Photochemically Induced Dynamic Nuclear Polarization Study on Flavin Adenine Dinucleotide and Flavoproteins†

Cees G. van Schagen, Franz Müller,* and Robert Kaptein

ABSTRACT: Flavin adenine dinucleotide and some flavoproteins ranging in relative molecular mass from 15000 to 100000 were investigated by the photochemically induced dynamic nuclear polarization (photo-CIDNP) technique. At neutral pH values, where FAD forms a strong intramolecular complex, no photo-CIDNP signal is observed. Lowering the pH values of an aqueous solution of FAD leads to the gradual appearance of photo-CIDNP signals. The pH dependence of the intensity of the signal follows that of the fluorescence quantum yield of FAD, indicating that in both types of experiments the same mechanism is operating. This is an example of where a photochemically active constituent of a complex is used to induce polarization in its partner. The flavoproteins investigated did not exhibit a photo-CIDNP signal in the absence of external free flavin so that information on aromatic amino acid residues located in the neighborhood of the bound prosthetic group could not be obtained. If intermediate biradical complexes are formed in these cases, they are not able to dissociate which is mandatory for the observation of CIDNP signals. Addition of free aromatic amino acids to solutions of flavoproteins yields the corresponding CIDNP signals of the amino acids upon excitation of protein-bound flavins. Addition of free flavins to solutions of flavoproteins leads to the observation of CIDNP signals of aromatic amino acid residues. Various flavodoxins and other flavoproteins were investigated by using as an external dye free flavins carrying different charges. In general positively and negatively charged free flavins give rise to CIDNP signals originating from different aromatic amino acid residues. It is shown that the technique allows assignments of proton resonances of aromatic amino acid residues where the sequence and preferably the three-dimensional structure of flavoproteins are known. This is demonstrated with a few flavodoxins from various sources.

It has been shown that free flavins are valuable as dyes in photo-CIDNP[1] experiments (Kaptein et al., 1978; Müller et al., 1980). The interaction of excited flavin with aromatic amino acids and the resulting photo-CIDNP spectra obtained at 360 MHz were described recently (Kaptein, 1978; Müller et al., 1980). The nuclear spin polarization recorded in photo-CIDNP spectra is induced in the following reactions given here for flavin (F) and tyrosine (TH):

$$F \xrightarrow{h\nu} {}^1F \rightarrow {}^3F \quad (1)$$

$${}^3F + TH \rightarrow \overline{FH\cdot + T\cdot} \quad (2)$$

$$\overline{FH\cdot + T\cdot} \rightarrow F + TH^* \quad (3)$$

$$2FH\cdot \rightarrow FH_2 + F \quad (4)$$

$$FH_2 + O_2 \rightarrow F + H_2O_2 \quad (5)$$

After light excitation of the flavin, rapid intersystem crossing occurs to the reactive triplet state. ^3F abstracts the phenolic hydrogen atom of TH, yielding a short lived radical pair (eq 2). Upon electron back transfer within the geminate radical pair, the tyrosine is nuclear spin polarized (TH*). This latter species leads to the observation of emission or absorption lines in the photo-CIDNP spectra. For more detailed information with respect to the reaction mechanism and an explanation for the observation of a particular photo-CIDNP spectrum for biologically relevant compounds, the reader is referred to published data (Kaptein, 1971, 1977). During the experiments the small molecular weight flavin is not destroyed by light and is probably recycled as indicated by eq 4 and 5.

The photo-CIDNP technique has been used to identify amino acid residues in proteins (Kaptein, 1978), facilitating the assignment of resonance lines in ^1H NMR spectra. Since the isoalloxazine ring system is part of the prosthetic group in flavoproteins, it was interesting to test the possibility that CIDNP spectra could be detected for amino acid residues located in the neighborhood of protein-bound, photoexcited flavin. In this study we report on the observation of photo-CIDNP spectra of flavoproteins varying in relative molecular mass from 15000 to 100000. For generation of photo-CIDNP spectra, various external small molecular weight flavin derivatives were used in order to study the possible influence of charged groups on the interaction with the polypeptide chains. In addition FAD was also studied as a function of pH and solvent polarity. Some preliminary results were published elsewhere (Müller et al., 1980).

Materials and Methods

The following flavin derivatives were used as external dyes and were synthesized according to published procedures: N^3-methyllumiflavin and N^3-carboxymethyllumiflavin (Hemmerich, 1964) and N^3-(ethylamino)lumiflavin (Vass, 1966). FAD was a product of Boehringer, Mannheim, Germany, and was used without further purification.

