Intramolecular Electron Transfer in Flavin Adenine Dinucleotide. Photochemically Induced Dynamic Nuclear Polarization Study at High and Low Magnetic Fields

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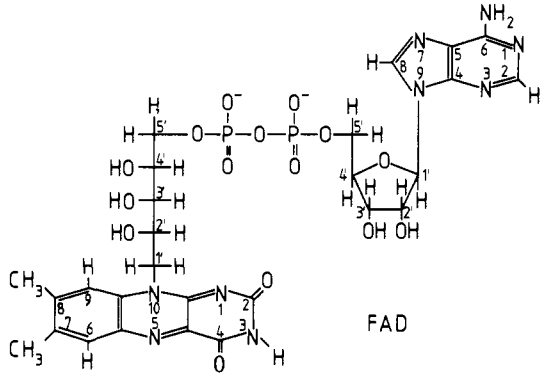
Abstract: Upon light excitation of the flavin moiety in flavin adenine dinucleotide (FAD) a biradical is formed via electron transfer from the adenine part. The photo-CIDNP effects arising from this biradical are presented. At high magnetic field the polarization is due to T_0–S mixing and shows a pronounced pH dependence similar to that of the fluorescence behavior. This is interpreted in terms of a shift of the stacking/destacking equilibrium of FAD toward the stacked form when going from acid to neutral pH. The complete absence of CIDNP at neutral pH indicates that the biradical is more completely stacked than the ground-state molecule. This gives rise to a strong exchange interaction that suppresses CIDNP at both high and low fields. A magnetic field dependent CIDNP study was made using a new falling tube system, which allows for very rapid sample transfer between the magnets used for generation and detection of the CIDNP effect, respectively. At low magnetic fields T_-–S polarization (emission for all protons) is observed with a pH dependence essentially the same as that at high field. The field-dependent CIDNP curves provide information on the open conformations of the FAD biradical present at acid pH. Evidence is obtained for at least two open conformations that interconvert slowly on the CIDNP time scale.

Flavins play an important role as coenzymes in biological systems and have therefore been studied extensively. Thus, the optical absorption and fluorescence properties of ground state, excited states, and various free radical forms have been well characterized.[2] Furthermore, the pK_a's of the acid–base equilibria of the various redox states are known. In particular in the case of the FAD molecule that contains two condensed aromatic ring systems the possibility of stacking interactions exists. Indeed, a variety of spectroscopic methods such as NMR,[3-6] UV–visible spectroscopy,[7,8] and fluorescence[7,9-12] have provided evidence for an equilibrium between stacked and unstacked states for FAD in solution.



In view of the possible biological role of the interactions between the flavin and adenine moieties of FAD we have performed a photo-CIDNP study of the coenzyme. Previously we have characterized the photo-CIDNP behavior of isolated flavin and adenine containing coenzymes such as 5′-adenosine monophosphate (5′-AMP).[13,14] Therefore, a comparison of these results with FAD, in which these moieties are covalently attached, could provide information on the equilibria between open and closed conformations. In particular, since for FAD a biradical intermediate is formed upon light irradiation, the magnetic field dependent CIDNP effect provides a sensitive probe of the distance between the radical centers[15,16] via the effect of electron spin exchange.

Preliminary observations of photo-CIDNP in FAD have been reported.[17] It was shown that the CIDNP effect has a pronounced pH dependence and furthermore depends on the polarity of the solvent, which was discussed in terms of stacking interactions between flavin and adenine ring systems. Here these observations are extended with field-dependent photo-CIDNP measurements.

We shall first present a brief review of the theory of magnetic field dependent CIDNP of biradical systems.

Theory of Biradical CIDNP

The magnetic properties of biradicals can be described by the spin Hamiltonian (in angular frequency units):

$$\hat{H} = (g_1\hat{S}_{1z} + g_2\hat{S}_{2z})\beta\hbar^{-1}B_0 - J(\tfrac{1}{2} + 2\hat{\vec{S}}_1\cdot\hat{\vec{S}}_2) + \sum_{i=1}^{N} A_i\hat{\vec{I}}_i\cdot(\hat{\vec{S}}_1 + \hat{\vec{S}}_2) \quad (1)$$

The first term describes the Zeeman interaction of the electron spins S_1 and S_2 with the magnetic field B_0, in which the CIDNP

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(16) (a) de Kanter, F. J. J.; den Hollander, J. A.; Huizer, A. H.; Kaptein, R. *Mol. Phys.* **1977**, *34*, 857-874. (b) The CIDNP time scale (i.e., the time in which the CIDNP effect is built up) is usually in the order of 10^{-9}–10^{-7} s. This is related to the inverse of the hyperfine coupling constant and for radical pairs to the time of the diffusional reencounters. For biradicals diffusion of course cannot play a role, but the nuclear-spin-dependent intersystem-crossing rate depends also on the exchange interaction.
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reaction occurs. The other symbols have their usual meaning. The second term represents the exchange interaction with the exchange integral J, and the third term the hyperfine interaction (hyperfine coupling constants A_i) with the nuclear spins I_i present in the biradical.

In both radical pairs and biradicals CIDNP airses through hyperfine-dependent mixing of triplet (T) with singlet (S) states. In the case of a freely diffusing radical pair the exchange interaction is effectively zero, and only matching of the T_0 and S states can occur. By contrast, for a triplet-generated biradical the presence of a finite exchange interaction allows T_-–S mixing, provided that the exchange integral has the usual negative sign (S state below the T states). This T_-–S mixing is most effective when S and T_- states match in energy, hence, when the condition

$$\frac{1}{2}(g_1 + g_2)\beta\hbar^{-1}B_0 = 2|J| \qquad (2)$$

holds. From the conservation of total spin angular momentum

$$\Delta m = \Delta m_1 + \Delta m_S = 0 \qquad (3)$$

it follows directly that $\Delta m_1 = -1$ (since $\Delta m_S = +1$) and, therefore, that only negative nuclear spin polarization will be observed. Note that for this T_-–S mechanism the sign of the CIDNP effect does not depend on the sign of the hyperfine coupling constants.

