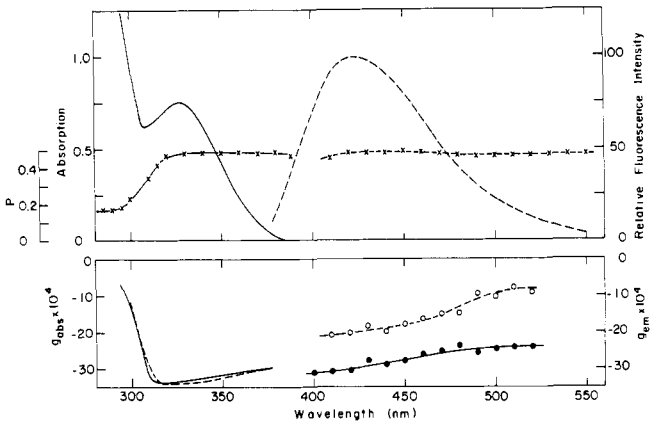

FIGURE 3: Spectroscopic results obtained for NADH in binary and ternary complexes with LADH. Upper part: absorption (solid line), emission (dashed line) and the spectra of linear polarization across the absorption and emission of the LADH:NADH:IBA ternary complex. Lower part: the anisotropy factors in absorption and emission of LADH:NADH (dashed lines) and of LADH:NADH:IBA (solid lines). The data points are indicated on the CPL and linear polarization spectra. Compositions of the complexes and experimental details are described in the text.

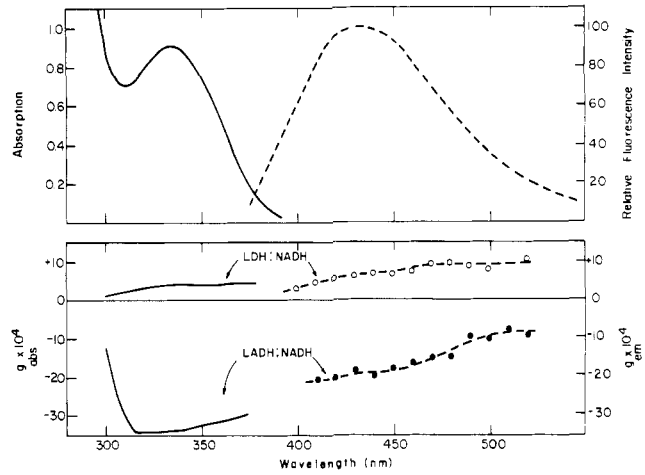

FIGURE 4: A comparison of spectroscopic results obtained for NADH in binary complexes with LDH and with LADH. Upper part: absorption and emission spectra of the LDH:NADH complex. Lower part: anisotropy factors in absorption and emission of LDH:NADH and of LADH:NADH. Data points are indicated on the CPL curves. Compositions of the complexes and experimental details are given in the text.

servations (Luisi et al., 1978), and decrease gradually in absolute magnitude, by about 10%, between 315 and 370 nm. Below 315 nm there is a rapid drop in g_{abs} similar to the one observed in the spectrum of linear polarization. In contrast to the CD, the CPL spectra of the binary and the ternary complexes differ markedly, the latter being 1.5–3 times larger and similar in its value to g_{abs}. The two CPL spectra decrease in magnitude with increasing wavelength but, while g_{em} for the ternary complex changes by about 20% across the emission band, that of the binary complex is reduced by a factor of 2.5. The spectra shown in Figure 3 were obtained for complexes in which the enzyme binding sites were saturated with coenzyme. Very similar CD and CPL spectra were obtained when only half of the LADH binding sites were occupied by NADH.

Figure 4 compares the results obtained for LADH:NADH with those of LDH:NADH. The CD and CPL spectra of the LDH:NADH:oxalate ternary complex were found to be identical with those of the binary complex. The most striking feature of Figure 4 is the opposite sign which both the CPL and CD spectra of NADH assume when bound to the two dehydrogenases. This finding is in sharp contrast to the CPL spectra of ϵNAD$^+$ when bound to the same two enzymes (Schlessinger et al., 1975), and its possible implications will be discussed below. The CD spectrum of the LDH:NADH (and LDH:NADH:oxalate) complex does not change significantly across the absorption band above 320 nm. The CPL spectra assume somewhat larger values than the CD and increase in value toward the long wavelength edge of the emission band.

The CD and CPL spectra of binary and ternary complexes with LDH were found to be independent of the degree of binding site occupation by coenzyme, similar to what was reported above for the complexes with LADH.