The flavoproteins were isolated and purified as described in the literature: old yellow enzyme from brewer's bottom yeast (Abramowitz & Massey, 1976), riboflavin-binding protein from egg yolks (Murthy et al., 1979), and D-amino acid oxidase from pig kidneys (Massey et al., 1961). The

† From the Department of Biochemistry, Agricultural University, De Dreijen 11, 6703 BC Wageningen, The Netherlands (C.G.v.S. and F.M.), and the Department of Physical Chemistry, State University of Groningen, Nijenborgh 16, 9747 AG Groningen, The Netherlands (R.K.). *Received July 1, 1981.* This work was supported in part by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO). This work was presented in part at the 6th International Symposium on Flavins and Flavoproteins, Kobe, Japan, March 1978.

[1] Abbreviations: photo-CIDNP, photochemically induced dynamic nuclear polarization; DSS, 4,4-dimethyl-4-silapentane-1-sulfonate; NMR, nuclear magnetic resonance; FAD, flavin adenine dinucleotide; Me_2SO (DMSO in figures), dimethyl sulfoxide; Lfl, lumiflavin; EDTA, ethylenediaminetetraacetic acid.

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Table I: Relative Photo-CIDNP Signal Intensity of C(6) H and C(8) CH_3 of the Isoalloxazine and C(8) H of the Adenine Moiety of FAD as a Function of the pH[a]

	rel photo-CIDNP signal intensity of		
pH	C(6) H	C(8) CH_3	C(8) H
1.3	0.4	0.4	1.9
1.6	0.6	1.3	2.7
2.1	1.6	1.8	5.0
3.3	0.9	1.2	3.2
5.7	0	0	0

[a] The intensities are given as ratios of the intensities of the signals obtained with and without illumination of the FAD solutions.

flavodoxin from *Megasphaera elsdenii* was isolated according to the procedure of Mayhew & Massey (1969). The flavodoxins from *Azotobacter vinelandii*, *Desulfovibrio vulgaris*, and *Clostridium MP* were a gift from Dr. S. G. Mayhew. Oxynitrilase and glucose oxidase were products of Boehringer, Mannheim, Germany.

CIDNP spectra were recorded on a Bruker HX 360 NMR instrument operating at 360 MHz. The instrumental setup was described earlier (Kaptein et al., 1978). The experimental conditions were as follows: 0.4-s light pulses and ±10-W laser light intensity; a delay of 7 s was used between the various 90° pulses. As compared to the published conditions (Kaptein et al., 1978), no presaturation pulse but a higher light intensity was used. Alternating "light" and "dark" free induction decays were collected and stored in the NIC-294 disk. After Fourier transformation, they were subtracted to yield the pure CIDNP difference spectrum. This procedure minimizes the effect of slow drift or temperature variations while permitting time averaging for improvement of the signal-to-noise ratio. To obtain the CIDNP spectra, 40–80 accumulations were necessary. The total volume in a flat-bottom 5-mm NMR tube was 0.2 mL. The protein concentration was about 1 mM. The final concentration of externally added flavin was usually also 1 mM. When external amino acids were added in place of small molecular weight flavins, the protein concentration was about 0.5 mM. The samples of flavoproteins with a small relative molecular mass were lyophilized several times in the presence of ^2H_2O; all other flavoproteins were dialyzed several times against a buffer solution prepared in ^2H_2O (99.95 atom %, Merck, Darmstadt, Germany). The chemical shifts are reported in parts per million relative to internal DSS.

Results and Discussion

Flavin Adenine Dinucleotide. We have shown previously (Kaptein et al., 1979) that CIDNP can be observed in the photoreaction of flavin with adenosine 5′-phosphate. In contrast, FAD in which the isoalloxazine and adenine moieties are covalently attached does not show a CIDNP signal in aqueous solution at neutral pH. However, a decrease of the pH leads to the gradual appearance of a CIDNP signal. At about pH 2.1, the signal is maximal and decreases again at lower pH values (Table I). The CIDNP spectrum exhibits absorption peaks due to the C(6) H and C(8) H of the adenine moiety and an emission line due to C(6) H of the isoalloxazine moiety of FAD. In addition a strong positive polarization due to CH_3(8) of the isoalloxazine moiety of FAD is also observed. Furthermore, a weak positive enhancement is observed at about 4.8 ppm. This latter line is probably due to the N(10) CH_2 group of FAD. Addition of about an equal volume of Me_2SO to a neutral aqueous solution of FAD yields an identical spectrum (Figure 1) as obtained in aqueous solution at pH values $\gtrsim$ 2.1. The emission line observed at about 2.1 ppm of the CIDNP spectrum of FAD (Figure 1) is probably due to a photochemical product of FAD and has not been investigated further.