In general the exchange interaction in a biradical will depend on the distance between the radical centers and their relative orientation. In addition, through-bond contributions could also play a role. However, often an approximate functional form, in which only the distance is taken into account, describes the CIDNP effects reasonably well. Thus, the exchange integral can be approximated by an exponential function:

$$J(r) = J_0 e^{-\alpha r} \qquad (4)$$

By variance of the magnetic field, a maximum in the CIDNP intensity corresponds to optimal matching of T_- and S states, and by use of eq 3 and 4 this can be related to an average distance between radical centers.[16a] In principle more than one maximum may occur in the field-dependent CIDNP curves, provided the biradical has distinct conformations that interconvert slowly on the CIDNP time scale.[16b]

While biradical CIDNP is usually associated with the T_-–S mechanism, it is nevertheless possible to observe effects due to T_0–S mixing as well. Since this latter mechanism is a spin-sorting process, in which no net nuclear spin polarization is generated, it can be effective only when an "escape" route is possible such as, for instance, a fast scavenging reaction,[16a] product-dependent spin–orbit coupling,[18] or decay of "escape" polarization by nuclear spin relaxation.

Materials and Methods

For the measurement of magnetic field dependent CIDNP effects the photoreaction was carried out in an auxiliary magnet with a variable magnetic field, while the CIDNP spectrum was measured at high field in a Varian XL-100 NMR spectrometer. For this a novel sample-transfer system was designed. Special features of this system are that sample transfer takes place by gravity in a *spinning* NMR tube. This makes very rapid transfer possible (minimum time between end of light irradiation and measurement of free induction decay (FID) was 0.4 s). Figure 1 shows the experimental setup, and a detailed description of the system is given in the legend.

The pulse sequence to obtain low-field photo-CIDNP spectra, using a slightly modified Varian FT-NMR program, is shown in Figure 2. The sequence is (PD-LP-LD-PW-AT), where PD is a pulse delay, LP is the length of the light pulse, LD is a light delay in which sample transfer takes place, PW is the length of the radio frequency pulse, and AT is the acquisition time. The light pulse is obtained from the homospoil gate from the Varian XL-100 which in this experiment controls the relay of a mechanical shutter. At the end of the light pulse the descending potential of the homospoil pulse is used to trigger the relay attached to the plastic rod (9 in Figure 1) on which the rotating sample tube is located, causing the tube to fall in the lower spinner (5 in Figure 1) within 0.4 s. Generally a time of 0.5 s for LD was used. Preliminary experiments to determine the length of the light pulse giving optimal CIDNP effect for the adenine-2 proton resonance of FAD led to a value of 1.0 s.

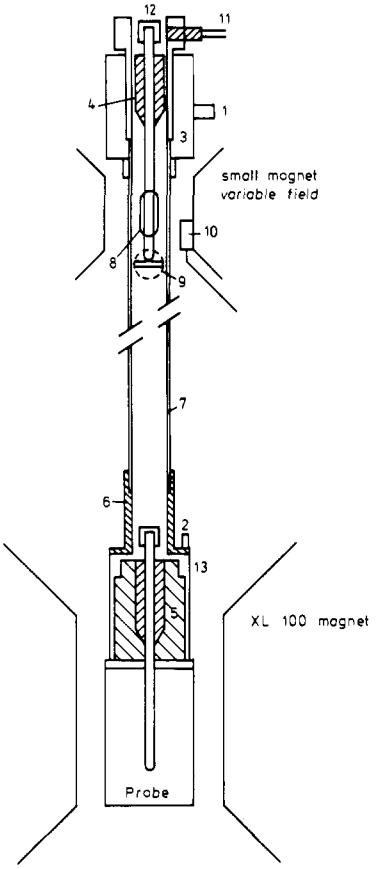

Figure 1. Falling tube system for field-dependent photo-CIDNP. The photo-CIDNP effect is generated in the small magnet (Oxford N100, stabilized power supply KSM type SCT 200-15) and detected in the Varian XL-100 magnet, stabilized by external water (^1H_2O) lock. To generate CIDNP, a sample was placed inside the small magnet and irradiated with light from a superhigh-pressure mercury lamp of 1000 W (SP 1000WQ, Philips). A $CuSO_4 \cdot 5H_2O$ (65 g/L, 5 cm) filter solution was placed between two semiconcave lenses focusing the light, via a computer-controlled shutter, onto a quartz rod guiding the beam to the sample via a hole (8) in the copper tube (7). The field strength of the small magnet was measured by using a Varian Gauss meter and a Hall element (10). The sample tube, inserted into a slim spinner (4) with a tapered end, was placed in a spinner house (3) mounted on the copper tube (7) that was connected to a proton probe of the XL-100 through an adapter (6). The sample tube with spinner (4) is rotating by compressed air coming into the spinner house through an air inlet (1), and its speed is monitored by an optoelectronic sensor (11), which receives light pulses through a reflecting strip on the tube cap (12) and is controlled by regulating the air pressure. The sample tube is kept in position by a removable plastic rod (9). After irradiation the plastic rod (9) is withdrawn by a computer-controlled relay, and the sample tube falls through the copper tube (7) into the second spinner (5) inside the spinner house (13) of the XL-100 probe. The slim spinner with its tapered end (4) fits precisely into the large one (5). The spinner rates are adjusted to a rate of about 20 Hz for both spinners. When the experiment is finished, the sample tube with the slim spinner is transferred to the upper spinner assembly (3) by compressed air through the air inlet (2).

