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Flavodoxin from *Anabaena* 7120: Uniform Nitrogen-15 Enrichment and Hydrogen-1, Nitrogen-15, and Phosphorus-31 NMR Investigations of the Flavin Mononucleotide Binding Site in the Reduced and Oxidized States†

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ABSTRACT: Interactions between flavin mononucleotide (FMN) and apoprotein have been investigated in the reduced and oxidized states of the flavodoxin isolated from *Anabaena* 7120 (M_r ~21 000). ^1H, ^{15}N, and ^{31}P NMR have been used to characterize the FMN–protein interactions in both redox states. These are compared with those seen in other flavodoxins. Uniformly enriched [^{15}N]flavodoxin (>95% isotopic purity) was isolated from *Anabaena* 7120 grown on $K^{15}NO_3$ as the sole nitrogen source. ^{15}N insensitive nucleus enhanced by polarization transfer (INEPT) and nuclear Overhauser effect (NOE) studies of this sample provided information regarding protein structure and dynamics. A ^1H-detected ^{15}N experiment allowed the correlation of nitrogen resonances to those of their attached protons. Over 90% of the expected N–H cross peaks could be resolved in this experiment.

Flavodoxins constitute a group of low molecular weight (M_r 14 000–23 000), FMN[1]-containing flavoproteins that mediate electron transfer at low redox potential between the prosthetic groups of other proteins (Mayhew & Ludwig, 1975). In some organisms, flavodoxin is produced constitutively, while in others it is produced only under conditions of limiting iron. Flavodoxins serve as a replacement for the iron-containing protein, ferredoxin, in electron-transfer reactions (Tollin & Edmondson, 1980).

The FMN cofactor serves as the redox carrier in flavodoxins. The coenzyme can exist in three oxidation states, two of which have variable protonation states: oxidized (FMN), one electron reduced or semiquinone ($FMNH^\bullet$ or $FMN^{\bullet-}$), and two electron reduced or hydroquinone ($FMNH_2$ or $FMNH^-$). The ability for flavin-containing proteins to conduct one- or two-electron transfers permits them to mediate between one-electron- and two-electron-transfer pathways. All three redox states of flavodoxin exist in vitro, but redox reactions probably only occur between the reduced and semiquinone states in vivo (Simondson & Tollin, 1980).

The redox potentials of both transitions in FMN are altered by its association with the apoprotein. The transition from reduced to semiquinone states in flavodoxin has a midpoint potential of −400 to −500 mV, as compared to −124 mV in free FMN (Simondson & Tollin, 1980; Sykes & Rogers, 1984). Coenzyme in different redox states appears to interact differently with the apoprotein. The redox potential for the

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[1] Abbreviations: BIRD, bilinear rotation decoupling; COSY, correlated spectroscopy; FMN, flavin mononucleotide; INEPT, insensitive nucleus enhanced by polarization transfer; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser spectroscopy; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; TSP, (trimethylsilyl)propionic acid.

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reduced to semiquinone transition could be lowered by destabilizing the reduced form and/or by stabilizing the semiquinone form (Simondson & Tollin, 1980). The ability of the protein to manipulate the redox potential of its cofactor allows nature to fine tune flavodoxins to function in specific reactions.

Different types of apoprotein–FMN interactions have been proposed to account for the altered redox potential: ring strain, charge–charge, burial of a charge in a hydrophobic environment, and hydrogen bonding. If a bent conformation of reduced FMN was favored in solution, then holding the reduced coenzyme is a planar orientation would destabilize it (Massey & Hemmerich, 1980). Recent work by Moonen et al. (1984a), however, indicates that reduced FMN exists in a planar conformation in solution. Moonen et al. (1984b) further proposed that interactions between the negative charge on the FMN phosphate and the negative charge on the N1 atom of the reduced isoalloxazine ring could play a major role in altering flavodoxin redox potentials. Hydrogen-bonding changes with redox state have also been proposed to account for the altered redox potentials (Müller, 1972).

Multinuclear NMR spectroscopy provides a powerful approach to studies of properties of proteins in solution. NMR studies of larger proteins are facilitated by enriching their spin $^1/_2$ isotopes that have a low natural abundance: ^{13}C and ^{15}N. Proteins from cyanobacteria, such as *Anabaena* 7120, can be enriched economically since the organism can fix nitrogen in the form of nitrate and carbon in the form of carbon dioxide. Thus growth media consisting of $K^{15}NO_3$ and/or $^{13}CO_2$ as the sole nitrogen and/or carbon source result in proteins (and all other cell components) uniformly enriched in ^{15}N and/or ^{13}C.