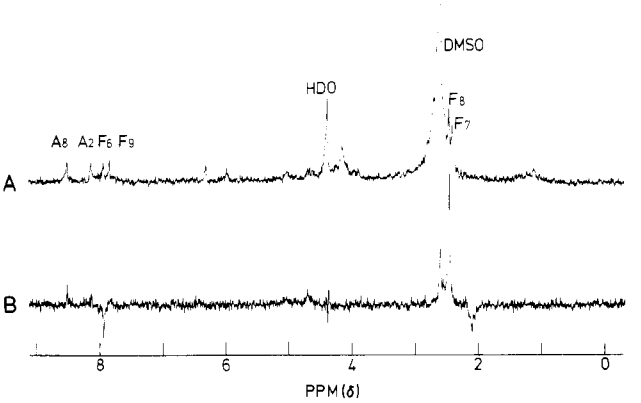

FIGURE 1: 360-MHz ^1H FT NMR spectra (25 pulses) of 3 mM FAD in a mixture of ^2H_2O and Me_2SO (3:2 v/v). (A) Dark spectrum; (B) difference spectrum between light and dark spectra. Resonance lines labeled A and F refer to the adenine and flavin moieties of FAD, respectively.

From fluorescence studies it is known that FAD forms a strong intramolecular complex in neutral aqueous solution (Wahl et al., 1974). This complex can be disrupted by either lowering the pH or adding an organic solvent to an aqueous solution of FAD (Tsibris et al., 1965). To observe a CIDNP signal, it is mandatory that the intermediate radical pair formed upon excitation can separate to distances where the exchange interaction between the electron spins is diminished. Thus our results provide independent evidence for the existence of a strong intramolecular complex in FAD and are in accordance with the fluorometric studies. In the presence of organic solvents or at acid pH values, the complex is disrupted so that the conditions for the observation of CIDNP signals is fulfilled. This indicates that the stability of the complex formed is many orders of magnitude smaller than that in neutral aqueous solution. The fact that below pH 2.1 the intensity of the CIDNP signal decreases drastically indicates that an additional mechanism is operating. One possible explanation may be the formation of a small amount of flavin radical cation which is rather stable against oxidation by molecular oxygen (Müller et al., 1981). The protonation reaction of the neutral flavin radical yielding the cation shows a pK_a of 2.3 (Land & Swallow, 1969). The drastic decrease of signal intensity below pH values of about 2 is, however, not caused by the generation of stable radicals since the width of the NMR lines is unaffected. The pH dependence of the fluorescence of all flavin compounds shows a drastic decrease of the quantum yield below pH values of about 2.0 (Tsibris et al., 1965). The explanation for this observation is that increasing concentrations of protons in solution lead to a very efficient radiationless deactivation of the excited singlet state of flavin. This very efficient process affects the intersystem crossing reaction so that the yield of flavin in the triplet state (precursor of the spin polarization reaction) decreases very rapidly with increasing hydrogen ion concentration. The curves of the pH dependence of the photo-CIDNP signal and fluorescence quantum yields of FAD coincide, indicating that in both types of experiments the same initial mechanism is operating. The generation of CIDNP signals upon light excitation of FAD is an example of where flavin as a part of a molecule is used to induce polarization within the molecule via the excited isoalloxazine moiety.

Flavoproteins. A similar, if not identical, situation can be envisaged in a flavoprotein with respect to bound flavin as

described above for FAD. In principle, the excited isoalloxazine moiety of the prosthetic group could induce polarization on neighboring amino acids. However, no flavoproteins studied so far have shown any CIDNP signals. This observation can be explained from the fact that the prosthetic group and neighboring amino acid residues in flavoproteins are not free to associate or dissociate in the same manner as the corresponding free constituents in an aqueous solution. One of the requirements to observe CIDNP signals is the formation *and* dissociation of the intermediate radical pair (cf. above). This latter reaction has to occur within the time scale of the polarization process, i.e., $\sim 10^8$ s^{-1}, which for obvious reasons is not possible in flavoproteins because the constituents are limited in their rotations and translations. It is known that flavoproteins can be reduced photochemically by electron donors, e.g., EDTA (Palmer & Massey, 1966). This reaction occurs via the triplet state of flavin (Visser et al., 1977), showing that the CIDNP active state can still be reached in flavoproteins. So that the proposal could be tested, further experiments were conducted in which the free amino acids Tyr and Trp were added to solutions of flavoproteins. Upon excitation of the protein-bound flavin, CIDNP signals of the corresponding free amino acids were easily detected. The results further indicate that the active centers, i.e., the prosthetic group, of the flavoproteins investigated in this paper are accessible to small molecular weight organic compounds.

