Acid–Base Equilibria of the Oxidized β-Nicotinamide Adenine Dinucleotide–Pyruvate Adduct in the Ground and Electronically Excited States. A Proton Transfer Probe for Proteins†

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ABSTRACT: Proton transfer reactions of the oxidized NAD^+–pyruvate adduct in its electronically excited singlet state were studied in aqueous solution and when bound to several proteins. The ionization constant of the adduct in the excited state was found to differ by several orders of magnitude from its value in the ground state. A rapid deprotonation reaction was found to follow electronic excitation if a suitable proton acceptor is present. The deprotonation is accompanied by a marked change in the fluorescence spectrum of the adduct and is therefore easy to detect and follow. The free adduct in aqueous solution was found to be in equilibrium between open and folded conformations of which only the open one is fluorescent. The equilibrium constant between these two forms was evaluated. A complex dependence of the fluorescence on the pH in the range of 1–7 was found, which originates in a shift of the equilibrium constant toward the open conformation and in protonation of acid groups of the adduct. Interaction of the adduct with four proteins was studied. Each had a different effect on the proton transfer reaction, reflecting differences in the microenvironment of the bound adduct. A possible use of the adduct as an excited-state proton transfer probe for proteins is presented.

The adducts formed upon addition of certain carbonyl compounds to NAD^+ [1] have been characterized and studied by several authors (Burton & Kaplan, 1954; Burton et al., 1957; Dolin & Jacobson, 1964; Lee et al., 1966; Lee & Winer, 1967; Di Sabato, 1968, 1970; Everse et al., 1971; Arnold & Kaplan, 1974). Of particular interest are the adducts formed between NAD^+ and natural substrates of NAD^+-linked dehydrogenases since these adducts are related to the abortive ternary complexes formed between the enzymes and the oxidized forms of the coenzyme and substrate. The adducts may be formed upon incubation of NAD^+ and the carbonyl compound at alkaline pH. The preparation of the NAD^+–pyruvate adduct has been described in detail by Everse et al. (1971) and is shown schematically in Figure 1. Pyruvate addition to NAD^+ first leads to the formation of the adduct in its reduced form which is easily oxidized to the more stable oxidized form. Both the reduced and oxidized forms of the adduct were found to bind to several NAD^+-dependent dehydrogenases with various affinities (Lee & Winer, 1967; Everse et al., 1971).

The oxidized NAD^+–pyruvate adduct is weakly acidic. From the changes in its absorption with the pH, the value of its pK_a was determined to be 9.6 (Di Sabato, 1970; Everse et al., 1971). Dolin & Jacobson (1964) reported a similar value (9.5) for the pK_a of the oxidized NAD^+–acetone adduct. In neutral aqueous solution, therefore, practically all the molecules are protonated and exist in equilibrium between the keto and enol forms (see Figure 1). It is well established that the equilibrium between such two tautomers of isoquinolinol derivatives is shifted, almost exclusively, toward the keto form (Mason, 1958; Evans et al., 1967; Katritzky & Lagowski, 1963). For 1-hydroxyisoquinoline in aqueous solution, the ratio between the keto and enol tautomers is about 70 000 (Mason, 1958). Thus, only the amide form of the adduct is present in neutral aqueous solution.

The pK_a values of many aromatic acids and bases, in their excited singlet state, markedly differ from their values in the ground electronic state (Weller, 1961; Loken et al., 1972; Schulman & Capomaccia, 1975; Gafni et al., 1976; Gafni & Brand, 1978; Laws & Brand, 1979). These molecules will therefore tend to reach a new equilibrium by proton transfer, following electronic excitation. When the aromatic molecule is bound to a biological system, these excited-state proton transfer reactions may be used to probe the microenvironment of the binding site and in particular the presence of proton accepting and donating groups (Loken et al., 1972; Laws et al., 1979). In the present study the possible use of the oxidized NAD^+–pyruvate adduct as a proton transfer probe was explored. The excited-state reactions of the adduct were studied in aqueous solution by absorption, fluorescence, and fluorescence decay measurements. The rate constants of these reactions were evaluated and a reaction scheme, which fits all the experimental results, was constructed. A preliminary study was also made of the effects of adduct binding to several proteins on its fluorescence. Specific interactions with the binding sites were revealed, in most cases leading to changes in both the ground-state and excited-state proton transfer reactions.

Materials and Methods

NAD^+ and sodium pyruvate were purchased from Sigma Chemical Co. Snake venom phosphodiesterase (from *Crotalus terrificus* terrificus) was obtained from Boehringer Mannheim Co. as a solution in 50% glycerol. Bovine serum albumin, crystallized and lyophilized, was obtained from Sigma and used without further purification. 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 were dissolved in 0.1 M phosphate buffer of

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[1] Abbreviations used: NAD^+, β-nicotinamide adenine dinucleotide; ϵNAD^+, nicotinamide 1,N^6-ethenoadenine dinucleotide; FAD, flavin adenine dinucleotide; εFAD, flavin 1,N^6-ethenoadenine dinucleotide; BSA, bovine serum albumin; LDH, beef heart lactate dehydrogenase; LADH, horse liver alcohol dehydrogenase; MDH, pig heart malate dehydrogenase; GPDH, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase.