As the first step in a multinuclear NMR analysis of *Anabaena* 7120 flavodoxin, one- and two-dimensional ^1H, ^{31}P, and ^{15}N NMR spectroscopies have been used here to investigate interactions between oxidized and reduced FMN and the protein. This flavodoxin has a molecular weight of 21 000 (SDS–polyacrylamide gel electrophoresis). Recently, Vervoort et al. (1986) published a comparative ^{13}C, ^{15}N, and ^{31}P NMR study of flavodoxins from four species (*Megasphaera elsdenii*, *Clostridium MP*, *Azotobacter vinelandii*, and *Desulfovibrio vulgaris*). They discussed their results in terms of hydrogen bonding and the electronic nature of the FMN binding site in oxidized and reduced flavodoxins. Results of the present study are compared and contrasted with their results. Information concerning protein dynamics and side-chain solvent accessibility was obtained from NOE and INEPT ^{15}N NMR experiments.

Materials and Methods

Growth of Cyanobacteria. Seventy-liter batches of *Anabaena* 7120 were grown on a slight modification of medium C (Kratz & Myers, 1955). Each batch of cyanobacteria was grown for 6 days under constant illumination and agitation. The temperature was maintained at 23 ± 2 °C. Since flavodoxin is produced only under iron-limiting conditions in this organism, the iron content was lowered to 60% (560 μg/L) of that of the original medium. At this iron level, about equal quantities of flavodoxin and ferredoxin were produced. To obtain flavodoxin uniformly enriched with ^{15}N, $K^{15}NO_3$ (98+% isotopic purity) was used as the sole nitrogen source.

Flavodoxin Purification. Flavodoxin was purified by the procedure of D. W. Krogmann (personal communication). Typically, 100 mg of flavodoxin was obtained from 600 g of wet cell paste. Protein fractions with A_{466}/A_{276} ratio greater than 0.14 were considered to be pure.

Chemicals. $K^{15}NO_3$ (98+%) was purchased from the Mound Facility of the Monsanto Research Corp. Other chemicals were reagent grade or better.

NMR Spectroscopy. ^1H NMR spectra (400 MHz) were obtained in the Fourier transform mode with a Bruker AM-400 wide-bore spectrometer. Samples consisted of 1.5 mM flavodoxin in 150 mM phosphate buffer at pH 7.5. The total volume was 0.4 mL in a 5-mm NMR sample tube. Flavodoxin studied in ^2H_2O was previously lyophilized and resuspended in ^2H_2O 3 times. Samples studied in H_2O contained 10% ^2H_2O to provide the field/frequency lock signal. Reduction of the flavodoxin was accomplished by adding solid sodium dithionite to an argon-flushed solution of flavodoxin in the NMR tube. ^1H chemical shifts are referenced to an internal standard of TSP (0.0 ppm). See the figure legends for additional experimental parameters.

^{15}N NMR spectra (50.68 MHz) were obtained on a Bruker AM-500 spectrometer, using a 5-mm broad-band probe. The oxidized [^{15}N]flavodoxin sample was 4.4 mM in 100 mM phosphate buffer at pH 7.5. The reduced [^{15}N]flavodoxin sample was 2.1 mM in 100 mM phosphate buffer at pH 7.0. Reduction was achieved as described above. Each sample had a total volume of 0.4 mL in a 5-mm NMR sample tube. The solvent was 90% H_2O/10% ^2H_2O. ^{15}N chemical shifts are referenced to liquid ammonia (0.0 ppm); experimental shifts were determined with respect to an external standard of $(^{15}NH_4)_2SO_4$ in 1 M HNO_3, which was assigned a chemical shift of 22.3 ppm.

One-dimensional nitrogen observe spectra were recorded of oxidized and reduced [^{15}N]flavodoxin. Proton–nitrogen couplings were collapsed by using WALTZ-16 (Shaka et al., 1983) decoupling gated on during acquisition only. The acquisition time was 393 ms, followed by a 6-s relaxation delay. The long delay was needed to diminish the NOE on lysine ε-amino resonances arising from water saturation that occurs during broad-band decoupling.

Nitrogen resonances from both oxidized and reduced [^{15}N]flavodoxin also were observed by using the INEPT pulse sequence. WALTZ-16 decoupling was used during acquisition to decouple attached protons. The relaxation delay was 6 s. The variable delay was chosen to be $^3/_8J_{NH}$ (4.17 ms), with J_{NH} assumed to be 90 Hz. This resulted in singly and triply protonated nitrogens having positive intensities, while doubly protonated nitrogens had negative intensities.

The nitrogen spectrum of oxidized [^{15}N]flavodoxin was obtained with full NOE by power-gated ^1H decoupling. Low power (0.5 W) was used to generate an NOE during the 6-s delay time, and WALTZ-16 decoupling was used during acquisition to collapse ^1H–^{15}N coupling.

