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Preparation and Characterization of ^{15}N-labeled *Desulfovibrio vulgaris* Flavodoxin in the Presence of Macromolecular Crowding Agents Using 2D ^{1}H-^{15}N NMR Spectroscopy

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Abstract

Protein structure and function are typically investigated using dilute purified protein in aqueous buffer solutions. However, proteins have evolved to function in the crowded environment of the cell. Characterizing structure and function in conditions that mimic this crowded environment may thus provide a more relevant description of proteins. Crowded conditions in the cell can be mimicked by using long chain polysaccharides such as Ficoll and Dextran. The goal of this work was to characterize the impact of crowding agents on the structure of *Desulfovibrio vulgaris* flavodoxin using NMR spectroscopy. Flavodoxin is a small electron transport protein that has 147 amino acid residues and a molecular weight of 18 kDa. It contains four α-helices, five parallel β-sheets, and a flavin mononucleotide cofactor bound to two surface loops. The protein functions as an electron transfer agent in anaerobic, aerobic and photosynthesis bacteria. Since the electron transfer properties are dependent on structure, determining the structure of the protein in conditions that mimic physiological conditions may provide insights into its function that have not been identified in previous studies. ^{15}N-labeled *D. vulgaris* flavodoxin was expressed in *E. coli* grown using ^{15}N-labeled media. FPLC was used to purify the flavodoxin. First, an anion exchange column was run using an NaCl gradient for elution. The resulting yellow fractions containing flavodoxin where then concentrated and run on a gel filtration column. Gel electrophoresis indicated that the protein was >95% pure and suitable for NMR studies. One-dimensional ^{1}H and 2D ^{1}H-^{15}N NMR spectra were acquired first on the ^{15}N-labeled flavodoxin in dilute solution. Comparisons with similar spectra in the literature validated that the protein was identical and correctly folded. Then a series of 2D ^{1}H-^{15}N NMR spectra were collected with increasing amounts of Ficoll 70, up to a final concentration of 400 mg/mL, at temperatures of 298 K and 308 K. Only very slight changes were observed in the resonances at either temperature, indicating that this crowding agent does not appreciably impact the structure of the protein. Similar titrations are currently in progress using Dextran as well as other proteins as crowding agents.

Keywords: Flavodoxin, Molecular Crowding, NMR Spectroscopy

1. Background

The cell interior is very crowded with a large variety a proteins and other macromolecules[1,2]. When the structure of an individual protein is studied, however, the protein is typically purified away from these conditions. The question arises then as to whether the structure of the purified protein is the same as the structure of the protein in crowded conditions. It is important to determine the structure of proteins in conditions that most closely reflect the physiological conditions because structure determines the function of proteins. Crowding agents such as Ficoll, a hydrophilic polysaccharide that takes up space in solution, can be used to mimic proteins and other macromolecules consistent with physiological conditions.

The protein of interest for this research is flavodoxin. It is a low molecular protein that contains a single noncovalently bound flavin mononucleotide (FMN) prosthetic group. Flavodoxin from *Desulfovibrio vulgaris* contains 147 amino acid residues[3,4]. It functions as an electron transport protein and its redox potentials are determined by protein-FMN interactions. If these interactions change as a result of cellular crowding, then the redox potentials will change, too. The structure of *D. vulgaris* flavodoxin[5] is shown in Figure 1.