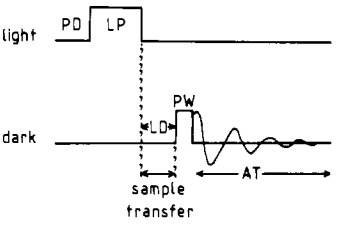

Figure 2. Pulse sequence used to obtain the CIDNP spectra at low magnetic field. The abbreviations are explained in the text.

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High-field photo-CIDNP experiments were performed as described previously.[19] Photo-CIDNP difference spectra were obtained by using a light *minus* dark cycle. At high field the samples contained 0.5 mM FAD and 10 mM citrate, except for the pH titration where no buffer was used. 2,2-Dimethyl-2-silapentane-5-sulfonate (DSS, 0.1 mM) was used as internal reference. At low field the FAD concentration was 1.0 mM, and no buffer was used. FAD and 5′-AMP were obtained from Sigma (St Louis, MO). Deuterated acetic acid, sodium chloride, and citric acid were obtained from Merck (Darmstadt, FRG). N_3-(carboxymethyl)-lumiflavin (FI) was a gift of Dr. F. Müller (Sandoz, Basel). FAD was freeze-dried three times from D_2O (99.75%, Merck); all other chemicals were used without further purification. The pH values were not corrected for the isotope effect. Samples were not degassed as it was noted that this hardly affects the CIDNP intensities.

Results

Effect of pH on FAD Chemical Shifts. Early NMR studies on FAD including the effect of solvent, concentration, temperature, and pH have been reported by several authors.[3-6] We have remeasured the pH dependence of the ^1H chemical shifts of FAD in D_2O solution under the conditions of the CIDNP experiments to correlate these results with those of the CIDNP work. Figure 3 shows the pH titration of FAD. The strongest pH dependence is observed for the adenine H8, H2, H1′, and flavin H9 and 8-methyl protons. The curves are all characterized by the pK_a of 3.6 of the adenine moiety.[20] In the low pH range the chemical shifts of FAD are similar to those of the corresponding protons of riboflavin and 5′-AMP at low pH.

FAD Photo-CIDNP at High Magnetic Field. Figure 4 shows 360-MHz photo-CIDNP spectra of a 0.5 mM solution of FAD in D_2O at pH 2.44. The difference spectrum (Figure 4a) was taken with one light–dark cycle, while the dark spectrum (Figure 4b) was a sum of 20 scans. Polarized lines are observed for the adenine H8 and H2 protons and the flavin H6 and 8-methyl protons (7-methyl protons are weakly polarized). The polarization pattern is identical with that observed for 5′-AMP and free flavin (FI) at low pH.[13]

The effect of pH on the photo-CIDNP signal of FAD is shown in Figure 5. A maximum is observed at pH $\approx$ 2.4 with a sharp decrease at both low and high pH. No change in sign occurred for the adenine H8 proton at high pH as was observed for 5′-AMP.[13]

For comparison we have drawn in Figure 5 also the pH-dependent CIDNP results for 5′-AMP and free flavin. Furthermore, the fluorescence measurements on a solution of FAD in H_2O[7] are shown scaled to the maximum of the CIDNP curve. The similarity of the fluorescence and CIDNP behavior in the low pH region is striking.

To investigate the mechanism of the CIDNP effect, we performed experiments on FAD in the presence of extra 5′-AMP at pH 1.0 and 6.5. The polarization of the 5′-AMP H8 proton is indicated in Figure 5 by the black squares 1 and 2, respectively. At pH 6.5 the flavin moiety of FAD is able to induce polarization in 5′-AMP although it was absent in FAD itself. Similarly, polarization can be induced in tyrosine, using the flavin moiety of FAD as the photoexcited dye at neutral pH (data not shown). At low pH no increase in adenine CIDNP was observed in the presence of 5′-AMP. These results show that the decrease of CIDNP in the high pH region is not due to a low yield of triplet flavins but is an inherent property of the biradical nature of the effect as will be discussed below in more detail. By contrast, in the low pH region the reduction of CIDNP intensity follows that of the fluorescence quenching and cannot be recovered by adding 5′-AMP. Therefore, it is likely to be due to radiationless decay of the flavin excited singlets with concomitant reduction of the flavin triplet yield.

Addition of sodium chloride decreases the photo-CIDNP effect contrary to the experiments with FI and 5′-AMP,[13] where no salt effect was observed. With free 5′-AMP one can reverse the sign