The conditions used to excite the prosthetic group of flavoproteins did not lead to measurable destruction of the proteins, except for old yellow enzyme. This enzyme underwent an irreversible photoreaction, yielding a faint purple product. It is suggested that a cysteinyl residue is placed close to the prosthetic group which reacts with the excited flavin in an irreversible reaction.

Addition of external flavin to solutions of flavoproteins led to the generation of CIDNP signals of amino acid residues upon excitation of the flavin. Even with flavoproteins possessing a relatively high molecular mass, CIDNP signals could be observed despite the apparent large line width. For compensation for the light absorbed by the flavoprotein itself, the external flavin was added in about equimolar amounts with respect to bound flavin. The results are summarized in Table II and discussed below.

Flavodoxins. Flavodoxins are electron-transferring proteins possessing a relatively small molecular mass. This property makes them suitable for a NMR study. Such a study is currently under investigation (C. G. van Schagen and F. Müller, unpublished results). The flavodoxins contain a relatively small number of aromatic amino acids. Despite the fact that the flavodoxin from *M. elsdenii* contains only two tyrosines, four tryptophans, and four phenylalanines, the conventional NMR spectrum is still rather complex in the aromatic region, making assignment tedious (van Schagen & Müller, 1977). The photo-CIDNP technique should be very helpful in unraveling the ^1H NMR spectrum of *M. elsdenii* flavodoxin (Figure 2A). When the negatively charged lumiflavin derivative was used as an external dye, absorptions were observed in the aromatic region of the photo-CIDNP spectrum (Figure 2B). Since the protein contains no histidine residues, the absorptions must originate from tryptophan residues. An interesting observation is the fact that depending on the concentration of externally added flavin, one or two tryptophan residues were observable in the CIDNP spectrum. Thus when a concentration of external dye which was lower than that of the flavodoxin was used, only one tryptophan residue could be observed (result not shown). The CIDNP spectrum consisted of three absorption and one emission lines (Figure 2B, peaks numbered 1). Increasing the concentration of external dye produced another set of three lines (set 2 in Figure 2B) absorbing at higher fields than those of set 1. The absorption lines of set 1 exhibit chemical shift values closely related to those of free tryptophan whereas the lines of set 2 appear about 1.5 ppm toward higher field compared to those

Table II: ^1H Chemical Shifts of Amino Acid Residues Observed in Various Flavoproteins by the Photo-CIDNP Technique

flavoprotein	chemical shift (ppm)[a]	amino acid residue	dye
M. elsdenii	7.77	Trp-100	Lfl-CH_2COO^-[b]
flavodoxin	7.52	Trp-100	
(M_r 15 000)	7.07	Trp-100	
	3.32	Trp-(β-CH_2)	
	7.77	Trp-100	Lfl-CH_2COO^-[c]
	7.32	Trp-100	
	7.07	Trp-100	
	6.72	Trp-91	
	6.32	Trp-91	
	5.47	Trp-91	
	3.32	Trp-(β-CH_2)	
	7.32	Trp-96	Lfl-$CH_2CH_2NH_3^+$
	7.07	Trp-96	
	6.67	Tyr-6	
	6.32	Trp-91	
	5.47	Trp-91	
	3.14	Trp-96 (β-CH_2)	
	3.32	Trp-91 (β-CH_2)	
M. elsdenii	7.65	Trp	Lfl-CH_3 or
apoflavodoxin	7.33	Trp	Lfl-CH_2COO^-
	6.90	Tyr	
	3.30	Trp-(β-CH_2)	
	7.59	Trp	Lfl-$CH_2CH_2NH_3^+$
	7.22	Trp	
	3.51	Trp (β-CH_2)	
	3.37	Trp (β-CH_2)	
Clostridium MP	7.59	Trp-6 or -95	Lfl-$CH_2CH_2NH_3^+$
flavodoxin	7.21	Trp-6 or -95	
(M_r 15 800)	6.81	Tyr-88 (−106?)	
	3.13	Trp-(β-CH_2)	
	6.8	Tyr-5 (−106?)	Lfl-$CH_2CH_2COO^-$
Desulfovibrio	7.16	Trp-60	Lfl-$CH_2CH_2COO^-$
vulgaris	6.94	Trp-60	
flavodoxin	6.72	Tyr-98	
(M_r 16 300)	3.14	Trp-60 (β-CH_2)	
Azotobacter	7.56	Trp	Lfl-CH_3
vinelandii	7.42	Trp	
flavodoxin	7.00	Tyr	
(M_r 19 900)	3.20	Trp-(β-CH_2)	
glucose oxidase	8.0	His	Lfl-CH_3
(M_r 186 000)	7.2	His (Trp?)	
	6.9	Tyr	
old yellow enzyme	6.9	Tyr	Lfl-CH_3
(M_r 98 000)			
D-amino acid	7.93	His	Lfl-$CH_2CH_2NH_3^+$
oxidase	7.82	His	
(M_r 80 000)	7.03	Tyr	
	6.79	Tyr	
	8.0	His	Lfl-CH_2COO^-
	7.9	His	
	7.04	Tyr	
	6.79	Tyr	
	3.08	Tyr-(β-CH_2)	
oxynitrilase	6.8	Tyr	Lfl-CH_3
(M_r 75 000)			
riboflavin-binding	7.90	His	Lfl-CH_3 or
protein	7.80	His	Lfl-CH_2COO^-
(M_r 36 000)	7.15	Trp[d]	
	6.90	Tyr	