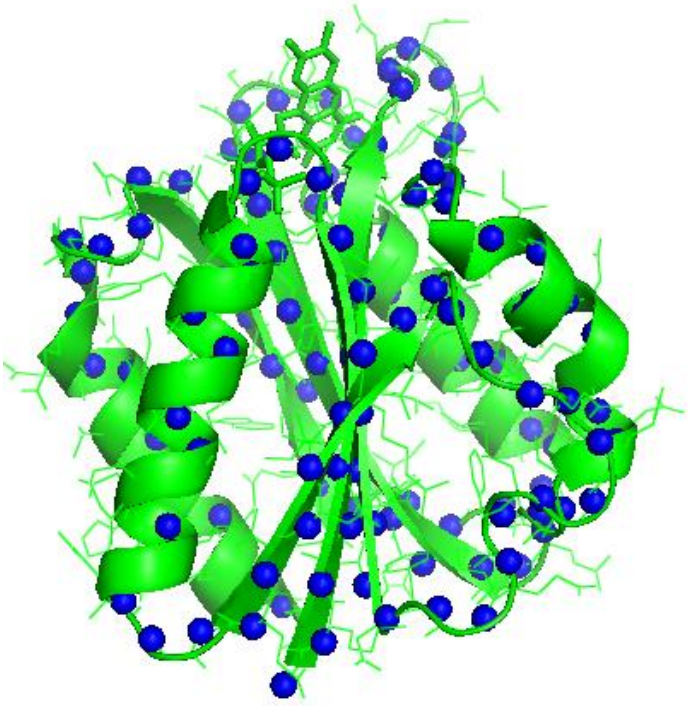

Figure 1. Ribbon diagram of the crystal structure of *D. vulgaris* flavodoxin.

Figure 1 was generated from the coordinates in Protein Data Bank file 2fx2 using PyMOL[6]. The four α-helices and five β-sheet strands are represented by coils and arrows, respectively. The FMN cofactor is shown in stick format. Peptide bond amide NH groups are indicated by colored blue spheres.

2. Materials and Methods

Flavodoxin was expressed in *E. coli* cells grown on either LB rich or M9 minimum media to prepare unlabeled and ^{15}N-labeled protein, respectively. In the latter, $^{15}NH_4Cl$ provided the only source of nitrogen and thus all flavodoxin produced was ^{15}N-labeled throughout, including the FMN. Cells were harvested by centrifugation and ruptured using a combination of freeze/thaw, lysozyme treatment, and sonication. Cellular debris and membranes were removed by centrifugation at 20K rpm followed by ultracentrifugation at 50K rpm resulting in a supernatant solution containing a mixture of all cellular proteins. Flavodoxin was separated and purified from the other proteins using Fast Protein Liquid Chromatography (FPLC). Two types of columns were used in sequence. The first column was an Uno Q column. This strong anion exchange column separates proteins based on their overall charge (flavodoxin is a negatively-charged protein). A NaCl gradient was used to elute the flavodoxin. The second column was a Sephadex column. This size exclusion column separates proteins based on molecular weight. Yellow fractions from the Uno Q column were pooled, concentrated, loaded onto the Sephadex column, and eluted with buffer. Purity of the samples from each column was analyzed by SDS-PAGE.

After elution from the Sephadex column the protein was concentrated and transferred into 100 mM phosphate buffer with 35 mM potassium chloride at pH 6.8. NMR samples contained 270 μM flavodoxin and 10% deuterium oxide. A Ficoll stock solution was prepared that contained 500 mg/mL Ficoll PM 70 (Sigma) dissolved in 100 mM phosphate buffer with 35 mM potassium chloride at pH 6.8. The NMR sample with Ficoll was prepared by

combining flavodoxin and Ficoll stock solutions to obtain 270 μM flavodoxin and 300 mg/mL Ficoll, along with 10% deuterium oxide. NMR data sets were collected on a Bruker AvanceIII 500 MHz NMR spectrometer.

3. Results

SDS-PAGE was used to follow the purification process as shown in Figure 2. Numerous proteins remaining after the Uno Q column (lane 2) were removed by the Sephadex column (lane 1) resulting in a single protein band with

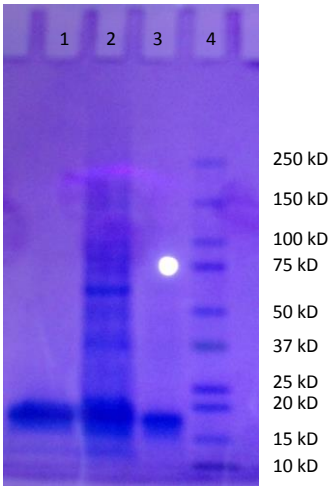

Figure 2. SDS-PAGE analysis of FPLC purification steps.