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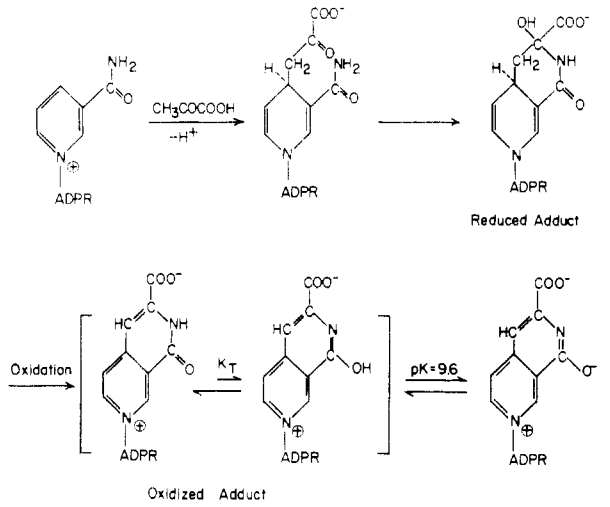

FIGURE 1: Schematic representation of the reactions which lead to the formation of the oxidized NAD^+–pyruvate adduct. The protonated (neutral) species is in equilibrium between the keto and enol tautomers while the ionized species assumes the enolate form only.

the desired pH and dialyzed against several changes of the same buffer for 48 h. Glyceraldehyde-3-phosphate dehydrogenase was a gift from A. Levitzki and Y. Henis (The Hebrew University, Jerusalem). The apoenzyme was prepared following the procedure of Henis & Levitzki (1977) and was substantially free from NAD^+ as evidenced by the A_{280}/A_{260} ratio of 1.8 to 1.9. Enzyme concentrations were determined from the optical density at 280 nm (Sund & Theorell, 1963; Velick, 1958; Fox & Dandliker, 1956).

The oxidized NAD^+–pyruvate adduct was prepared by the method described by Everse et al. (1971). The adduct was stored desiccated at −20 °C in the dark. Cleavage by phosphodiesterase was performed by adding 40 μg of the enzyme to 2 mL of $\sim 10^{-4}$ M adduct solution in 0.01 M phosphate buffer (pH 7.3) and keeping the mixture at room temperature ($\sim$23 °C) for 2 h. The reaction was followed by monitoring the increase in fluorescence intensity at 430 nm.

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. Fluorescence decay measurements were done with an instrument built in this laboratory and described elsewhere (Hazan et al., 1974). The excitation wavelength was 340 nm, and the fluorescence decay was observed either through a blue glass filter transmitting light in the wavelength range of 390–460 nm (where the protonated adduct emits) or through a Schott KV filter transmitting above 500 nm (where the emission is due mainly to the deprotonated species). The decay curves were analyzed by using the method of nonlinear least squares (Grinvald & Steinberg, 1974).

The effects of acetate concentration on the emission spectrum of the adduct were studied by using a 2×10^{-6} M adduct solution in 1 mM phosphate buffer (pH 7.3). Acetate was added from a 4 M stock solution (acidified to pH 7.3 by HCl) which also contained 2×10^{-6} M adduct. The concentration of the latter in the sample was therefore unchanged upon acetate additions. The effect of the pH on the adduct's emission was studied in a similar way by adding the appropriate amounts of HCl from 0.01, 0.1, or 1 M stock solutions which contained the same concentration of adduct as the sample being studied.

Complexes between the adduct and proteins were prepared by adding the appropriate volume of adduct stock solution to the protein solution, followed by incubation of the mixture for about 5 min at 20 °C. All measurements were done at 20 °C.

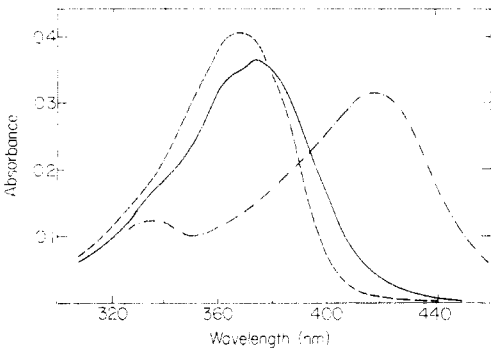

FIGURE 2: Absorption spectra of the oxidized NAD^+–pyruvate adduct in aqueous solution at pH 1 (---), at pH 7.3 (—), and in 0.1 N NaOH (–·–). The three spectra were recorded by using the same adduct concentration. The wavelengths of maximal absorptions are summarized in Table I.

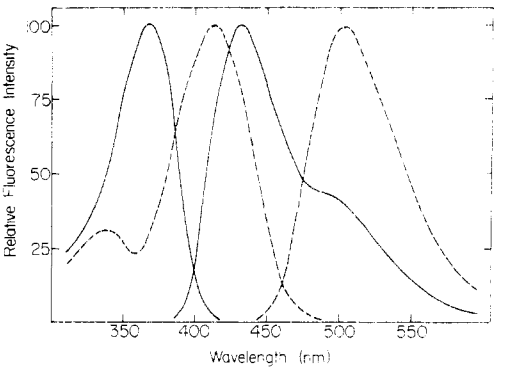

FIGURE 3: Fluorescence and fluorescence excitation spectra of the oxidized NAD^+–pyruvate adduct. (—) In 0.001 M phosphate buffer, pH 7.3; excitation wavelength of the fluorescence was 360 nm; emission wavelength used to record the excitation spectrum was 430 nm. (---) In 0.1 N NaOH; fluorescence was excited at 415 nm; excitation spectrum is for emission at 500 nm. All the spectra are normalized to the same peak height.

Results

Figure 2 presents the absorption spectra of the oxidized NAD^+–pyruvate adduct at neutral pH, at pH 1, and in 0.1 N NaOH. The spectrum in the acid pH is shifted toward shorter wavelengths compared to the absorption at pH 7.3 and is somewhat increased. The two spectra cross at 378 nm. The absorption spectrum in alkaline solution is shifted to longer wavelengths and has its maximum at 418 nm. The absorption spectrum at pH 1 is identical with the spectrum obtained at neutral pH after cleavage of the adduct by phosphodiesterase. Figure 3 presents the fluorescence and the fluorescence excitation spectra of the adduct in neutral aqueous solution and in 0.1 N NaOH. The emission from the neutral solution has a maximum at 433 nm and a shoulder at about 500 nm, while the fluorescence of the basic solution has a single peak at 505 nm. The excitation spectrum of the adduct in 0.1 N NaOH resembles the absorption of the same solution (see Figure 2) while the excitation spectrum for fluorescence of the neutral solution is shifted to shorter wavelengths relative to the absorption and coincides with the absorption spectrum of the adduct in pH 1 solution (and, hence, also with the absorption of the cleaved adduct at neutral pH).