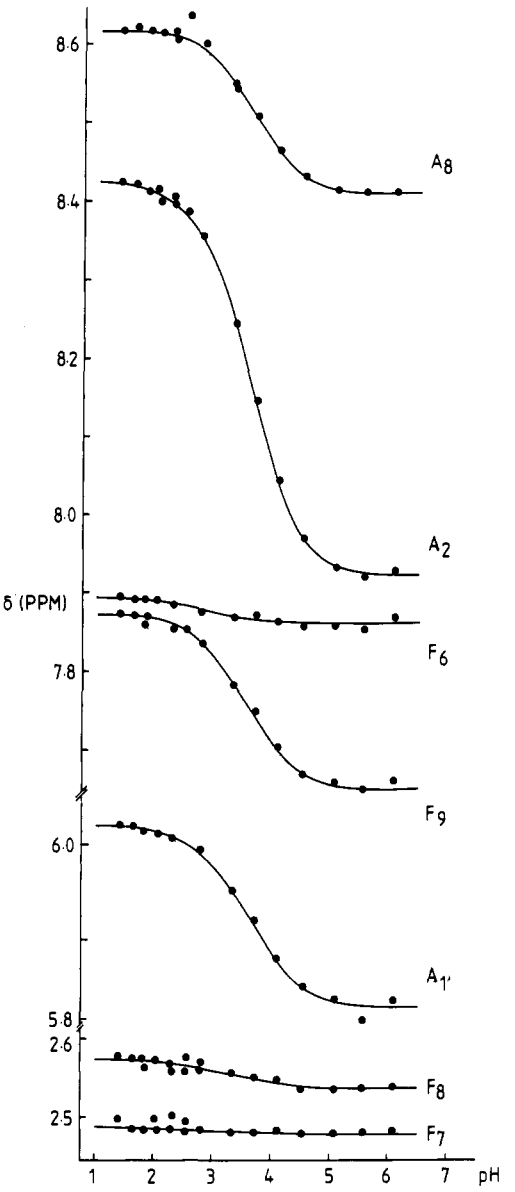

Figure 3. pH titraton of FAD. The chemical shifts of the adenine H8, H2, and H1′ protons (A_8, A_2, and $A_{1'}$, respectively) and the flavin H6 and H9 and 7- and 8-methyl protons (F_6, F_9, F_7, and F_8) are plotted as function of pH. The experiments were carried out on a 0.5 mM FAD solution in D_2O at room temperature.

of the CIDNP effect by adding buffer. This is caused by a buffer-catalyzed deprotonation of the adenine radical cation in the radical pair, generating a new radical pair with a Δg of opposite sign.[13] This sign change did not occur for FAD in the presence of acetate buffer at pH 4.8.

Low Magnetic Field Dependent Photo-CIDNP Spectra. Field-dependent photo-CIDNP provides unique information on the conformation and dynamic properties of biradical species.[15,16a,18,21-26] Thus far, in these experiments the photoreaction is carried out in a separate magnet with variable magnetic field, while detection takes place in the NMR spectrometer. The sample is then transferred either manually[15,18,21] or by using a flow system.[22-25,27] Manual sample transfer takes a time of at least

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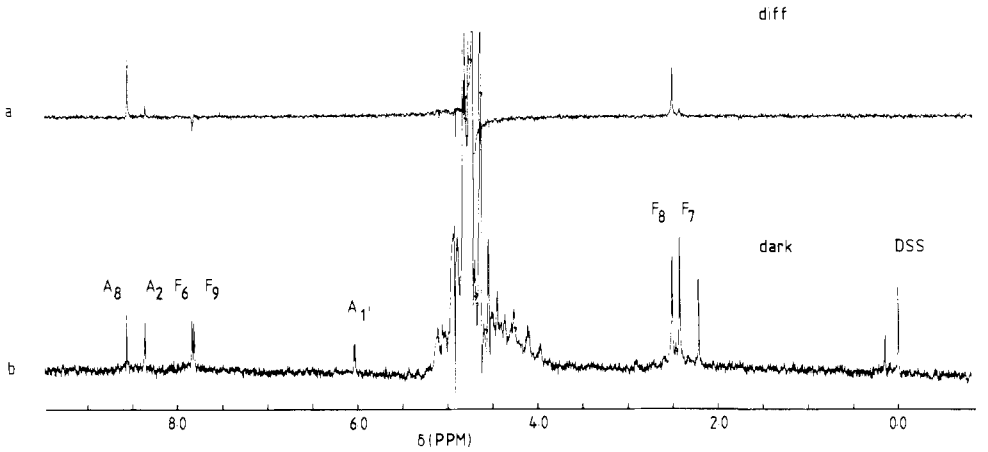

Figure 4. The 360-MHz ^1H photo-CIDNP spectrum of 0.5 mM FAD in D_2O at pH 2.44, no buffer added: (a) difference spectrum obtained by one light–dark cycle; (b) dark spectrum (20 scans). The resonances are labeled as in Figure 3. Impurities are present at 0.15 and 2.21 ppm.

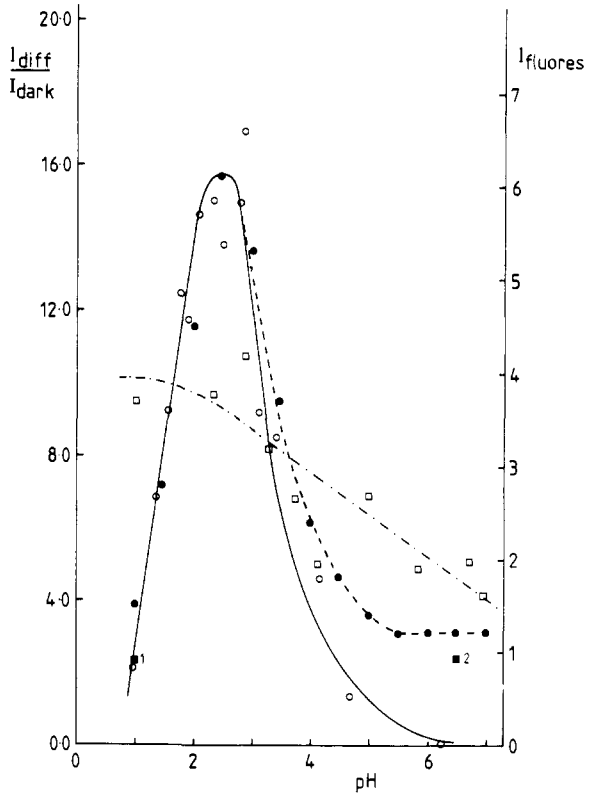

Figure 5. Photo-CIDNP and fluorescence of FAD versus pH. The intensities are plotted as the ratio of the peak heights of the adenine H8 proton in the difference spectrum and the dark spectrum. The following curves are displayed: drawn line and open circles, photo-CIDNP of 0.5 mM FAD in D_2O; dashed line and closed circles, fluorescence intensities of 1.44 μM FAD at 30° in 0.2 M sodium phosphate buffer in arbitrary units.[7] For comparison is also shown a dash-dot curve (–·–) and open squares: the photo-CIDNP intensities of free flavin and AMP (0.4 mM FI and 2 mM 5′-AMP in D_2O, unbuffered). Finally, control photo-CIDNP experiments are indicated by closed squares. FAD in the presence of additional 5 mM 5′-AMP at pH 1.0 (1) and at pH 6.5 (2).