Figure 2 lane contents are as follows: Lane 1, pooled fractions from Sephadex column; Lane 2, pooled fractions from Uno Q column; Lane 3, flavodoxin standard; Lane 4, protein molecular weight standards.

the correct molecular weight. The 1D ^{1}H NMR spectrum of the purified unlabeled flavodoxin is shown in red in Figure 3. The resonance dispersion observed is indicative of a folded protein. For example, the resonances in the negative ppm range will only occur if a protein is folded. A denatured protein would also have a simpler spectrum of mostly overlapping resonances

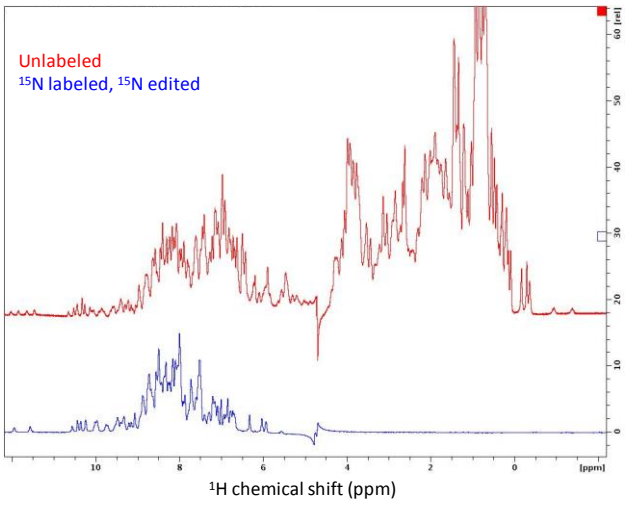

Figure 3. Comparison of 1D ^{1}H NMR spectra in the absence of Ficoll.

In Figure 3, the 1D ^{1}H NMR spectrum of unlabeled flavodoxin is shown in red while the ^{15}N-edited 1D ^{1}H NMR spectrum of ^{15}N-labeled flavodoxin is shown in blue.

The ^{15}N-labeling strategy provides three advantages compared to the unlabeled protein: spectra are simplified, the ^{15}N chemical shift adds a 2nd dimension, and the large ^{1}H resonances from the Ficoll can be eliminated. Figure 3 compares the 1D ^{1}H NMR spectrum of the unlabeled flavodoxin (red) with the ^{15}N-edited 1D ^{1}H NMR spectrum of the ^{15}N labeled flavodoxin (blue). In the edited spectrum, resonances are observed for only protons directly attached to nitrogen atoms. Figure 4 spreads this information into two dimensions. Each circular correlation observed in the 2D ^{1}H-^{15}N NMR spectra represents an individual NH group in the protein. This spectrum matches that in the literature[7,8] and provides absolute proof that the flavodoxin is properly folded. The locations of the NH groups of each peptide bond are colored blue in the three-dimensional crystal structure of flavodoxin shown in Figure 1. The amide NH groups are spread throughout the structure of the protein. Since the positions of the correlations in Figure 4 depend on the structure of the protein, they provide a means to monitor structural changes induced by crowding agents.