The effects of cleavage by phosphodiesterase on the fluorescence of the adduct are very pronounced, as seen in Figure 4, and result in a large increase of the fluorescence intensity and a relative enhancement of the long-wavelength

Table I: Absorption and Fluorescence Maxima of the Intact and Phosphodiesterase-Cleaved NAD^+–Pyruvate Adduct

	intact adduct				cleaved adduct			
	absorption		emission		absorption		emission	
	λ (nm)	ν_a (cm^{-1})	(nm)	ν_f (cm^{-1})	λ (nm)	ν_a (cm^{-1})	λ (nm)	ν_f (cm^{-1})
pH 1	368	27 170	433	23 090			433	23 090
			503	19 880			503	19 880
pH 7.3	374	26 740	433	23 090	368	27 170	433 (500)	23 090
0.1 N NaOH	418	23 920	505	19 800	418	23 920	505	19 800

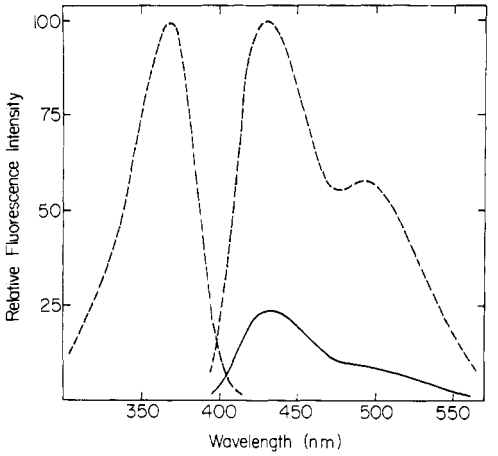

FIGURE 4: Fluorescence of the oxidized NAD^+–pyruvate adduct (solid line) and of the same sample after cleavage by phosphodiesterase (broken line). The spectra were recorded in 0.001 M phosphate buffer, pH 7.3; the excitation wavelength was 378 nm. The excitation spectrum shown is for emission at 530 nm from the cleaved adduct and was normalized to the height of the emission maximum. This excitation spectrum and the spectra obtained for emission at 430 nm of both cleaved and intact adduct are identical.

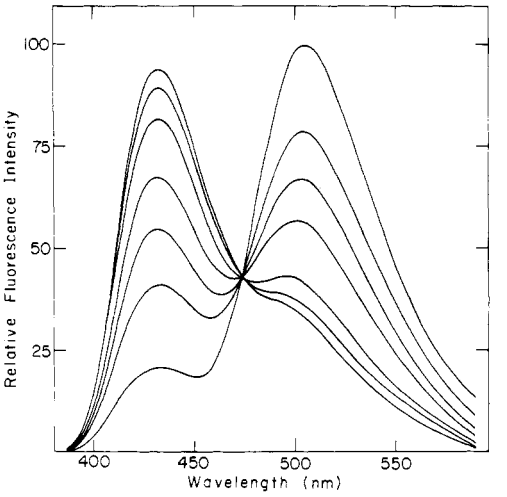

FIGURE 5: Emission spectra of the adduct in neutral aqueous solution in the presence of the following concentrations of sodium acetate (in order of decreasing heights of the 433-nm peaks): 0.01, 0.04, 0.11, 0.33, 0.60, 1.04, and 2.67 M. The spectra were excited at 365 nm.

shoulder (the fluorescence intensity at 433 nm increases 4.3 times while at 505 nm the increase is by a factor of 6.9). As will be shown under Discussion, these spectroscopic differences between intact and cleaved species result from the fact that the majority of molecules of the intact adduct assume a nonfluorescent folded conformation which is absent in the cleaved adduct.

Table I summarizes some of the spectroscopic data of the intact and cleaved adduct at pH 1, at pH 7.3, and in 0.1 N NaOH. Since the pK_a of the NAD^+–pyruvate adduct is 9.6 (Di Sabato, 1970; Everse et al., 1971), the only species present in neutral aqueous solution is the protonated one while the only species present in 0.1 N NaOH is the ionized form. From the ground-state pK_a and the data presented in Table I, the value of the pK_a in the electronically excited singlet state (pK_a^*) may be calculated by using the Förster cycle (Weller, 1961; Bartok et al., 1962)

$$pK_a^* = pK_a - \frac{0.625}{T}\,\frac{\nu_a^{AH} + \nu_f^{AH} - \nu_a^{A^-} - \nu_f^{A^-}}{2} \qquad (1)$$

where ν_a^{AH} and ν_f^{AH} are the transition frequencies (in cm^{-1}) of the long-wavelength absorption and of the fluorescence of the protonated molecule while $\nu_a^{A^-}$ and $\nu_f^{A^-}$ are the corresponding values for the ionized species. By use of the appropriate values from Table I, it is found that $pK_a^* = 2.6$. In the excited state, at neutral pH, the adduct will therefore tend to release a proton into the aqueous environment. The observation that emission from the protonated species (at 433 nm) predominates indicates that the rate of proton release is slow compared with the (combined) rate of depopulation of the excited state via other channels, i.e., fluorescence and nonradiative deexcitation processes.