3–5 s, and exact repeatability is a problem, especially when the relaxation time T_1 of the polarized product is short. With use of a flow system the time between the end of the reaction period and detection can be 2–3 s, but this method requires a much larger sample volume. We should finally mention the interesting possibility of detection directly in the low and variable magnetic field.[26] A disadvantage of this experiment is that the chemical shift dispersion is lost and the total polarization is measured in a single resonance line.

In the case of FAD we found that the T_1 relaxation times measured by the inversion–recovery method at 100 and 360 MHz

Table I. T_1 Values (seconds) of Protons in FAD at 100 and 360 MHz

proton	100 MHz	360 MHz	proton	100 MHz	360 MHz
8	0.13	0.80	F9	0.13	0.51
A2	0.63	2.62	F7-Me	0.19	0.83
F6	0.25	2.0	F8-Me	0.19	0.74

are rather short, in particular at 100 MHz (see Table I). For this reason we designed a falling tube system for sample transfer between the auxiliary magnet and spectrometer probe (cf. Figure 1). This system is fast (minimum transfer time is ca. 0.4 s) and requires only a small sample volume (ca. 0.3 mL as in a normal NMR tube). The system has been described in detail in the Materials and Methods section and in the caption of Figure 1. We note that a special feature is the use of a separate small sample spinner that fits in the spinner of the NMR probe. Thus, the sample is spinning at all times at the correct spinning rate. This decreases the transfer time considerably without loss of spectral resolution. We note that at low magnetic fields it is not necessary to take light *minus* dark difference spectra, because the intensity of the dark spectrum is practically zero and requires a time of several T_1's to establish.

Figure 6 shows photo-CIDNP spectra at various pH values of a 1.0 mM solution of FAD in D_2O taken with the falling tube system. The photoreaction was carried out in the auxiliary magnet at a magnetic field of 3.0 mT (30 G). In the acid pH range polarization is observed for the same nuclei (the adenine H8 and H2 protons and the flavin H6 and 8-methyl protons and the 7-methyl protons weakly) that show CIDNP at high field (Figure 4). However, the sign of the polarization is now negative for all lines, indicating that a T_–S mechanism is responsible for the effect at low field. Also, the pH profile is very similar to that observed at high field (cf. Figure 5). Different intensity ratios of the H8/H2 polarizations are observed at low versus high field due to the shorter T_1 value of H8 compared to H2, which leads to stronger loss of polarization for H8 during sample transfer.

A new feature is the relatively strong emission line at 4.7 ppm. The chemical shift position is that of the HDO line, but the origin of the polarization is not completely clear. The most likely explanation is that it is due to H–D exchange with the polarized adenine H8 proton. This proton is known to exchange with D_2O, albeit at a slow rate.[28,29] It is possible that the exchange rate is much enhanced when the adenine moiety is in its radical cation form. Of course, the H–D exchange would also occur at high field, but the HDO polarization is then masked by the strong dark intensity of this line and could not be distinguished from a subtraction artifact. An alternative assignment of the emission line

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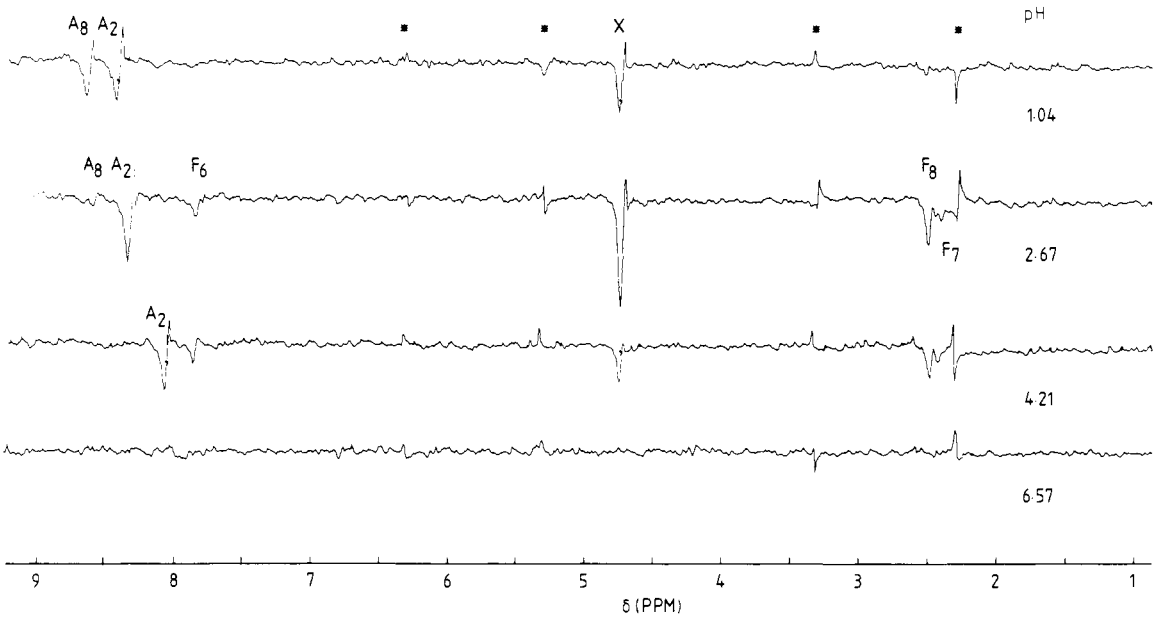


Figure 6. Low-field (3.0 mT) photo-CIDNP spectra of FAD recorded with the experimental setup shown in Figure 1. Spectra were taken at four pH values as indicated. Instrumental artifacts are indicated by asterisks. Polarization × is discussed in the text.

at 4.7 ppm to a flavin H1′ proton reported to resonate at 4.73 ppm[5] is not likely because of the lack of multiplet structure.