A two-dimensional proton-detected nitrogen experiment was carried out with [^{15}N]flavodoxin. This experiment, which is known variably as a $^{15}N\{^1H\}$ heteronuclear multiple quantum or $^{15}N\{^1H\}$ reverse (or inverse) experiment, correlates nitrogen resonances with those of their attached protons and provides higher detection sensitivity of the heteronucleus than direct observation. The pulse sequence used was that described by Müller (1979). The experiment uses a Bruker reverse probe, in which the proton pulses are applied to the inner proton coil, while the heteronuclear pulses are applied to the outer coil. Bruker reverse electronics is also employed. Protons are pulsed from the decoupler, while the standard Bruker transmitter pulses nitrogen. The decoupler and receiver have a common reference frequency in this experiment. A BIRD (Garbow et al., 1982) sequence was employed to eliminate long-range coupling and to suppress signals from protons not coupled to a heteronucleus. ^{15}N decoupling was not carried out during acquisition, resulting in an antiphase doublet signal for each

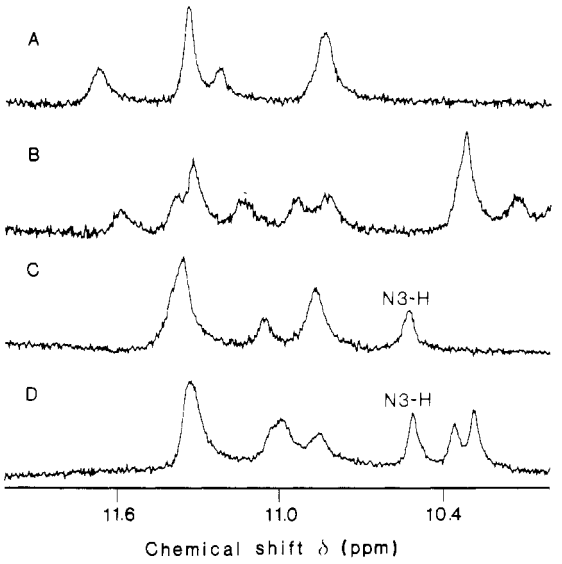

FIGURE 1: Low-field region of ^1H NMR spectra of flavodoxin in 150 mM phosphate at pH 7.5: (A) reduced in ^2H_2O; (B) reduced in H_2O; (C) oxidized in ^2H_2O; (D) oxidized in H_2O. All spectra are 128 transients, with a 60° pulse and a spectral width of 6024 Hz. The acquisition time was 680 ms, followed by a 2-s relaxation delay. Chemical shifts are referenced to (trimethylsilyl)propionic acid.

correlated resonance. Proton resonances are referenced to TSP, while nitrogen resonances are referenced to liquid ammonia. Referencing was determined by aligning the two-dimensional cross peaks with the corresponding one-dimensional spectra shown to the side and bottom of Figure 5.

^{31}P NMR spectra (161.98 MHz) were recorded on a Bruker AM-400 wide-bore spectrometer using a 10-mm broad-band probe. The sample consisted of 0.8 mM flavodoxin (oxidized or reduced) in 100 mM Tris at pH 8.0 in a total volume of 2.0 mL in a 10-mm NMR sample tube. Reduction was achieved as described above. The solvent was 90% H_2O/10% ^2H_2O. ^{31}P chemical shifts are referenced to 85% phosphoric acid (0.0 ppm).

RESULTS

^1H NMR of Reduced and Oxidized Flavodoxin. Figure 1 shows a comparison of the low-field regions of reduced and oxidized flavodoxin in H_2O and ^2H_2O. The resonance at 10.5 ppm in oxidized flavodoxin is assigned to the proton on N3 of the flavin ring via a ^1H–^{15}N correlation experiment (see below). Since this resonance was also seen after a 3-day exposure to ^2H_2O solution, it must be hydrogen bonded and/or solvent inaccessible in the oxidized state. Changes in the low-field region occur upon reduction, but the resonances have not been assigned.

^{31}P NMR on Reduced and Oxidized Flavodoxin. Figure 2 shows phosphorus NMR spectra of oxidized and reduced flavodoxin. Since only one major resonance is seen in each spectrum, it is clear that it belongs to the phosphate group of the FMN cofactor. The line widths are narrower when protons are decoupled (spectra not shown). The phosphorus chemical shift is indicative of the dianionic form of the monoester (Jones & Katritzky, 1962), but in both redox states the chemical shift is downfield slighlty from that of free FMN. It is clear from the single major resonance observed that this flavodoxin does not have an additional covalently bound phosphate group as has been observed in *A. vinelandii* flavodoxin (Edmondson & James, 1979).

Table I shows a comparison of the phosphorus chemical shifts of five flavodoxins. It can be seen that the flavodoxin from *Anabaena* 7120 has a greater change in chemical shift upon reduction than do the other flavodoxins; all five shift in the same direction (downfield) upon reduction. This may be indicative of a small conformational change occurring at the phosphate binding site. It has been demonstrated that distortions in the O–P–O bond angle of phosphate esters cause changes in the chemical shift of the phosphorus atom (Gorenstein, 1975). Changes in the electronegativity and π-bonding of the phosphorus also result in chemical shift changes (Letcher & Van Wazer, 1966).

The shoulder seen on the phosphorus resonance of the reduced flavodoxin probably arises from a small percentage of flavodoxin in the semiquinone form. This spectrum is similar to that seen for other flavodoxin solutions that have contained small amounts of the semiquinone form (Moonen & Müller, 1982). Since the semiquinone resonance is nearer to the reduced than to the oxidized resonance, the phosphate binding conformation of the semiquinone more closely resembles that of reduced flavodoxin than that of oxidized flavodoxin.