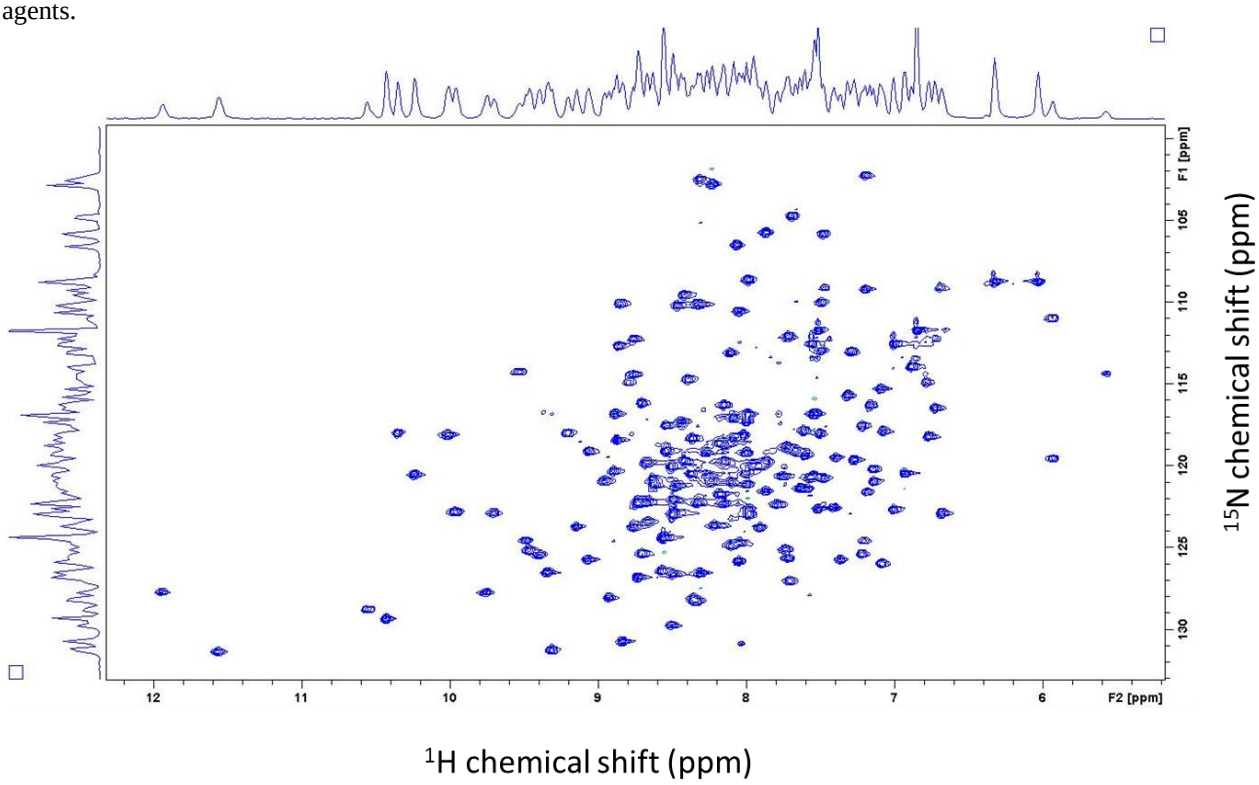

Figure 4. Two-dimensional ^{1}H-^{15}N NMR spectrum of ^{15}N-labeled flavodoxin.

The 2D spectrum shown in Figure 4 was collected in the absence of Ficoll. The 1D ^{1}H and ^{15}N projections are shown along the top and side of the 2D spectrum.

The 1D ^{1}H NMR spectrum of the Ficoll-containing sample is shown in Figure 5 (red). Since Ficoll is a polymer, its repeating structural units give rise to a very large ^{1}H signals. With ^{15}N-editing, however, the Ficoll proton signals are easily eliminated since these protons are not bonded to nitrogen. By contrast, the desired flavodoxin amide NH signals are readily observed as indicated by the blue spectrum in Figure 5. A region of the 2D ^{1}H-^{15}N spectra of flavodoxin in the presence of Ficoll is shown in Figure 6. All ^{1}H-^{15}N correlations, including that for FMN, experienced a small, systematic chemical shift change in the same direction. However, several of the ^{1}H-^{15}N correlations were observed to shift to a greater extent. Two of these correlations that appear in this region of the spectrum are indicated by the red circles in Figure 6. The correlations with the greatest Ficoll-induced shifts correspond to the backbone NH groups of Ala29, Asp63, Glu66, Gly103, and Gly128. The locations of these backbone NH groups in the three-dimensional crystal structure of flavodoxin are shown in Figure 7. None of these residues are located in regions of regular secondary structure that comprise the rigid scaffold of the protein.

However, four of the residues are located in the flexible loop regions that interact with FMN. This may suggest that this region of the protein is more susceptible to structural changes induced by interactions with other proteins. Understanding these structural changes may be important since physiological electron transfer involves protein-protein interactions that may have similar effects and thus may alter redox potentials.