The magnetic field dependence of the FAD polarization was measured at pH 1.04, 2.67, and 4.21 (see Figure 7). For this the polarization of the adenine H2 proton was monitored because this proton has the longest T_1 value (0.63 s at 100 MHz; cf. Table I), so that a minimum loss of polarization occurred during sample transfer. Although the scatter in the experimental points is rather large, in particular for the measurements at pH 1.04, the curves for the different pH values seem to have similar shapes composed of two contributions, one with an emission maximum at 3.5 mT (with a width at half-height of ca 4.0 mT) and a second much broader curve with a maximum estimated at 10 mT and a width of ca 14 mT.

Discussion

The stacking interactions between the flavin and adenine moieties of FAD have been extensively studied by using NMR,[3-6] optical spectroscopy,[7,8] and fluorescence techniques.[7,9-12] In aqueous solution at neutral pH an intramolecular stacking equilibrium exists, which is shifted about 80% toward the stacked form. By changing conditions such as temperature,[3-6] the addition of organic solvents,[4,6,17] or pH[17] the equilibrium may be shifted toward open conformations. The effect of pH can be seen in Figure 3. The large shift differences between the acid and neutral forms in particular for the adenine H8 and H2 and flavin H9 and 8-methyl protons can be explained in terms of ring-current effects in a stacked conformation. The magnitude of the shifts, for instance, much larger for H9 than for H6 of flavin, is in qualitative agreement with a suggested conformation.[5] The pH-dependent curves of Figure 3 closely follow the protonation of the adenine moiety at the N_1 atom with a pK_a of 3.6. At acid pH the stacking is completely absent, as judged from the chemical shifts which are similar to those of isolated adenine and flavin species. Presumably the increased solvation of the protonated adenine counteracts effectively the hydrophobic interaction between the aromatic rings.

The Photo-CIDNP Spectrum of FAD and Its pH Dependence. The photo-CIDNP effects observed for FAD at pH 2.4 at high magnetic field (see Figure 4) are qualitatively the same as those reported earlier for 5′-AMP and free flavin FI.[13] Thus, the polarization is of T_0–S type, despite the fact that it arises from a biradical rather than a radical pair. We have previously shown[13] that the CIDNP effects originate from electron transfer to a triplet flavin moiety. Taking into account the protonation states of the various species involved (adenine, AH, pK_a = 3.6 for the N_1 atom of ADP;[20] flavin radical, FH•, pK_a = 8.4;[30] adenine cation radical, pK_a = 4.0 ± 0.2 for 5′-AMP[13]), the reactions that lead to the observed polarization at pH 2.4 can be written as in the left-hand side of Scheme I with the corresponding chemical structures of the species involved in Scheme II.

Scheme I

pH < 3.6		pH > 3.6
$F{-\!\!-}AH_2^+$		$F{-\!\!-}AH$
↓ hν	(1)	↓ hν
$^1F{-\!\!-}AH_2^+$		$^1F{-\!\!-}AH$
↓ isc	(2)	↓ isc
$^3F{-\!\!-}AH_2^+$		$^3F{-\!\!-}AH$
↓	(3)	↓
$^3\overline{F^{\cdot-}{-\!\!-}AH_2^{\cdot 2+}}$		$^3\overline{F^{\cdot-}{-\!\!-}AH^{\cdot+}}$
↓	(4)	↓
$^3\overline{FH^{\cdot}{-\!\!-}AH^{\cdot+}}$		$^3\overline{FH^{\cdot}{-\!\!-}A^{\cdot}}$
↓	(5)	↓
$^1\overline{FH^{\cdot}{-\!\!-}AH^{\cdot+}}$		$^1\overline{FH^{\cdot}{-\!\!-}A^{\cdot}}$
↓	(6)	↓
$F{-\!\!-}AH_2^+$		$F{-\!\!-}AH$

Here, $F{-}AH_2^+$ represents FAD protonated at the adenine ring. After light excitation (eq 1) to the singlet state the flavin undergoes intersystem crossing to the triplet state, (2), from which an ionic biradical is formed by electron transfer, reaction 3. We then assume that intramolecular proton transfer (eq 4) occurs. This is likely considering the difference in pK_a of the flavin radical (8.4) and that of the adenine dication radical, which is not known but must be lower than that of the monocation radical determined to be 4.0 ± 0.2.[13] We note that we consider only *intramolecular* proton-transfer reactions, since we have previously shown[13] that solvent- or buffer-catalyzed (de)protonations are slow compared to the CIDNP time scale at low buffer concentrations. A similar

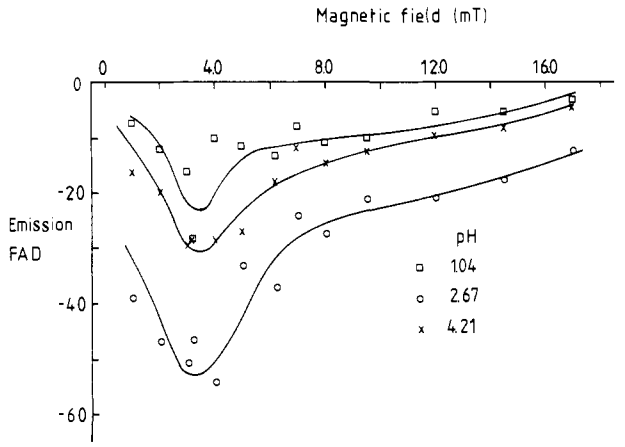


Figure 7. Magnetic field dependence of FAD photo-CIDNP. The peak area of the adenine H2 resonance of 1.0 mM FAD is shown at three pH values. Light pulse = 1 s and light delay = 0.5 s.

intramolecular proton transfer fast on the CIDNP time scale has been observed in the case of a photo-CIDNP study of pApA.[14] The next step is then intersystem crossing in the biradical (eq 5), which is at least partially induced through nuclear-spin-dependent interactions. Finally, polarized FAD is formed by back electron transfer from the singlet-state biradical (eq 6). No other polarized products are formed. Since the polarization is of T_0–S type, the spin-sorting nature of this mechanism requires that the *escape* polarization leaks away by spin–lattice relaxation and, therefore, that the biradical is rather long lived.