^{15}N NMR on Reduced and Oxidized [^{15}N]Flavodoxin. One-dimensional nitrogen spectra were obtained of reduced (Figure 3A) and oxidized (Figure 3B) [^{15}N]flavodoxin. The resonances from flavin ring nitrogens have been assigned in both redox states on the basis of their chemical shifts in free FMN in solution and in other flavodoxins (Moonen et al., 1986). These chemical shifts are given in Table II. N3 and

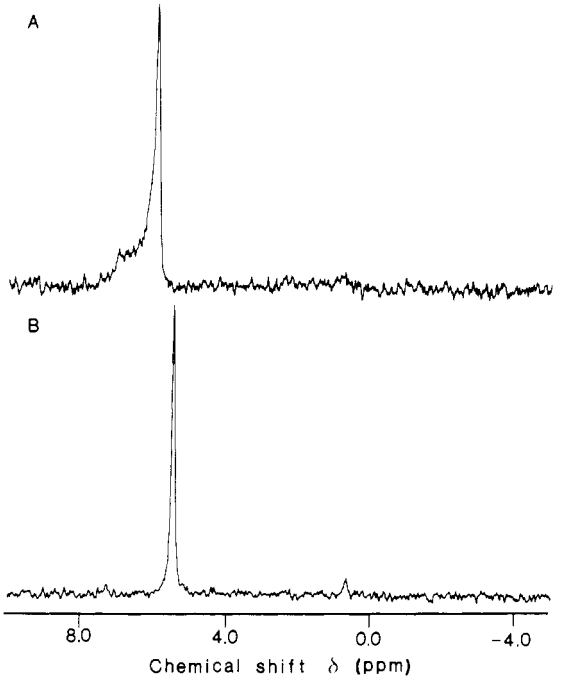

FIGURE 2: ^{31}P NMR spectra of flavodoxin in 100 mM Tris at pH 8.0 in the (A) reduced and (B) oxidized states. Both spectra are the result of 35 000 transients, obtained with a spectral width of 4854 Hz. The aquisition time was 0.84 s, followed by a 1-s relaxation delay. Chemical shifts are referenced to 85% phosphoric acid.

Table I: Comparison of Phosphorus Chemical Shifts of FMN in Reduced and Oxidized States of Several Flavodoxins

flavin	^{31}P chemical shift (ppm)[a] reduced	oxidized	flavin	^{31}P chemical shift (ppm)[a] reduced	oxidized
free FMN	5.1	5.1	*M. elsdenii*[c]	5.4	5.3
Anabaena 7120[b]	5.9	5.4	*D. vulgaris*[c]	5.5	5.4
			A. vinelandii[c]	6.4	6.3
Clostridium MP[c]	5.8	5.7			

[a] All chemical shifts are relatived to 85% phosphoric acid. [b] Present study. [c] From Vervoort et al. (1986).

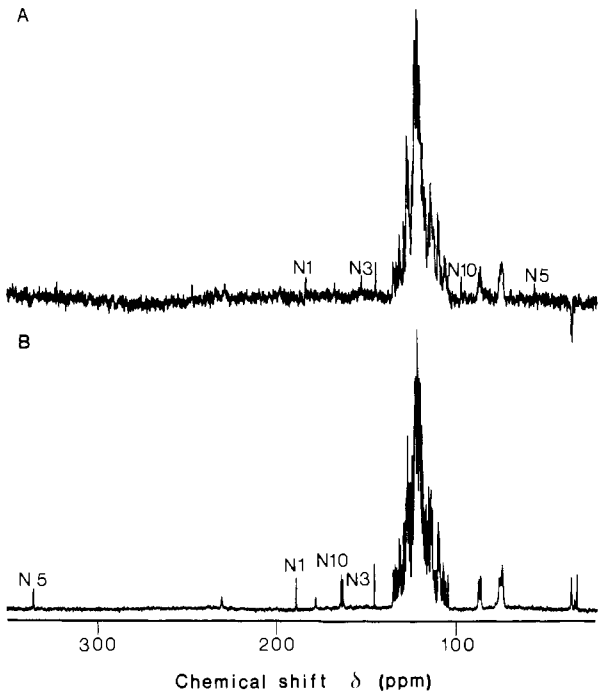

FIGURE 3: ^{15}N NMR direct observation spectra of [^{15}N]flavodoxin in the (A) reduced (6000 transients) and (B) oxidized (10 500 transients) states. A 90° pulse and a spectral width of 20 833 Hz were used. The acquisition time was 393 ms, followed by a 6-s relaxation delay. Chemical shifts are referenced to liquid ammonia.