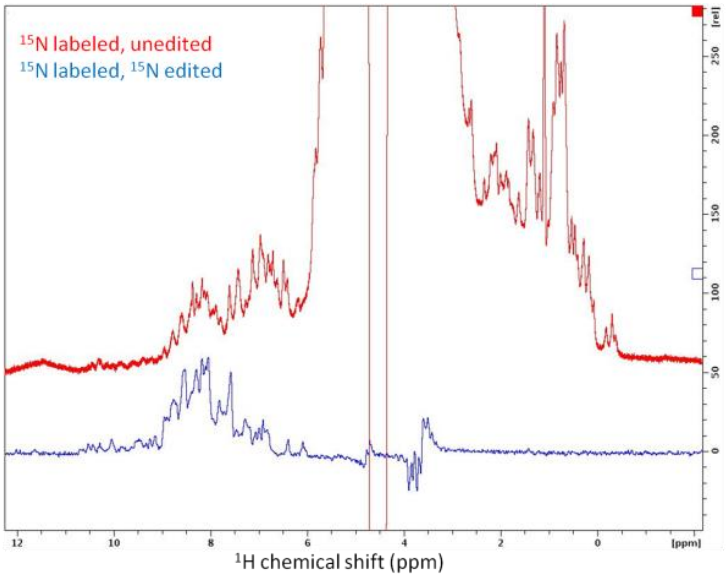

Figure 5. Comparison of 1D ^{1}H NMR spectra in the absence of Ficoll.

Both spectra shown in Figure 5 were collected on ^{15}N-labeled flavodoxin in the presence of Ficoll. The unedited spectrum is shown in red while the ^{15}N-edited spectrum is shown in blue.