The pH profile of the FAD polarization (Figure 5) shows a maximum at pH 2.4 with a sharp decrease at both low and high pH. There is a striking similarity with the pH dependence of the fluorescence of FAD (also shown in Figure 5), except that in the high pH region the fluorescence levels off at about 20% of the maximum value, while the CIDNP effect vanishes. The pH-dependent CIDNP behavior of FAD is quite distinct from that of 5′-AMP and free flavin, which is also shown in Figure 5 for comparison. We note that this confirms that the effects observed for FAD are truly intramolecular in origin.

A number of experiments were performed to clarify the origin of the observed effects. Thus, addition of 5′-AMP to a FAD solution did not increase the adenine polarization at pH 1.0, while it did so at pH 6.5. It is therefore likely that the low pH decrease of both fluorescence and CIDNP effect has the same origin. The same radiationless deactivation processes that, by depletion of the flavin excited singlet, cause quenching of the fluorescence are also responsible for a lower concentration of triplets and, therefore, biradicals. Interestingly, the situation is different at high pH. The increase of CIDNP upon the addition of 5′-AMP at pH 6.5 (Figure 5) shows that there is still FAD with its flavin moiety in the triplet state around which can be trapped by 5′-AMP (and also by *N*-acetyltyrosine, experiment not shown). Apparently the steep decrease at the high-pH side is due to the biradical nature of the polarization. It seems most likely that it is associated with increased stacking interactions in the biradical at the higher pH. In these stacked conformations the biradical centers are in close proximity, resulting in a large exchange integral, which in turn renders the nuclear-spin-dependent T_0–S mixing ineffective.

The corresponding reactions at high pH (pH > 3.6) are shown in the right-hand side of Scheme I. Now we start from FAD with an unprotonated adenine moiety (indicated as F–AH). The reactions are analogous to those at low pH. Again we consider only intramolecular proton-transfer in step 4, made possible by the difference in pK_a of the flavin and adenine cation radical, 8.4 and 4.0, respectively.[31] However, in this case it leads to a biradical with neutral flavin and adenine groups for which increased stacking interactions are expected. The large exchange integral makes T_0–S mixing impossible, so that the intersystem-crossing step 5 now occurs by other mechanisms such as spin relaxation or spin–orbit coupling. It can be noted that in the case of free adenine and flavin species the neutral adenine radical would give negative polarization for the H8 proton.[13] The addition of acetate did not result in a sign change in the case of FAD, because at a pH around 5 the polarization is already very small due to the strong stacking interaction.

We can now make a further comparison with the fluorescence studies[32] and the present CIDNP results. It was shown[32] that the fluorescence quenching at neutral pH in fact reflects the conformational equilibrium of *ground-state* FAD, since the lifetime of the singlet-state flavin is too short to allow for an equilibrium to be attained. We explain the pH-dependent CIDNP behavior also on the basis of a ground-state property of FAD, in this case the pK_a of 3.6 of the adenine moiety. The reason is that now the protonation–deprotonation equilibria are slow compared to the CIDNP time scale of 10^{-7}–10^{-9} s (except for intramolecular proton transfer) so that the pH dependence is governed by the protonation state of the precursor molecule (FAD). The complete suppression of CIDNP at neutral pH indicates that the stacking equilibrium in the $FH^{\bullet}$–$A^{\bullet}$ biradical is shifted completely to the stacked form before T–S mixing can occur, in contrast to that of FAD itself where open conformations are present for about 20%.

Low-Field Photo-CIDNP. The spectra of Figure 6 show that at low magnetic fields the polarization of FAD is of T_-–S type, which is characteristic for a biradical species. The pH profile approximately follows that of the high-field CIDNP effects (Figure 5), with vanishing polarization at neutral pH. This shows that in the stacked conformation of the biradical the exchange integral is too large even for the T_-–S mechanism to become effective.

The field-dependent CIDNP effects shown in Figure 7 give information on the extended conformation of the FAD-derived biradical. In all three curves two contributions can be discerned with the maxima at 3.0 and 10.0 mT, respectively. This reflects two distinct conformations of the biradical in the open form, which apparently are the same at the three pH values 1.04, 2.67, and 4.21. It is interesting to note that this is the first example where fine structure is observed on the field-dependent CIDNP curves. This has been anticipated in a theoretical study on biradical CIDNP[16a] and is due to the fact that the conformational transitions are not fast with respect to the rate of T_-–S mixing. This phenomenon is similar to that of slow exchange in conventional NMR and ESR spectroscopy.

From fluorescence experiments rate constants for the stacking and destacking conformational transitions were measured to be around 10^9 s^{-1}.[32] Similar rate constants used in the theory of biradical CIDNP were defined in a different way so that a comparison is difficult. Nevertheless, an order of magnitude calculation can be attempted.