Proton release from excited adduct molecules may be facilitated by the presence of a suitable proton acceptor, i.e., an acid whose pK_a is above the pK_a^* of the adduct. In the present study the acetate ion ($pK_a = 4.75$) was used as proton acceptor. The emission spectra of the adduct in the presence of various concentrations of acetate are shown in Figure 5. The emission of the protonated species at 433 nm is seen to decrease with increasing acetate concentration while the emission from the ionized adduct (505 nm) is enhanced. The presence of an isoemissive point, at 473 nm, clearly indicates that only two species (protonated and ionized) contribute to the emission.

The fluorescence excitation spectra of the adduct were studied in neutral aqueous solution in the absence and in the presence of 2.7 M acetate, as well as for the cleaved adduct in neutral solution with no acetate. In all three cases the excitation spectra for emission at 430 nm and at 530 nm were found to be identical. Thus, as expected, the ionized species emitting in the long-wavelength band is generated in the excited state.

The quenching of fluorescence of the protonated adduct by acetate is presented in Figure 6 and is seen to follow the Stern–Volmer equation:

$$F_0/F = 1 + k_Q C\tau \qquad (2)$$

Here F_0 and F are the fluorescence intensities in the absence and in the presence of quencher, k_Q is the bimolecular rate constant for quenching, C is the concentration of quencher, and τ is the fluorescence decay time in the absence of quencher. The latter was found to be 0.85 ns for the NAD^+–pyruvate adduct, and from this value and the slope of the straight line in Figure 6 k_Q was calculated to be $1.5 \times 10^9\ M^{-1}\ s^{-1}$. Proton transfer from the excited adduct to acetate is thus a diffusion-controlled process. Similar values for rate constants of proton transfer to acetate have been reported for 2-naphthol [$2.9 \times 10^9\ M^{-1}\ s^{-1}$ (Weller, 1961)] and for 2-

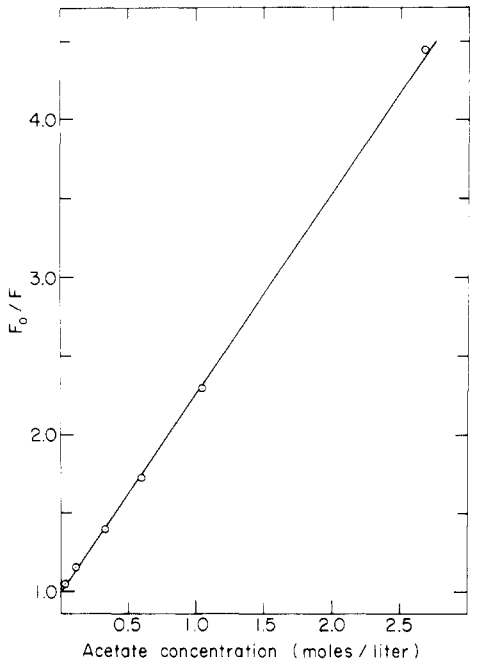

FIGURE 6: Stern–Volmer plot for the quenching of the fluorescence of the adduct at 430 nm by acetate.

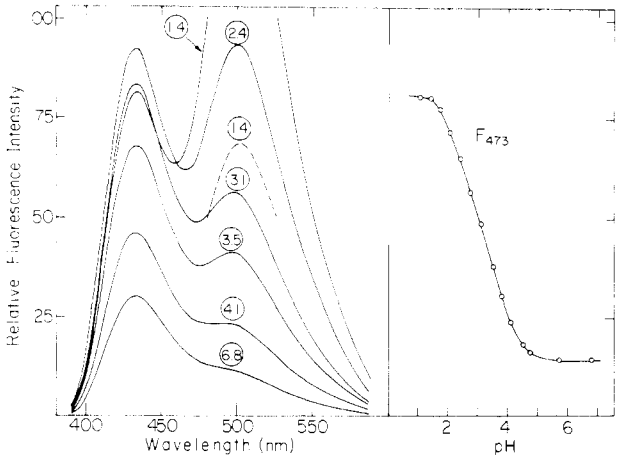

FIGURE 7: Fluorescence spectra of the oxidized NAD^+–pyruvate adduct in aqueous solution at the pH values listed. The same adduct concentration was used throughout, and the fluorescence was excited at the isosbestic point (378 nm). The dashed line presents the twofold reduced fluorescence at pH 1.4. The pH dependence of the fluorescence intensity at 473 nm (the isoemissive wavelength of protonated and ionized adduct molecules) is shown on the right side of the figure.

hydroxy-1-naphthaleneacetic acid [1.7×10^9 M^{-1} s^{-1} (Gafni et al., 1976)]. As was mentioned before, practically only the keto tautomer of the adduct (see Figure 1) is present in neutral aqueous solution. The fast rate of proton transfer to acetate indicates that the deprotonation (in which an enolate is formed) occurs directly from the excited keto tautomer.

The large increase in the fluorescence quantum yield upon cleavage of the adduct by phosphodiesterase (Figure 4) is not accompanied by a similar increase in the fluorescence decay time which was found to be 1.0 ns for the cleaved adduct. This finding is indicative of static quenching in the intact adduct and will be dealt with under Discussion.