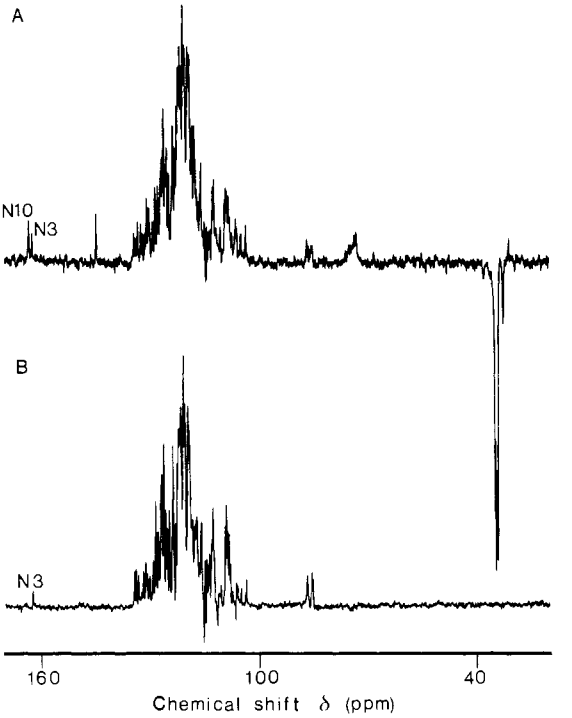

FIGURE 4: ^{15}N NMR spectra of [^{15}N]flavodoxin: (A) single pulse sequence with applied NOE (868 transients); (B) INEPT pulse sequence (1000 transients). A 90° pulse and a spectral width of 20 833 Hz were used in each experiment. The acquisition time for the INEPT experiment was 197 ms, followed by a 6-s relaxation delay. The acquisition time for the NOE experiment was 393 ms, also followed by a 6-s relaxation delay. Chemical shifts are referenced to liquid ammonia.

N10 in the oxidized protein cannot be assigned in this manner but are distinguishable with an INEPT experiment (see below).

Only two resonances are observed in the histidine region (177 and 231 ppm for oxidized and 165 and 229 ppm for reduced flavodoxin). Amino acid analysis (data not shown) suggests the presence of a single histidine residue in this flavodoxin. This indicates that both histidine resonances arise from the same side chain and that no other histidine resonances are expected. The τ- and π-resonances cannot be assigned unequivocally on the basis of their chemical shifts in free histidine (Blomberg et al., 1977) because hydrogen bonding to these nitrogens may significantly alter their chemical shifts, as has been observed for the catalytic triad histidine of α-lytic protease (Bachovchin & Roberts, 1978). Other interesting regions in both spectra are as follows: 105–135 ppm, amide backbone nitrogens and glutamine and asparagine side-chain nitrogens; 84–88 and 72–77 ppm, arginine δ- and η-side-chain nitrogens, respectively; and 31–36 ppm, lysine ϵ-amino nitrogens and N-terminal nitrogen.

Figure 3 shows that the relaxation delay was sufficient with oxidized flavodoxin to remove a negative NOE on the lysine ϵ-amino nitrogens arising from chemical exhange of saturated water protons. However, the same delay time was not sufficient to remove the negative NOE on the lysine ϵ-amino nitrogens of reduced flavodoxin.

Figure 4B shows INEPT spectra of oxidized [^{15}N]flavodoxin. Only resonances from nitrogens with attached protons whose exchange rate with solvent protons is slower than J_{NH} are observed. Thus the lysine ϵ-amino nitrogen, arginine η-side-chain nitrogen, and histidine resonances are not seen. Also, the well-resolved resonance at 147 ppm disappears. This suggests that this signal may be a main-chain proline nitrogen (no attached protons). As can be seen in Figure 4B, resonances of both positive and negative intensity are present in the region from 105 to 120 ppm. This provides a way to distinguish main-chain nitrogens (positive intensity) from asparagine and glutamine side-chain nitrogens (negative intensity) since their chemical shift regions overlap. Only one of the flavin ring nitrogen resonances of oxidized flavodoxin is seen in the INEPT spectrum. It must correspond to N3, since it is the only protonated nitrogen. This assignment allows the adjacent resonance in the one-dimensional spectrum (Figure 3B) to be assigned to N10.

Figure 4A shows the spectrum of oxidized [^{15}N]flavodoxin with an NOE applied. The lysine side-chain nitrogens show a large and negative NOE. Smaller NOE's are also seen for the N-terminal and asparagine and glutamine side-chain nitrogens. When the spectrum of reduced flavodoxin is compared with this spectrum, it is clear that only a small NOE is being manifested in the reduced spectrum. It is also seen that some, but not all, of the asparagine and glutamine side-chain nitrogens experience an NOE (compare this spectrum with the INEPT spectrum of oxidized flavodoxin in Figure 4B).

Figure 5 shows the proton-detected ^{15}N spectrum for oxidized [^{15}N]flavodoxin. Figure 5 clearly shows that almost all of the resonance overlap, present in the amide proton and nitrogen regions of the respective one-dimensional spectra, has been removed. One also sees cross peaks arising from the arginine δ-nitrogens (7.0, 85.0 ppm) and the N3 nitrogen of the isoalloxazine ring (10.5, 162.5 ppm). This latter cross peak is critical since it allows the assignment of the N3 proton of oxidized flavodoxin in Figure 1.