[a] Relative to internal DSS. [b] Concentration of dye was lower than that of the protein. [c] Concentration of dye was equal or higher than that of the protein. [d] The β-CH_2 group could not be observed.

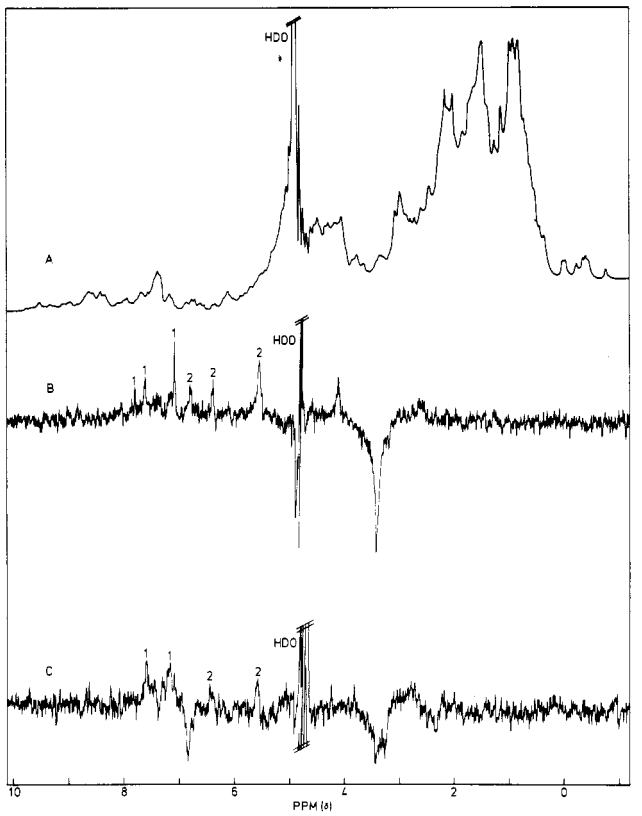

FIGURE 2: 360-MHz ^1H FT NMR spectra of 1 mM flavodoxin from *M. elsdenii* in 0.1 M sodium phosphate, p^2H 7.1. (A) Conventional spectrum. Photo-CIDNP spectra obtained in the presence of the negatively (B) and the positively (C) charged flavin as an external dye.

of free tryptophan. Moreover the width of the resonance lines of set 1 is smaller than that of the resonance lines of set 2. This suggests that the mobilities of the two tryptophan residues in question are different. It is tempting to suggest that set 1 is due to a tryptophan residue located on the surface of the protein and that the other set is due to a somewhat more buried tryptophan residue. This suggestion would explain the different widths of the resonance lines of the two tryptophan residues and the concentration dependence of the spectrum. This suggestion is supported by conventional ^1H NMR experiments where it could be shown that the resonances of set 2 belong to a tryptophan residue located in the neighborhood of the prosthetic group (paramagnetic broadening in the spectrum of the semiquinone form) (C. G. van Schagen & F. Müller, unpublished results). James et al. (1973) assigned in a similar way the proton chemical shifts at 6.32 and 7.07 ppm in the NMR spectrum of *M. elsdenii* to Trp-91. As far as the chemical shift at 6.32 ppm is concerned, the results of James et al. (1973) are in agreement with our data and interpretation. With regard to the value of 7.07 ppm, it must be concluded from our data that this resonance is due to a tryptophan residue other than Trp-91. The polypeptide chain of *M. elsdenii* contains tryptophan residues at positions 7, 91, 96, and 100 (Tanaka et al., 1973). As already mentioned above, the resonance lines at lower field (set 1, Figure 2B) are not broadened in the spectrum of flavodoxin flavosemiquinone (C. G. van Schagen and F. Müller, unpublished results). According to the structure proposed by James et al. (1973) for *M. elsdenii* flavodoxin, Trp-7 is located within a distance of 10 Å from the isoalloxazine ring of FMN, which should lead to broadening of the corresponding resonance lines in the spectrum of the semiquinone. Therefore, the low-field tryptophan resonances must be assigned to either Trp-96 or Trp-100 in *M. elsdenii* flavodoxin (see also below). The strong emission line at about 3.5 ppm in the spectrum of Figure 2B is due to the β-CH_2 group of the tryptophan residues. In addition to the resonance lines of tryptophan residues also an absorption line at about 4.5 ppm is observed in Figure 2B, which could be due to the N(10) CH_2 group of FMN, since no CIDNP signals are expected in this spectral region from aromatic amino acid residues (Müller et al., 1980). However, this latter line was not always observed in the CIDNP spectra, and its origin is therefore unclear.