Figure 7 presents the effects of increased acidity on the fluorescence of the adduct. A marked enhancement in the relative quantum yield is observed as the pH is lowered. This effect is accompanied by a large increase in the efficiency of deprotonation as reflected by the relative increase of the

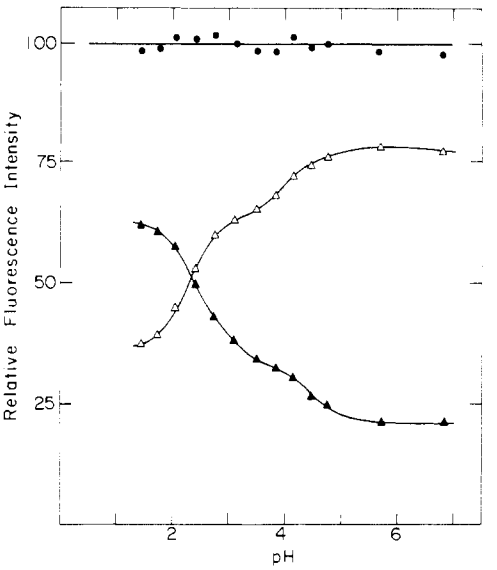

FIGURE 8: Dependence on the pH of the relative fluorescence intensities of the adduct at 433 (△) and 510 nm (▲) and their sum (●) when the fluorescence intensity at 473 nm is normalized to the same value throughout the pH range covered. The data are taken from the spectra shown in Figure 7.

long-wavelength emission band which originates in the deprotonated adduct. Below pH 2.4 this band becomes dominant. Figure 7 also presents the pH dependence of the emission intensity at the isoemissive point (473 nm). The titration curve shows a pK value of 3.2 to be involved in the enhancement of fluorescence. Between pH 7 and 1 F_{473} increases by a factor of 5.6 which is very similar to the 5.5-fold increase in F_{473} observed upon adduct cleavage at neutral pH. It should be noted that a similar ratio, i.e., 5.8, was found between the values of F_{473} of adduct solutions in 0.1 N NaOH and in neutral aqueous solution (having the same optical density at the excitation wavelength).

The acetate titration spectra of Figure 5 reveal that the decrease in the emission at 433 nm equals the enhancement observed at 510 nm. The same relation is found for the spectra at different pH values if their heights at the isoemissive point are normalized to the same value as shown in Figure 8 (as will be explained under Discussion, the increase in the total fluorescence intensity of the adduct at acid pH, to which F_{473} is proportional, is due to a shift in the equilibrium between nonfluorescent and fluorescent species toward the latter; keeping F_{473} constant is thus equivalent to having a fixed concentration of fluorescent molecules). Figure 8 also shows the effect of pH on the individual values of F_{433} and F_{510}. An increase in the rate of excited-state deprotonation with decreasing pH is observed, evidenced either from the increase in F_{510} or from the decrease in F_{433}. This rate increase shows a complex dependence on the pH, having two apparent pK values at pH 4–4.5 and at pH ~2.5.

A preliminary study was made of the interaction between the NAD^+–pyruvate adduct and several proteins. Binding of the adduct was evidenced from the changes in its spectra; however, the binding constants were not determined; hence, nonbound adduct may have contributed to the observed fluorescence and excitation spectra.

Figure 9 presents the effect of a large molar excess of BSA on the fluorescence of the adduct. The fluorescence was excited outside the protein's absorption and its maximum increased 1.5 times compared to that of free adduct at the same concentration. No shift in λ_{max} was observed. The long-wavelength shoulder is less pronounced than that of the free

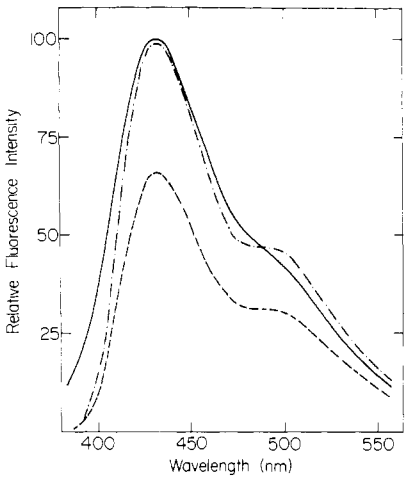

FIGURE 9: Emission spectra of 5×10^{-6} M oxidized NAD^+–pyruvate adduct solution in 0.01 M phosphate buffer (pH 7.3) (---) and of the same solution in the presence of 3×10^{-4} M BSA (—). The fluorescence of free adduct, normalized to the same height as that in the presence of BSA, is also shown (–·–). The spectra were excited at 360 nm.

adduct, indicating a small efficiency of excited-state proton release from the bound adduct.

Figure 10 summarizes the fluorescence and fluorescence excitation spectra of the adduct in the presence of three dehydrogenases. With LADH, at the concentrations used, the fluorescence is enhanced about 1.5-fold and slightly shifted to shorter wavelengths (by 3 nm). No shift was observed in the excitation spectrum. The relative height of the long-wavelength shoulder is unchanged by the binding; hence, LADH has no effect on the proton transfer efficiency.

The interaction of the adduct with GPDH (Figure 10B) manifests itself by changes in both emission and excitation spectra. The emission peak increases in intensity and is slightly shifted to longer wavelengths; however, a more pronounced effect is revealed in the long-wavelength shoulder which is markedly enhanced upon binding of adduct to the enzyme. The excitation spectrum for emission at 515 nm, shown in Figure 10B, shows a second, long-wavelength band which indicates that the long-wavelength emission is partially due to adduct molecules ionized in the ground state. The contribution of these molecules to the 515-nm emission is, however, very small (i.e., most of the emission at this wavelength originates in molecules which have been deprotonated in the excited state) as the following consideration shows. The fluorescence described in Figure 10B was excited at 360 nm where the excitation efficiency of the ionized adduct is 4.3 times smaller than that at 415 nm (see Figure 3). Since the excitation spectrum shown in Figure 10B is 2.3 times higher at 360 nm than at 415 nm, it follows that direct excitation of the ionized species can, at most, be responsible for 10% of the observed fluorescence intensity at 515 nm. This is clearly much smaller than the contribution of the ionized species to the emission at this wavelength, proving that most of the ionized species is formed in the excited state.