Discussion

Interactions between the flavin coenzyme and apoflavodoxin are manifested in the chemical shifts of the isoalloxazine ring nitrogens. Hydrogen bonding to these nitrogens changes their resonance position. Pyrrole-type nitrogens, such as N3 and N10 in oxidized and all nitrogens in reduced FMN, are directly bonded to three other atoms in a plane. This results in the

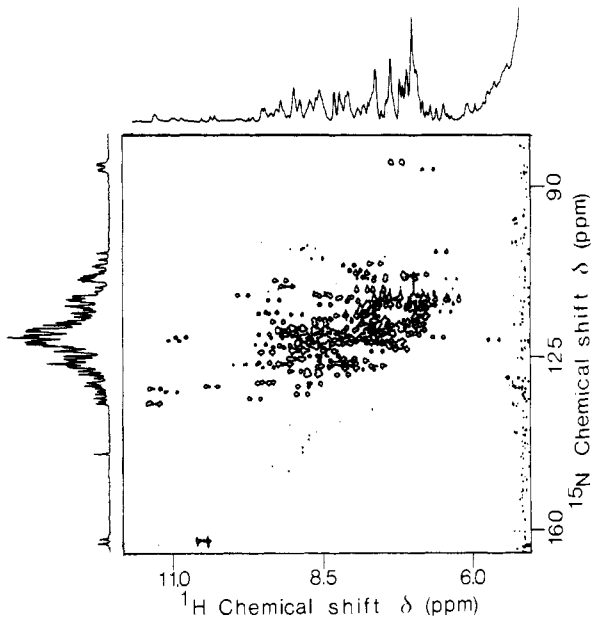

FIGURE 5: ^{1}H-detected ^{15}N correlation spectrum of oxidized [^{15}N]-flavodoxin. Ninety-degree nitrogen and proton pulses were used. The spectral width in each dimension was 4348 Hz. The positive and negative peaks of the antiphase doublets are displayed equivalently here. ^{1}H chemical shifts are referenced to (trimethylsilyl)propionic acid; ^{15}N chemical shifts are referenced to liquid ammonia.

lone pair of electrons on the nitrogen being involved in the conjugated π-system of the ring and not being accessible to the surroundings. Pyridine-type nitrogens, however, such as N1 and N5 of oxidized FMN, are bonded to only two atoms. Their lone pairs lie directly in the plane of the ring and do not contribute to the conjugated π-system. Pyridine-type nitrogens thus have exposed lone pairs and are very sensitive to hydrogen bonding; typical chemical shift changes on hydrogen bonding are 5–15 ppm upfield. Pyrrole-type nitrogens are less hydrogen bond sensitive, showing only small downfield shifts of 1–3 ppm (Witanowski et al., 1981).

Vervoort et al. (1986) have recently compared FMN nitrogen chemical shifts in various flavodoxins with those for free FMN or FMNH$^-$ in polar and for tetraacetylriboflavin (TARF) in apolar environments. The chemical shifts of the isoalloxazine ring nitrogens in these two environments represent the extremes for complete and no hydrogen bonding. Chemical shifts of flavodoxin-bound FMN tend to fall between or near these extremes, allowing the degree of hydrogen bonding to a particular nitrogen to be determined. Table II compares the isoalloxazine ring nitrogen resonances of *Anabaena* 7120 flavodoxin to those of free FMN and TARF in the oxidized and reduced states.

In flavodoxin from *Anabaena* 7120, the hydrogen bonding observed is different from any of the other flavodoxins so far studied. N1 appears to form a hydrogen bond in the oxidied state, although it is weaker than those in the other types of flavodoxin. In the reduced state, the chemical shift is dominated by the negative charge present, as in the other flavodoxins. N3 in the oxidized flavodoxin appears to form a stronger hydrogen bond than those of the other flavodoxins, as evidenced by its downfield chemical shift. It also appears to form a stronger hydrogen bond in the reduced state than in the other flavodoxins. The existence of this hydrogen bond in the oxidized state is also shown by the lack of exchange in $^{2}H_2O$ of the N3 proton (see Figure 1). N5 shows the greatest difference from the other flavodoxins. In the oxidized protein, the chemical shift is at least 6 ppm farther upfield than that of any of the other flavodoxins. Its chemical shift is almost

Table II: Comparison of ^{15}N Chemical Shifts of FMN in *Anabaena* 7120 Flavodoxin with Those of Model Compounds[a]

atom	*Anabaena* 7120[b]	FMN[c]	TARF[d]
	Oxidized Flavin		
N1	188.0	190.8	199.9
N3	162.5	160.5	159.8
N5	335.0	334.7	344.3
N10	163.5	164.6	150.2
	Reduced Flavin		
atom	*Anabaena* 7120[b]	FMNH$^-$[c]	$TARFH_2$[d]
N1	182.5	182.6	116.7
N3	151.5	149.3	145.8
N5	55.0	57.7	60.4
N10	95.6	97.2	72.2