Using a positively charged dye instead of the negatively charged one gave the CIDNP spectrum shown in Figure 2C. Besides the absorption lines due to tryptophan residues (Figure 2B), the spectrum contains additional lines at 6.67 ppm and at about 3.2 ppm. It should be noted that the intensity of the peaks is less than those in Figure 2B leading to the apparent loss of some peaks of the two tryptophan residues. The additional emission line at 6.67 ppm in Figure 2C originates from one of the two tyrosine residues (numbers 6 and 89) present in *M. elsdenii* flavodoxin (Tanaka et al., 1973). The resonance positions of the tyrosine residue are almost identical with those of free tyrosine (Table II). This indicates that this tyrosine residue is positioned at the surface of the protein and is, therefore, assigned to Tyr-6. The fact that the tyrosine residue is not observed in the photo-CIDNP spectrum when the negatively charged dye is used suggests the presence of negatively charged amino acid residue(s) in the neighborhood of the tyrosine residue. The sequence of *M. elsdenii* flavodoxin (Tanaka et al., 1973) shows a glutamic acid residue in position 3. The residues between the glutamic acid and tyrosine residues are isoleucine and valine. It is most likely that the tyrosine and glutamic acid residues are exposed to bulk solvent whereas the other two residues are less exposed to the solvent. This could offer an explanation for the fact that the negatively charged dye cannot interact with the tyrosine residue (repulsion due to the negative charge carried by glutamic acid residue). Tyr-89 is located close to the prosthetic group and can be expected that its chemical shifts would differ from those of free tyrosine. Therefore it is most likely that the CIDNP signal belongs to Tyr-6. The emission line at about 2 ppm in Figure 2C is, by analogy with the corresponding peak in Figure 1B, due to a photochemical product of the dye.

Although the chemical shifts of the low-field peaks of tryptophan shown in parts B and C of Figure 2 are apparently identical, it is possible that these are due to different tryptophan residues, i.e., numbers 96 and 100. The sequence shows (Tanaka et al., 1973) that Trp-96 has two negatively charged neighbors, namely, Glu-95 and Asp-98, whereas Trp-100 has positively charged neighbors, i.e., Lys-101 and Arg-103. Therefore it is reasonable to propose that the low-field resonances observed in the presence of the negatively charged dye are due to Trp-100 and those observed in the presence of the positively charged dye are due to Trp-96.

It would be interesting to obtain CIDNP spectra of *M. elsdenii* flavodoxin in the presence of a neutral dye to support the interpretation given above; i.e., Tyr-6 should be observed in such a spectrum. An attempt to obtain such spectra in the presence of N^3-methyllumiflavin failed, owing to the limited solubility of this dye in aqueous solution. The poorer quality of the spectrum of Figure 2C as compared to that of Figure 2B is a consequence of the limited solubility of the particular dye used.

The three-dimensional structure of *Clostridium MP* flavodoxin is available at 1.9-Å resolution (Burnett et al., 1974). In contrast to the related flavodoxin from *M. elsdenii*, the

CIDNP spectrum of *Clostridium MP* flavodoxin consists of only one emission line in the presence of the negatively charged dye (Table II). This emission line appears toward higher fields compared to that of free tyrosine, which can be taken as an indication that this residue is under the influence of ring-current effects from an aromatic compound, e.g., the prosthetic group of the protein. Inspection of the sequence of *Clostridium MP* flavodoxin reveals that tyrosine residues occur at positions 5, 88, and 106 (Tanaka et al., 1974). According to the X-ray structure (Burnett et al., 1974), Tyr-5 is located within a radius of 10 Å from the prosthetic group, explaining the small upfield shift of the observed resonances as compared to those of free tyrosine. Furthermore, a positively charged amino acid residue (Lys-2) is located close to Tyr-5. This fact could explain the observation that no tyrosine signal is observed in the CIDNP spectrum when the positively charged dye is used (Table II). According to the X-ray structure, Tyr-88 is within 10 Å of the prosthetic group of *Clostridium MP* flavodoxin and is in contact with bulk water. Because of its easy accessibility, Tyr-88 is a good candidate for the observed signal, but Tyr-106 cannot be excluded with certainty as the source of the signal.