Discussion

The high degree of the linear polarization of fluorescence observed for NADH bound to LADH (see Figure 3) or to LDH (Velick, 1961) when excited in the long wavelength absorption band of the reduced nicotinamide ring indicates that the absorbing and emitting oscillators are parallel. From the dependence of the linear polarization of NADH fluorescence on the viscosity of its aqueous sucrose solutions (Weber, 1957) and from the high degree of polarization observed for NADH in 1,2-propanediol (Scott et al., 1970) the same conclusion is reached for the free coenzyme in solution. Photoselection of the emitting molecules has been shown to affect the CPL only when the oscillators in absorption and in emission are not parallel (Steinberg & Ehrenberg, 1974; Ehrenberg, 1976) and thus has no effect on the results presented here since the fluorescence was excited at the long wavelength band of the absorption spectrum. As a result, the anisotropy factors in absorption and emission are expected to be equal if these two processes connect the same pair of quantum states of the NADH molecule. This is clearly not the case for the free coenzyme in aqueous solution since the absorption and emission anisotropy factors were found to differ by more than an order of magnitude (see Figure 2). Marked differences between g_{abs} and g_{em} are not uncommon and usually indicate the existence of different molecular geometries in the ground (absorbing) state and the excited (emitting) state (Steinberg, 1975, 1978; Richardson & Riehl, 1978). Different pairs of quantum states of the molecule are connected, in these cases, by absorption and emission. Heterogeneity of the absorbing and emitting species may also lead to differences between g_{abs} and g_{em} if the species involved have different emission spectra or quantum yields. NADH in aqueous solution has been shown to exist in equilibrium between an extended and two folded conformations. In the latter two, the nicotinamide and adenine rings are intramolecularly stacked to form left or right-handed helical structures (Saenger et al., 1978). Thus there are three distinct species which absorb light and if these differ in their fluorescence spectra or quantum yields g_{abs} and g_{em} may assume different values. Upon cleavage of the NADH molecule with phosphodiesterase to form NMNH, the two folded conformations disappear because the possibility of stacking between adenine and nicotinamide is removed. (Intermolecular complexes between AMP and NMNH are not formed at the concentrations used in this study.) The unstacking is reflected in the fluorescence excitation spectrum by the disappearance of the 260-nm band which is due to absorption of light by adenine. The observation that the CD and CPL spectra are not affected by the cleavage clearly shows that the differences between these spectra are not due to the existence of the extended and folded conformations. Moreover, the quenching of NADH fluorescence, under various experimental conditions, has been shown to be a purely dynamic process (Scott et al.,

1970; Gafni & Brand, 1976) and it was concluded that both in absorption and in emission of light single species of NADH were involved. It may therefore be concluded that the dramatic difference between the optical activities in the ground and emitting states of NADH originates from changes, in the excited state, in the conformation of the nicotinamide ring, and in its interaction with the ribose to which it is linked. Even small changes in molecular conformation may induce marked changes in the CPL spectrum (Schlessinger et al., 1974). Syn and anti configurations of nucleotides are known to exist (Egan et al., 1975; Rhodes & Schimmel, 1971) and to interconvert in aqueous solution on the nanosecond time scale, thus presenting one possible mechanism of excited state conformational change.

The absorption anisotropy factors of the LADH:NADH and LADH:NADH:IBA complexes were found to be identical. This is in agreement with the finding of Luisi et al. (1978) who concluded that there were no conformational differences (as sensed by the nicotinamide ring) between the two complexes. While this seems to be the case for the coenzyme in its ground state, it does not hold for the excited state conformation. The results presented in Figure 3 show that, when the nicotinamide ring is electronically excited, some conformational changes take place in the binary complex while the conformation of the ternary complex is unchanged. Thus, although the nicotinamide ring senses the same environment in the ground state of the binary and ternary complexes with LADH, the latter complex is more rigid and retains its conformation following electronic excitation of the reduced nicotinamide ring. Since no significant differences were observed between the anisotropy factors, in absorption or in emission, of binary and ternary complexes of NADH with LDH it is concluded that in this case both complexes have similar rigidities. The differences observed between g_{abs} and g_{em} are indicative of the fact that both complexes are not fully rigid but change conformation upon electronic excitation.

The anisotropy factors, in absorption and in emission, of NADH bound to LDH are opposite in sign to those of LADH-bound coenzyme. It has been suggested (Luisi et al., 1978) that the sign of the CD spectrum of NADH reflects the stereospecificity of the dehydrogenase to which it is bound. Our results clearly show that this cannot be a general rule since LADH and LDH both have A type stereospecificity towards NADH (Velick, 1961). The extrinsic optical activity induced in the absorption and emission bands of the reduced nicotinamide chromophore thus appears to be extremely sensitive to the structure and chirality of its binding site.

It is interesting to compare the CPL spectra of NADH bound to dehydrogenases with those of bound ϵNAD. In the latter case no differences could be detected between the CPL spectra of the coenzyme analog when bound to LADH and LDH (and a few other dehydrogenases) (Schlessinger et al., 1975). The conclusion was thus drawn that the adenine binding sites on these enzymes were very similar, if not identical, which is of interest from the point of view of the evolution of dehydrogenases. The results obtained in the present work clearly show that the nicotinamide binding sites on LDH and LADH differ both in structure and rigidity. These structural differences undoubtedly result from the proximity of the nicotinamide to the substrate binding sites. The latter may be expected to differ, being constructed to accommodate very different substrates.

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