[a] All ^{15}N chemical shifts are relative to liquid ammonia and are expressed in ppm. [b] Present study. The solvent was 100 mM potassium phosphate; the pH was 7.5 for oxidized and 7.0 for reduced flavodoxin. [c] From Vervoort et al. (1986). The solvent was 100 mM potassium pyrophosphate at pH 8.0. [d] From Vervoort et al. (1986). TARF and $TARFH_2$ are the oxidized and reduced forms of tetraacetylriboflavin, respectively. The solvent was $CHCl_3$.

the same as that for free FMN in solution. In the reduced flavodoxin, N5 is also shifted upfield by more than 6 ppm as compared to the other flavodoxins and again resonates near free FMNH$^-$ in solution.

The fact that N3 remains strongly hydrogen bonded in both redox states could be explained simply by having a functional group of the protein nearby with which to hydrogen bond. Since N3 remains protonated in each redox state, the same functional group could be hydrogen bonding in both cases. This cannot be true for N5, however. The protonation state of N5 varies with redox state. N5 is a hydrogen bond donor in the reduced state and a hydrogen bond acceptor in the oxidized state. Three explanations can be considered: (1) Hydrogen bonding in both redox states is to a group on the protein. If so, the group must be different in each redox state. The hydrogen-bonding change could be effected by changes in the electrostatic environment induced by a redox-related conformational change that results in different functional groups being located in the vicinity of N5. The X-ray crystal structure data for the smaller molecular weight *Clostridium MP* flavodoxin (Ludwig et al., 1975) indicate that only small conformational changes occur upon reduction. (2) Hydrogen bonding in one redox state is to protein and in the other redox state is to water. Since the dimethylbenzene edge of FMN is known from crystal structures to be exposed to solvent in *Clostridium MP* (Burnett et al., 1974), *D. vulgaris* (Watenpaugh et al., 1972), and *Anacystis nidulans* flavodoxin (Smith et al., 1983), this may be reasonable. Also, in *Clostridium MP* flavodoxin, the proton on N5 of the semiquinone forms a hydrogen bond to the protein (Smith et al., 1977). Thus in *Anabaena* 7120 flavodoxin, N5 may be hydrogen bonded to the protein in the reduced state and to water in the oxidized state. (3) Hydrogen bonding in both redox states is to water. N5 may be solvent accessible in both redox states.

The INEPT and NOE-applied spectra of oxidized flavodoxin give a qualitative description of the lysine, glutamine, and asparagine side-chain environments. The size of an NOE effect depends on, among other things, the correlation time of the nitrogen–proton internuclear vector. Since the correlation times for side-chain nitrogens exposed to solvent can be considerably shorter because of their increased mobility and/or increased proton exchange rates relative to those nitrogens buried within the protein, the observed negative NOE's probably indicate that the lysine side-chain nitrogens and some of the asparagine and glutamine side-chain nitrogens are on

the surface of the protein. This is also true for the N-terminal nitrogen. In the INEPT experiment, no signals are observed for the lysine side-chain nitrogens because the protons are in fast exchange (lifetime $\ll 1/J_{NH}$) with solvent. The large negative NOE's experienced by the lysine side-chain nitrogens result because their correlation time is shortened relative to that of the protein by rapid proton exchange and/or increased mobility. Further experiments will be needed to determine the contribution of each to the shortened correlation time. The INEPT experiment shows about twice as many negative peaks corresponding to asparagine and glutamine side-chain residues as does the experiment with NOE. This may indicate that those side chains that exhibit a negative NOE are on the protein surface and those that do not are in the protein interior and/or are hydrogen bonded. Since the asparagine and glutamine side-chain nitrogens are observed in the INEPT spectrum, their attached protons are not in fast exchange with solvent. Thus the small negative NOE's observed result from increased mobility relative to overall protein tumbling and not from increased proton exchange. The magnitudes of the glutamine and asparagine side-chain NOE's are smaller than the lysine side-chain NOE's because proton exchange probably contributes to the lysine NH correlation times only. It is clear that information concerning the structure and dynamics of proteins can be obtained from nitrogen NOE experiments (Smith et al., 1987).

$^1H\{^{15}N\}$ multiple quantum spectroscopy has been carried out at natural abundance with smaller proteins (Ortiz-Polo et al., 1986) and on other proteins uniformly enriched with ^{15}N (McIntosh et al., 1987). Uniform ^{15}N enrichment provides the higher sensitivity needed for larger or less soluble proteins. Excellent resolution of the N–H signals was obtained in the present work with [^{15}N]flavodoxin (see Figure 5). Over 90% of the expected peaks have been detected as discrete cross peaks.