Figure 10C presents the results obtained in the presence of LDH. The effects of this dehydrogenase on both fluorescence and excitation spectra are dramatic. The emission, excited at 378 nm, is mainly due to the ionized species and is maximal at 488 nm (compared to 505 nm for the free adduct). The fluorescence excitation spectrum for the emission at 430 nm, where only the protonated adduct emits, is similar to that observed for the free adduct but shifted by 3 nm to longer wavelength. The excitation spectrum for emission at 500 nm

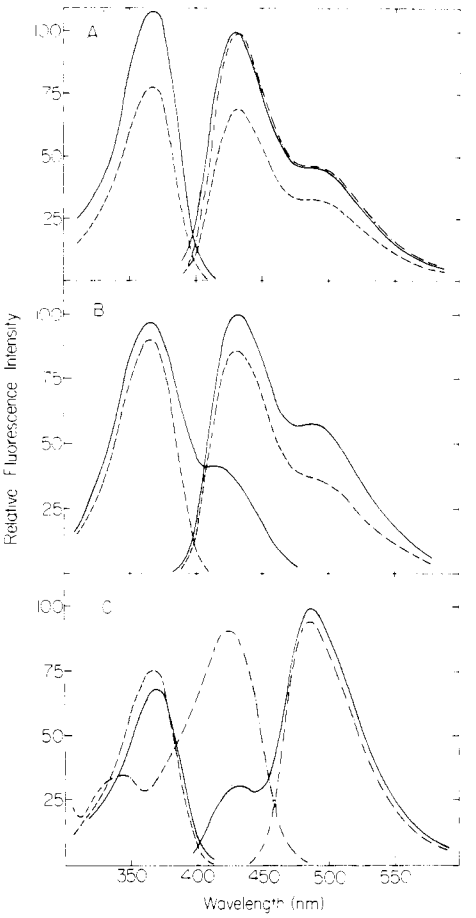

FIGURE 10: Fluorescence and fluorescence excitation spectra of the oxidized NAD^+–pyruvate adduct in the presence of dehydrogenases. (A) (---) 6×10^{-6} M adduct in 0.01 M phosphate buffer (pH 7.3); excitation wavelength was 378 nm; fluorescence excitation spectrum for the emission was at 430 nm. (—) 6×10^{-6} M adduct in the presence of 3.6×10^{-5} M LADH. Fluorescence was excited at 378 nm while the excitation spectrum is for emission at 430 nm (an identical spectrum is obtained for the emission at 500 nm). (–·–) Fluorescence of the free adduct brought to the same height as that in the presence of LADH to allow easy comparison. (B) (---) 2.6×10^{-5} M adduct in 50 mM Hepes buffer plus 10 mM EDTA (pH 7.3); fluorescence was excited at 360 nm; excitation spectrum is for 430-nm emission. (—) 2.6×10^{-5} M adduct in the presence of 2.7×10^{-5} M GPDH; same Hepes plus EDTA buffer; fluorescence was excited at 360 nm; the excitation spectrum (515-nm emission) is increased by a factor of 2. (C) (–·–) 6.3×10^{-6} M adduct in the presence of 1.25×10^{-5} M LDH in 0.1 M phosphate buffer (pH 7.3); fluorescence was excited at 415 nm; the excitation spectrum is for emission at 500 nm. (—) Same adduct–LDH mixture; fluorescence spectrum was excited at 378 nm and increased 2.3-fold while the excitation spectrum (430-nm emission) is increased 5-fold. (---) Excitation spectrum of free adduct (2×10^{-5} M) for the emission at 430 nm.

resembles the corresponding spectrum of the free adduct in 0.1 N NaOH (Figure 3); however, its maximum is at 426 nm compared to 415 nm for the free adduct. Also, the ratio between the height at the peak to that at the 360-nm minimum is 3.1 compared to the value of 4.3 obtained for the adduct in basic solution. This indicates some contribution from protonated adduct molecules to the excitation spectrum for emission at 500 nm. This may be due both to emission from protonated molecules and to emission from ionized molecules formed by excited-state proton transfer.

Discussion

The absorption and fluorescence spectra of the oxidized NAD^+–pyruvate adduct display a complex dependence on the

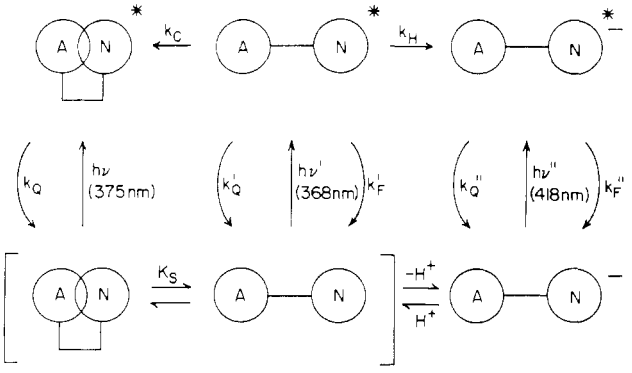

FIGURE 11: Kinetic scheme of reactions of the oxidized NAD^+–pyruvate adduct in aqueous solution. Details of the various species and rate constants involved are described in the text.

pH. In 0.1 N NaOH solution emission is observed, as expected, only from the ionized species. However, a marked increase in fluorescence intensity is observed at acid pH values or upon cleavage by phosphodiesterase. It is well established that NAD^+, as well as other dinucleotides, exists in aqueous solution in equilibrium between open and folded conformations (Weber, 1957; Shifrin & Kaplan, 1961; Velick, 1961; Spencer & Weber, 1972; Barrio et al., 1973). In the folded conformation interaction between the two bases of the dinucleotide often leads to quenching of its fluorescence. Thus, the fluorescence of FAD and ϵFAD originates in the open conformation only (Spencer & Weber, 1972; Barrio et al., 1973). Similarly, the fluorescence of ϵNAD^+ in its folded conformation is strongly quenched compared to that of ϵNAD^+ fragments which lack the nicotinamide or of the coenzyme analogue when bound to several dehydrogenases (Lee & Everse, 1973; Luisi et al., 1975; Gruber & Leonard, 1975; Gafni, 1979). In the latter case no interaction between ethenoadenine and nicotinamide is possible since the coenzyme analogue is bound in the open conformation (Luisi et al., 1975; Gafni, 1977). The large increase in fluorescence of the NAD^+–pyruvate adduct which accompanies its cleavage by phosphodiesterase indicates that interactions between adenine and the modified nicotinamide ring, which lead to quenching of the fluorescence of the latter, have to be invoked to explain the observations made in the present study.