In the restricted diffusion model first a distribution function for the end-to-end distance of the biradical was calculated.[16a] This function is divided in segments of equal probability centered around a distance r_i. The motion of the biradical is then described by jumps between neighboring distance segments, with rates

$$W_i = D'(\Delta r_i)^{-2}$$

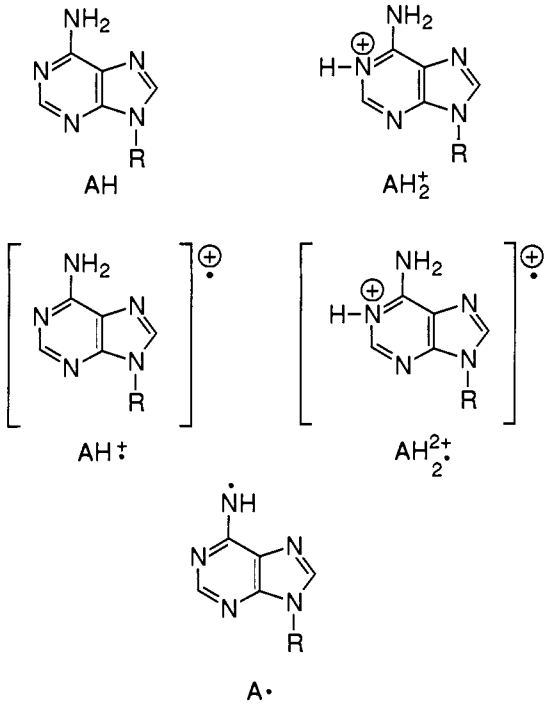
where Δr_i is the distance between the centers of two segments and D' an effective diffusion constant. Equating W_i with 10^9 s^{-1} (ref 32) and taking values for Δr_i between 1 and 3 Å, the diffusion constant D' is in the range 10^{-6}–10^{-7} cm^2 s^{-1}, which indeed corresponds to a slow-exchange type of behavior.[16a]

Qualitatively we may note that the two extended conformations of the FAD biradical corresponding to the observed maxima in the field dependence of CIDNP correspond to different average distances between the radical centers, with the most extended form belonging to the maximum at 3.0 mT. Using eq 4 and 5, we estimate these distances to be 8.8 and 9.4 Å, for the maxima at

(31) We note that a direct hydrogen atom transfer step instead of reactions 3 and 4 is unlikely, because for free 5′-AMP electron transfer as the primary step is strongly implicated.[13] Whether a similar H-atom transfer occurs at acid pH (left side of Scheme I) is not known but would not affect the conclusions.

(32) Visser, A. J. W. G. *Photochem. Photobiol.* **1984**, *40*, 703–706.

Scheme II



10.0 and 3.0 mT, respectively, on the basis of values for J_0 and α obtained from simulation of field-dependent CIDNP experiments on polymethylene biradicals.[16a,18] However, in applying eq 4 and 5 several assumptions are involved, such as the exclusive r dependence of the exchange integral and the fact that we have delocalized radicals in contrast to the ketone derived radicals used as calibration. Therefore, in view of these uncertainties the distances mentioned should be considered only as rough estimates.

Conclusions

The high-field photo-CIDNP spectrum of FAD at acid pH is similar to that obtained from 5'-AMP and free flavin FI and can be explained by T_0–S mixing in a biradical formed by intramolecular electron transfer from the adenine to the triplet flavin moiety.

The pH dependence shows a pronounced maximum at pH 2.4 and is very similar to the pH dependence of the fluorescence of FAD. At low pH the decrease in both fluorescence and CIDNP probably has the same origin, i.e., the radiationless decay of the excited singlet of flavin. At high pH the decrease in CIDNP is due to a shift from the protonated form of the biradical $FH^{\bullet}$–$AH^{\bullet+}$ to the neutral form $FH^{\bullet}$–$A^{\bullet}$ with concomitant increased stacking interactions (and therefore strong exchange interaction) in the neutral form. The pH-dependent curve follows the pK_a of ground-state FAD (protonation of the adenine moiety), because the protonation–deprotonation equilibria are slow on the CIDNP time scale except for intramolecular proton transfer, reaction 4 in Scheme I.

CIDNP experiments at low magnetic field carried out with a specially designed falling tube system showed that the polarization at low field arises from T_-–S mixing in the biradical with the same pH dependence as that observed at high field. The field-dependent CIDNP curves in the acid pH region show two maxima at 3.0 and 10.0 mT corresponding to two open (destacked) conformations of the biradical $FH^{\bullet}$–$AH^{\bullet+}$, which interconvert slowly on the CIDNP time scale.

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Intermolecular Resonance Coupling of Solute and Solvent Vibrational Modes

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Abstract: A theoretical derivation is presented of vibrational resonance coupling (Fermi resonance) between a solute Raman mode and the collective vibrational excitation of N equivalent surrounding solvent molecules. The perturbed intensities and frequencies of the coupled vibrations are shown to depend on the number of molecules in the solvation sphere. The effect of this sort of vibrational coupling on resonance Raman excitation profiles is considered, and it is shown that the effect has the potential to reveal the number of perturbing solvent molecules. The theory is applied to the analysis of previously reported resonance Raman data on cobalt porphyrin complexes.

I. Introduction

In Raman spectroscopy, intensity borrowing between vibrational modes on different molecules can result from vibrational coupling in the ground electronic states or from coupling of the excited electronic states responsible for the Raman intensity. The former effect, which is the subject of this paper, requires a close match of the vibrational energy levels and an appropriate dependence of the intermolecular potential on the normal coordinates for the two vibrational modes. Unlike intramolecular Fermi resonance,[1] which involves coupling between a fundamental and an overtone or combination vibration, intermolecular Fermi resonance can involve the interaction of fundamentals on two different molecules. The reason for the difference is that the coupling of two fundamentals is made possible by a perturbation of the type $(\partial^2 V/$

(1) (a) Fermi, E. *Z. Z. Physik.* **1931**, *71*, 250. (b) Herzberg, G. *Molecular Spectra and Structure*; Van Nostrand Reinhold: New York, 1945; Vol. II, pp 215-217.

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