The positively charged dye leads to the generation of CIDNP signals due to tryptophan and tyrosine residues of *Clostridium* flavodoxin (Table II). If the suggested correlation between the observation of a CIDNP signal of a particular amino acid residue and the charges of the neighboring amino acid residues is correct, then the tyrosine residues observed in the two sets of experiments might be due to two different tyrosine residues in the sequence. The two resonance lines observed in the CIDNP spectrum (Table II) must be assigned to either Trp-6 or Trp-95 since Trp-90 resonates at 6.39 and 5.95 ppm (James et al., 1973). The final assignment must await further NMR experiments included in our future program.

The CIDNP spectrum of *D. vulgaris* flavodoxin exhibits resonances of one tryptophan and one tyrosine residue. The sequence of the protein (Dubourdieu & Fox, 1977) contains two tryptophan residues, i.e., Trp-60 and Trp-140. Trp-60 and Tyr-98 are located in the isoalloxazine binding pocket of the protein (Watenpaugh et al., 1973). Favaudon et al. (1980) assigned the resonance at 6.82 ppm in the ^{1}H NMR spectrum of *D. vulgaris* to Tyr-98 and a resonance line at 7.12 ppm, observed in the two-electron reduced protein, to Trp-60. The chemical shifts observed in the CIDNP spectrum (Table II) are in good agreement with the values reported by Favaudon et al. (1980), and the upfield shift of the resonance as compared to the corresponding free amino acids suggests that the corresponding amino acid residues are located in the vicinity of the prosthetic group.

The CIDNP spectrum of *A. vinelandii* shows resonances due to tryptophan and tyrosine residues (Table II). The sequence of the protein contains five tyrosine and three tryptophan residues (Tanaka et al., 1977). No other structural data to aid assignment are available. Nevertheless, the resonances observed are identical with those of the corresponding free amino acids, indicating that they probably do not belong to amino residues constituting the active center of the protein.

The conventional ^{1}H NMR spectrum of *M. elsdenii* apoflavodoxin differs from that of the holoprotein at high and low fields (Figure 3A vs. Figure 2A). It is, therefore, interesting to apply the photo-CIDNP technique to the apoflavodoxin. The CIDNP spectrum obtained in the presence of the neutral dye is presented in Figure 3B and shows two absorption peaks due to tryptophan residues and two emission lines at about 7 and 3.5 ppm. The resonance line at about 7 ppm belongs to

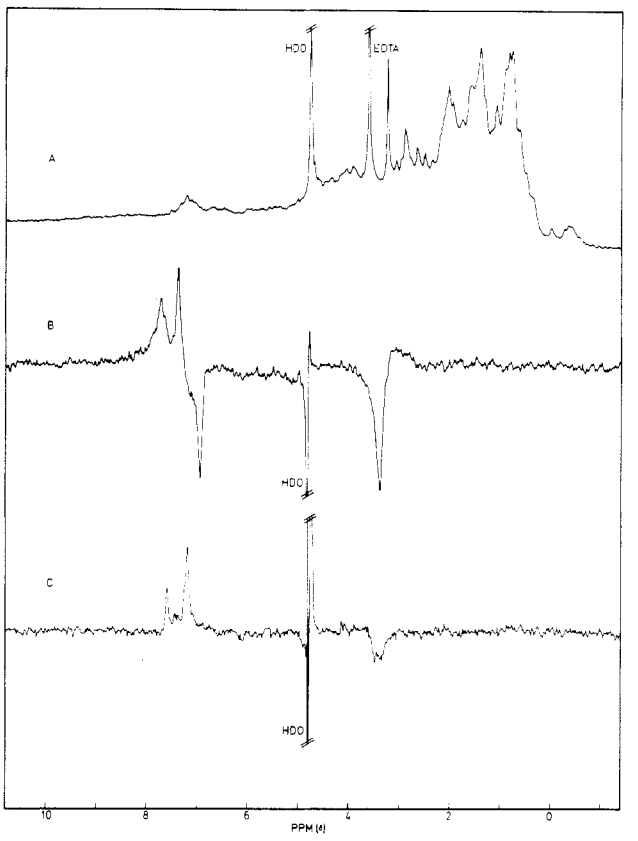

FIGURE 3: 360-MHz ^{1}H FT NMR spectra of 1 mM *M. elsdenii* apoflavodoxin in 0.1 M sodium phosphate, p^{2}H 7.1. (A) Conventional spectrum. Photo-CIDNP spectra obtained in the presence of the neutral (B) and in the presence of the positively charged dye (C).