The experimental results will be described with reference to the reaction mechanism shown in Figure 11. The oxidized adduct, at neutral pH, is in equilibrium between folded and open conformations, the equilibrium constant being K_S. The absorption spectra of these two species differ somewhat, having an isosbestic point at 378 nm (here, and in the following discussion, the assumption is made that the spectroscopic properties of the open conformation and those of the cleaved adduct are identical; this assumption is consistent with all the observations made in this study as may be seen from the following discussion). When excited at the isosbestic wavelength, the initial concentrations of open and folded conformations in the excited state are proportional to their concentrations in the ground state. The excitation spectrum for the fluorescence of the adduct at neutral pH (Figure 3) coincides with the absorption (and excitation) spectrum of the cleaved adduct, indicating that only absorption of light by the open conformation leads to light emission. The folded conformation is therefore concluded to be nonfluorescent and, furthermore, not to be converted to the open one in the excited state. The rate constant of the folding reaction of the adduct in the excited state, k_c, may be evaluated from the fluorescence decay times of the intact and cleaved molecules (τ_i and τ_c, respectively):

$$\frac{1}{\tau_i} = k_F' + k_Q' + k_H + k_c$$

$$\frac{1}{\tau_c} = k_F' + k_Q' + k_H \qquad (3)$$

k_F' is the rate constant for radiative transition to the ground state, k_Q' is the combined rate constant for all the nonradiative deexcitation processes, and k_H is the rate constant of the excited-state proton transfer reaction. Since $\tau_i = 0.85$ ns and $\tau_c = 1.0$ ns, the rate constant for folding is $k_c = 1/\tau_i - 1/\tau_c = 1.8 \times 10^8\ s^{-1}$. Similar values have been reported before for the excited-state folding reactions of FAD, $1.9 \times 10^8\ s^{-1}$ (Spencer & Weber, 1972), and of ϵFAD, $1.7 \times 10^8\ s^{-1}$ (Barrio et al., 1973), while for ϵNAD^+ the rate constant was found to be larger, $4.3 \times 10^8\ s^{-1}$ (Gruber & Leonard, 1975).

The increase in the fluorescence quantum yield of the adduct upon cleavage may be used, along with the values of τ_i and τ_c, to calculate K_S by means of eq 4 as described by Spencer & Weber (1972):

$$K_S = \frac{[\text{folded}]}{[\text{open}]} = \frac{F_c}{F_i}\frac{\tau_i}{\tau_c} - 1 \qquad (4)$$

F_c/F_i is the ratio of fluorescence quantum yields of cleaved and intact species, its value being 5.5 as determined from the increase in fluorescence intensity at the isoemissive wavelength. The value of K_S calculated from eq 4 is 3.7. Seventy-nine percent of the adduct molecules are thus in the folded conformation in neutral aqueous solution as compared with ~30% in NAD^+ (Jardetzky & Wade-Jardetzky, 1966) and 82% in FAD (Barrio et al., 1973).

In its ground state the oxidized NAD^+–pyruvate adduct is a weak acid ($pK_a = 9.6$). Upon electronic excitation the acid–base equilibrium shifts and the value of pK_a^*, as calculated from the experimental data by use of eq 1, is 2.6. Only protonated adduct molecules are thus present in the neutral aqueous solution and these will tend to deprotonate when brought to the excited state. It should be noted that such dramatic changes in the pK values of aromatic acids and bases upon excitation to their first excited singlet state are not uncommon. Thus, the pK_a of 2-naphthol shifts from its value of 9.46 in the ground state to 2.8 in the excited state while for the base acridine the shift is from 5.45 to 10.65 (Weller, 1961).

Upon adduct cleavage, the fluorescence at 433 nm is increased 4.3-fold while that at the isoemissive wavelength increases by a factor of 5.5. This difference is indicative of the fact that in the cleaved adduct proton transfer takes place to a larger extent (due to the longer lifetime of the excited state), leading to a relative quenching in the fluorescence of the protonated species. Since the excited-state proton transfer is a dynamic process, one may write

$$F_{0,c}/F_c = \tau_{0,c}/\tau_c \qquad (5)$$

where $F_{0,c}$ is the fluorescence intensity of the protonated cleaved species that would have been observed in the absence of proton transfer and F_c is the observed fluorescence (i.e., $F_{0,c}/F_c = 5.5/4.3$). $\tau_{0,c}$ is the fluorescence decay time in the absence of proton transfer. From eq 5 $\tau_{0,c}$ is found to be 1.28 ns. The rate constant for proton transfer may now be evaluated: $k_H = 1/\tau_c - 1/\tau_{0,c} = 2.2 \times 10^8\ s^{-1}$. From this value, and that of the pK_a^*, the rate constant of the reverse reaction (i.e., excited-state protonation) was calculated to be $k_H^- = 8.8 \times 10^{10}\ L\ mol^{-1}\ s^{-1}$. This value is typical of diffusion-controlled reactions involving protons in water, and similar values were

obtained for excited-state protonation reactions in other systems (Weller, 1961; Loken et al., 1972; Laws & Brand, 1979).