The ^1H–^{15}N correlation results have important potential uses. (1) They provide a means of resolving overlap in the "fingerprint" region of a COSY map (Senn et al., 1987) used in the COSY–NOESY strategy of sequential assignment of backbone ^1H NMR peaks. Such overlaps are a limiting factor in the application of this strategy to larger proteins (Wüthrich, 1986). (2) In proteins the size of flavodoxin, it may be advantageous to base sequential assignments on $(^{13}C_O)_i$–$(^{15}N_\alpha)_{i+1}$ connectivities (Kainosho & Tsuji, 1982). ^1H–^{15}N correlation, in this case, provides a way to extend the backbone ^{15}N assignments to the proton spin system of each residue. (3) Another potential use for ^1H–^{15}N correlation data is in the application of isotope-directed NOE's (Griffey et al., 1985; Griffey & Redfield, 1987). Isotope-directed NOE's are expected to be useful in determining the solution structure of larger proteins and in investigating their interactions with other molecules.

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NMR Sequential Assignment of *Escherichia coli* Thioredoxin Utilizing Random Fractional Deuteriation†

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ABSTRACT: All non-proline residues except for the N-terminal dipeptide have been assigned in the 108-residue protein *Escherichia coli* thioredoxin. Central to these experiments has been the use of protein samples in which all carbon-bound hydrogen positions are substituted to 75% with deuterium by bacterial growth on partially deuteriated carbon sources and media. The dilution of the local proton density gives rise to narrower line widths with little loss in sensitivity. In addition, passive or secondary coupling to protons not directly involved in the coherence transfer process of correlation experiments is largely suppressed, thus significantly improving the resolution for side-chain couplings. Simultaneous multiresidue-type assignments have been obtained by incorporation of several amino acids with differing selective α- and/or β-deuteriation into a fractionally deuteriated background. Combined with several single residue type labeling experiments, these selective labelings have yielded direct residue type assignments for two-thirds of the protein. In addition to improved resolution, the amide to carbon-bound proton NOESY spectra offered equivalent sensitivity while the amide to amide NOESY spectra offered superior sensitivity to that observed for natural abundance samples. The resultant sequential assignment has an average number of nearest-neighbor NOE connectivities of 2.35 out of the possible 3 α-amide, β-amide, and amide-amide connectivities.

In the 5 years since the first protein NMR sequential assignment was reported (Wagner & Wüthrich, 1982), application of this technique to significantly larger proteins has proceeded rather slowly. As anticipated a number of years ago (Jardetzky et al., 1978), 10 kdaltons has remained an effective upper boundary for assignment in the absence of a high-resolution X-ray structure. Even in cases where an X-ray determination is available, progress on larger proteins has been slow although recent multiple quantum experiments are encouraging (Dalvit & Wright, 1987). The difficulty is easily rationalized in light of the fact that both resolution and sensitivity of 2D NMR analysis decrease as approximately the cube of the molecular weight. The most straightforward view of resolution in the contour plots standardly used in 2D analysis is that of total occupied area. The number of cross-peaks is generally proportional to the molecule weight. The average cross-section of individual cross-peaks is roughly proportional to the square of the molecular weight because the correlation time of the protein, and therefore the T_2 relaxation rates of most protons, increases nearly linearly with molecular weight, and hence, the effective line widths are broadened in both dimensions of the 2D experiment. The same factors cause the decrease in sensitivity since broadening of the peak implies a decrease in the peak height which determines sensitivity in the contour plot. Furthermore, the practical millimolar concentrations tend to decrease with increasing molecular weight.

Various aspects of particular experiments will modify the details of this first-order analysis. For instance, sensitivity of a NOESY experiment will be somewhat less dependent on molecular weight while that of a phase-sensitive COSY will have a somewhat higher dependence. Nevertheless, in the absence of a major increase in available field strength, extension of detailed assignment experiments to significantly larger proteins will require direct attack on the resonance number and resonance line width problems.

Two approaches to spectral editing are available: (1) pulse sequences which select subsets of all the spin coupling patterns present and (2) selective isotope labeling. Pulse sequences, such as the spin topological filtration technique (Levitt & Ernst, 1985), can in principle edit out the various different side-chain coupling systems in proteins. However, sensitivity problems resulting from the shorter T_2 relaxation times seen in moderate-sized proteins are presently a significant limitation on these techniques. Isotope labeling has proven better suited for spectral editing of these proteins. A number of protein NMR experiments using ^2H, ^{13}C and ^{15}N labeling have been reported [e.g., Crespi et al. (1968), Markley et al. (1968), Chan and Markley (1982), Griffey et al. (1985), LeMaster and Richards (1985), and McIntosh et al. (1987)]. However, these isotopic labeling experiments have not played a significant role in any of the sequential assignments previously published.

The resonance line width problem requires reduction of the proton T_2 relaxation rates. For carbon-bound protein protons (and to a lesser extent nitrogen-bound protons) relaxation is predominantly due to ^1H-^1H dipolar interaction. Isolation of individual protons by selective deuteriation has been seen to give rise to narrower resonances in a number of protein experiments. However, besides a brief 1D NMR study of 90% ^2H-labeled elongation factor TU (Kalbitzer et al., 1985), little

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