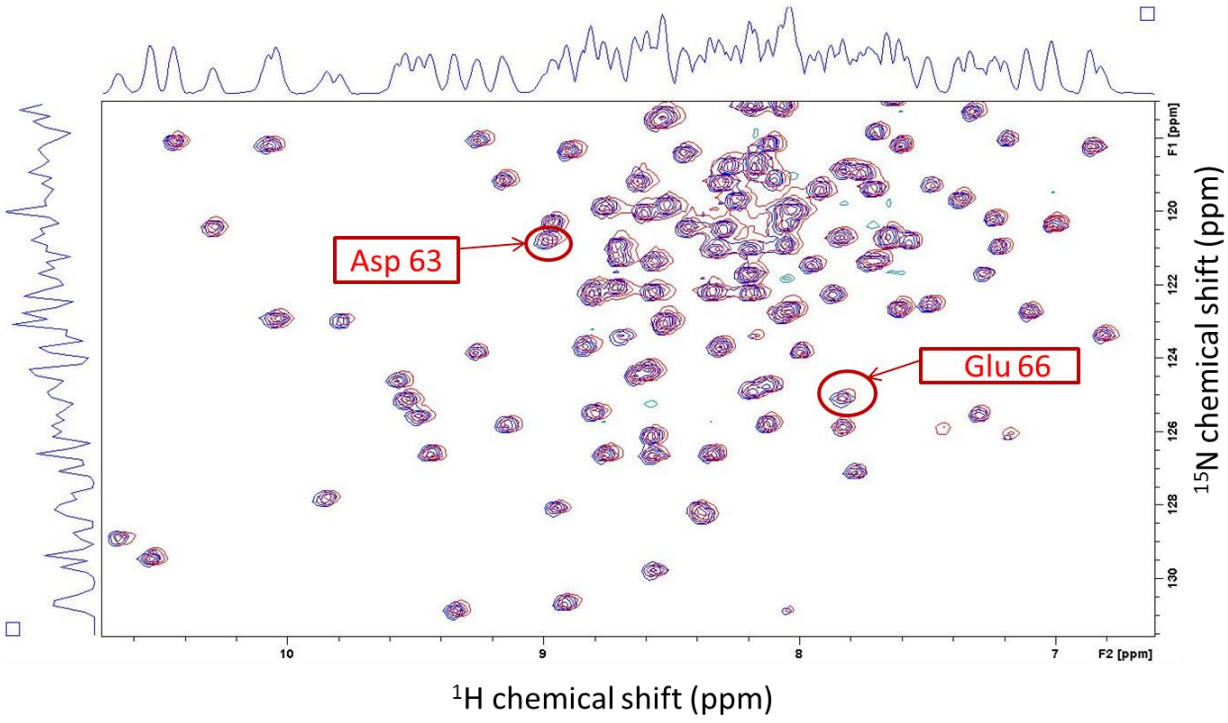

Figure 6. Overlay of 2D ^{1}H-^{15}N NMR spectra of ^{15}N-labeled flavodoxin.

In Figure 6, the spectrum shown in blue was collected in the absence of Ficoll while the spectrum shown in red was collected in the presence of Ficoll. The spectral region shown is a subset of that shown in Figure 4.

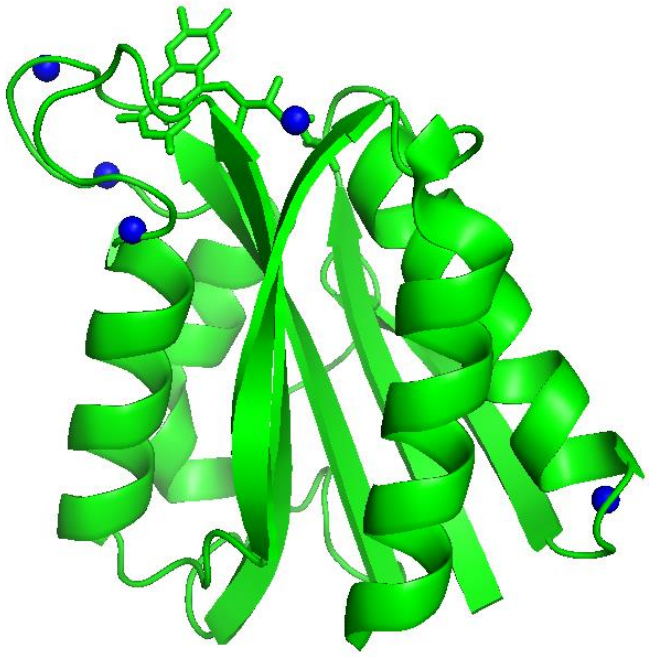

Figure 7. Ribbon diagram of the crystal structure of *D. vulgaris* flavodoxin.

Figure 7 is shown in the same orientation as Figure 1. The five residues with the most-shifted amide NH correlations in the presence of Ficoll are indicated by colored blue spheres.

4. Conclusions

The ^{15}N-labeling strategy provided an excellent means to acquire NMR spectra in the presence of large concentrations of crowding agents. More importantly, the 2D ^{1}H-^{15}N NMR spectra provided a sensitive method to monitor potential structural changes in any part of the protein. With the first crowding agent used, Ficoll, non-systematic chemical shift changes were observed for five NH groups in the protein. Interestingly, four of these NH groups are located close to the FMN binding site. These four changes in structure may impact the function of the protein since they may alter the protein-FMN interactions responsible for establishing the redox potential. The 5th NH group, Ala29, is located in a turn at the bottom of an α-helix and may reflect crowder-induced movement of this α-helix.

Future research will involve measuring other NMR parameters in addition to chemical shift in order to explore and better quantitate crowder-induced structural changes. This will include determining ^{1}H-^{15}N to ^{2}H-^{15}N exchange rates in the absence and presence of crowding agents that may be indicative of changes in hydrogen bonding interactions[9]. 3D NMR spectra will also be acquired in order to measure crowder-induced changes in ^{1}H-^{1}H distances derived from NOE intensities. Future research will also explore the effect of other artificial crowding agents such as Dextran and other proteins, and, ultimately, the cell contents themselves prepared as a cell-free extract. The efficient means to express and purify ^{15}N-labeled flavodoxin described here will leverage these studies.

5. Acknowledgments

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6. References

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