In 0.1 N NaOH solution, the adduct is fully in the ionized form. The 5.8-fold increase in fluorescence intensity at 473 nm, compared to the intensity at neutral pH, is similar to the increase observed upon cleavage of the adduct. It is therefore concluded that the ionized adduct assumes only the open conformation both in the ground and in the electronically excited state.

The complex dependence of the fluorescence of the adduct on the pH in the range 1–7 is due to a superposition of two effects: (a) enhancement of the (total) fluorescence as monitored by the increase in F_{473} and (b) increase in the efficiency of excited-state proton transfer (see Figures 7 and 8). The enhancement in F_{473} is quantitatively very similar to the one observed upon adduct cleavage, indicating that it is due to a shift in the equilibrium between folded and open conformations fully toward the latter (a similar change of the equilibrium takes place in the excited state since no refolding is observed following excitation). Similar unfolding reactions, upon acidification, have been found to occur in FAD (Bessey et al., 1949; Weber, 1950), in 5-amino-NAD^+ (Walter & Kaplan, 1963), and in the oxidized NAD^+–acetone adduct (Dolin & Jacobson, 1964). The pK_a values of these unfolding processes were in the range of 3.3–3.7 as compared to the value of 3.2 found for the NAD^+–pyruvate adduct in the present study. Unfolding upon acidification has been attributed to protonation of the amino group of adenine (Walter & Kaplan, 1963).

The observation that the efficiency of proton transfer is enhanced upon lowering the pH (Figure 8) is somewhat uncommon since for most organic acids the opposite behavior is observed. The two pK values which are revealed when the change in proton transfer efficiency with pH is followed are, most probably, due to protonation of acidic groups on the adduct, thereby eliminating negative charges and enhancing the rate of deprotonation. The smaller part of the effect is associated with a pK in the range of 4–4.5 and may result from protonation of the carboxylate group of the modified nicotinamide. A larger increase in the efficiency of proton transfer occurs at pH values below 3. The titration curve here is, undoubtedly, distorted since the excited-state deprotonation reaction whose $pK_a^* = 2.6$ becomes reversible and its efficiency tends to decline. The apparent pK of about 2.5 seen in Figure 8 is therefore above the real pK_a of the group being protonated. Since this pK_a is not known precisely, one cannot identify the group being protonated in an unambiguous way. However, it is very likely that the second phosphate group of the coenzyme analogue is the one involved. The phosphate groups of another NAD^+ analogue (5-amino-NAD^+) have indeed been shown to interact with the nicotinamide moiety electrostatically, thereby affecting its fluorescence, and the interaction disappeared upon protonation of the phosphate group (Walter & Kaplan, 1963). In the oxidized NAD^+–pyruvate adduct the same interaction, which brings the negative phosphate to the vicinity of the proton-releasing group, may be expected to slow the rate of deprotonation. Neutralization of the phosphate group will thus lead to the enhanced deprotonation which is observed.

The Adduct as a Proton Transfer Probe for Proteins. Excited-state proton transfer reactions of fluorescent chromophores bound to proteins have been used by several workers to study the microenvironment of the binding sites (De Luca et al., 1971; Loken et al., 1972; Laws et al., 1979). In contrast to the probes used in most of these studies, the oxidized NAD^+–pyruvate adduct is a derivative of the natural coenzyme and may be expected to bind to the coenzyme binding sites in NAD^+-linked dehydrogenases. Indeed, Lee & Winer (1967) reported binding constants in the range of 46–51 μN for binding of the adduct to LDH and MDH. Its fluorescence properties make the adduct a potential probe for proton-accepting groups and residues inside its bindings sites. The preliminary results reported here indeed demonstrate that the interactions between the modified nicotinamide ring and its binding site differ from one protein to the other and different rates of excited-state proton transfer are revealed. In one case, i.e., LDH, strong interaction with a negatively charged residue leads to deprotonation of the adduct also in the ground state. Lee & Winer (1967) made a similar observation and postulated an interaction with the imidazole of histidine to be involved.

It is well documented that NAD^+ and its analogues assume the open conformation when bound to all three dehydrogenases used in the present study (Velick, 1961; Lee et al., 1973; Luisi et al., 1975; Gafni, 1977). Binding to dehydrogenases may therefore be expected to induce an increase in the fluorescence quantum yield similar to the one observed upon cleavage of the dinucleotide by phosphodiesterase or upon its unfolding in acid or in base. Indeed, the increase in fluorescence of ϵNAD^+ upon binding to dehydrogenases has been reported to be very similar to the enhancement observed upon cleavage of the dinucleotide (Luisi et al., 1975). However, is some cases unfolding in solution and upon binding was found to have very different effects on the fluorescence (Gafni & Brand, 1976; Henis & Levitzki, 1977). Binding of the adduct to LDH was accompanied by a 5.7-fold increase in F_{473} (the optical density at the excitation wavelength being the same for the samples with and without enzyme). Thus, interactions between the modified nicotinamide and its binding site in LDH, while changing the rate of the proton transfer reaction, do not affect the total fluorescence of the adduct which increases as expected for an unfolded bound coenzyme analogue.

In the experiments involving LADH, GPDH, and BSA, the fluorescence enhancements observed were much smaller than that expected to accompany the unfolding of adduct. One possible reason for this behavior may be the partial quenching of fluorescence of bound (unfolded) adduct by amino acid residues in its binding sites. However, since the binding constants of the adduct to these proteins are not known, incomplete binding is possible and may be responsible for the small fluorescence enhancements. While the results reported here are preliminary, they demonstrate that differences in the adduct binding sites in various proteins are clearly reflected as changes in the rates of its excited-state proton transfer reactions.

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