a tyrosine residue. In the presence of the negatively charged dye, an identical CIDNP spectrum was obtained, whereas in the presence of the positively charged dye, a spectrum was observed exclusively due to tryptophan residues (Figure 3C and Table II). The absence of the high-field peaks of tryptophan (cf. Figure 2B, set 2) in the spectrum of the apoflavodoxin gives independent support to the assignment of the high-field peaks in the spectrum of the holoprotein to Trp-91. It is very difficult to assign the lines in the CIDNP spectra of the apoflavodoxin since the positively and negatively charged dyes discriminate differently between the tyrosine residues in the holo- and apoproteins. This is probably a consequence of conformational changes occurring in the flavin binding site upon preparation of the apoprotein. Nevertheless, the differences observed between the spectrum of Figure 3B and that of Figure 3C suggest that the dyes interact with different tryptophan residues. The observed chemical shifts support this suggestion (Table II). Moreover, the low-field peak (7.65 ppm) in Figure 3B is rather broad, whereas the peak at higher field (7.33 ppm) is rather sharp in comparison. This situation is reversed in Figure 3C. The fact that two tryptophan emission peaks are observed in Figure 3C, in contrast to Figure 3B, is strong evidence for this proposal.

Other Flavoproteins. With increasing molecular weight the line width in a conventional ^{1}H NMR spectrum also increases, leading to unresolved spectra. The "envelope" thus obtained does, however, contain sharp lines from amino acid residues possessing large internal freedom. It seemed of interest to explore the possibility for observing such sharp lines in proteins of molecular weights between 50 000 and 100 000. It was expected that the photo-CIDNP difference technique should make it possible to observe such lines. As demonstrated in Figure 4 for D-amino acid oxidase, this is indeed the case. It

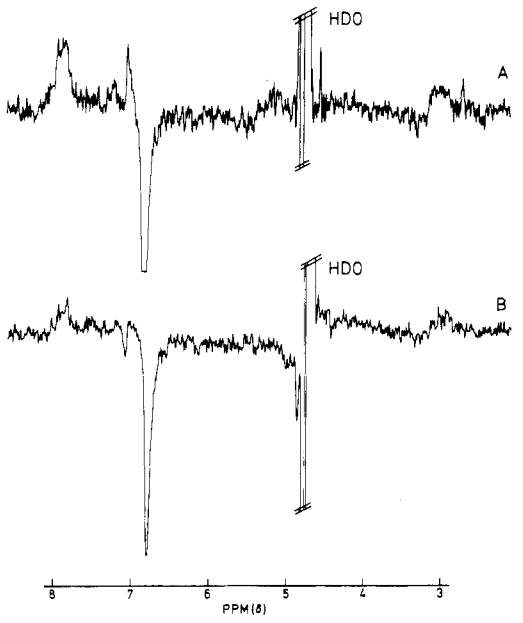

FIGURE 4: 360-MHz ^1H FT NMR spectra of 1 mM D-amino acid oxidase in 0.1 M sodium phosphate, p^2H 7.2. Difference spectra (light and dark) obtained in the presence of the negatively (A) and the positively (B) charged flavin as an external dye.

should be noted that the signal-to-noise ratio is less favorable than that for the smaller flavoproteins.

The signals observed in the CIDNP spectra of the various flavoproteins are collected in Table II. It is seen that various amino acid residues can indeed be observed in large flavoproteins. Although the resonance lines cannot be assigned yet, this information may be helpful in further studies of flavoproteins. Especially the ease of obtaining CIDNP spectra and the relatively low protein concentrations needed make this technique a valuable tool for chemical protein modification studies. The observation that differently charged dyes (cf. also Figure 4) can lead to CIDNP signals originating from different amino acid residues should make it possible to study the topography of proteins. Furthermore, it should be possible to investigate any influence of chemical modification of particular amino acids on the structure of the protein and to identify possible essential and nonessential amino acid residues. Such studies performed in the presence and absence of substrates or inhibitors may contribute to the unraveling of protein active centers.

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