Nuclear Magnetic Resonance Studies of Two-Iron-Two-Sulfur Ferredoxins. 3. Heteronuclear (^{13}C, ^{1}H) Two-Dimensional NMR Spectra, ^{13}C Peak Assignments, and ^{13}C Relaxation Measurements†

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ABSTRACT: Carbon-13 enrichment of ferredoxin, an electron transport protein in photosynthesis, was achieved by isolating the protein from *Anabaena variabilis* cells grown on [20% ^{13}C]CO_2 as the sole carbon source. This level of enrichment permitted a number of novel experiments to be carried out with 140-mg samples of the protein. Subspectra of the aromatic (115–160 ppm) region were generated, corresponding to ^{13}C resonances from either nonprotonated or singly protonated carbons. Heteronuclear (^{13}C, ^{1}H) two-dimensional NMR chemical shift correlation spectra obtained of the low- and high-field regions permitted facile cross assignment of a large number of ^{13}C and ^{1}H resonances from directly bonded C-H pairs in the protein. Single-frequency ^{1}H decoupling experiments were used to assign several nonprotonated carbon resonances. T_1 relaxation measurements of assigned carbon resonances indicate that the electron relaxation time of reduced ferredoxin, which is more paramagnetic, is shorter than that of oxidized ferredoxin, which is less paramagnetic.

Because of the low natural abundance of ^{13}C (1.1%), a ^{13}C spectrum of ferredoxin (about 160 mg of protein in 2.5 mL) with adequate signal-to-noise requires about 24 h of signal averaging. Proteins enriched in ^{13}C have been isolated from *Anabaena variabilis* cells grown with [20% ^{13}C]CO_2 as the sole carbon source (Chan & Markley, 1983). The ferredoxin II isolated was enriched with ^{13}C well above natural abundance, but below the level at which ^{13}C–^{13}C coupling of labeled neighboring carbons complicates the spectrum (London et al., 1975). After enrichment, the same amount of protein yielded a spectrum in only 15 min with a signal-to-noise ratio comparable to that of the spectrum of ferredoxin at natural abundance obtained in 24 h. Enrichment made the following experiments feasible: heteronuclear two-dimensional NMR spectroscopy of the protein (Chan et al., 1982; Chan & Markley, 1982); ^{13}C NMR studies of interactions between ferredoxin and ferredoxin-NADP$^+$ oxidoreductase (Chan et al., 1983b); and accurate T_1 relaxation measurements of individual carbon resonances.

We present here detailed assignments of ^{13}C peaks in the 110–160 ppm region of the ferredoxin spectrum. The peaks in this region correspond to aromatic ring carbons and to the guanidinium carbon of the single arginine (Arg^{42}; Chan et al., 1983a). The region can be separated readily into two subspectra: one containing only the nonprotonated carbon resonances and the other containing only the singly protonated carbon resonances (Chan et al., 1982). This spectral region was the easiest to interpret since the presence of fewer peaks in this region than in the carbonyl or aliphatic regions makes it better resolved and since the numerous ^{1}H NMR peak assignments in the aromatic region of oxidized and reduced ferredoxin (Chan & Markley, 1983; Chan et al., 1983a) could be used to make cross assignments based on heteronuclear two-dimensional NMR spectroscopy or coherent selective proton decoupling. The T_1 relaxation values for several resolved single-carbon resonances were determined and are discussed in light of their distance from the iron-sulfur center.

Experimental Procedures

Materials and Sample Preparation. *Spirulina maxima* ferredoxin (natural abundance) and *Anabaena variabilis* ferredoxin (uniformly enriched to 20% in ^{13}C) were isolated in our laboratory (Chan & Markley, 1983). Sources of chemicals used and procedures for preparing samples have been described (Chan & Markley, 1983).

Heteronuclear (^{13}C, ^{1}H) Two-Dimensional Chemical Shift Correlation Spectroscopy. The spectra were obtained on the NT-200 spectrometer at a ^{13}C frequency of 50.3 MHz and a ^{1}H frequency of 200 MHz, with a 20-mm ^{13}C variable temperature probe. The sample was placed in a spherical microcell (Chan & Markley, 1983). The pulse sequence used was adapted from that of Morris & Hall, (1981). A total of 128 sets of FIDs[1] each of 1K data points was obtained in a total time of 50 h. Data for the aromatic region and aliphatic region were collected in two separate experiments. This was possible because all ^{1}H–^{13}C coupled aromatic resonances are located in the low-field regions of the ^{1}H and ^{13}C spectra while the ^{1}H–^{13}C coupled aliphatic resonances are located exclusively in the high-field regions. It is desirable to obtain separate two-dimensional correlated spectra for each region in order to allow for better digital resolution in the proton chemical shift dimension. For each experiment the carbon carrier frequency was located at the center of the ^{13}C region (127 ppm for the low-field data set and 41 ppm for the high-field data set). Quadrature detection was employed. For the low-field

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[1] Abbreviations: 2Fe·2S*, the iron-sulfur center consisting of two iron atoms and two inorganic sulfur atoms; FID, free induction decay; pH*, pH meter reading of a solution in $^{2}H_2O$ uncorrected for the deuterium isotope effect; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; Me_4Si, tetramethylsilane; APT, attached proton test; ul, uniformly labeled.

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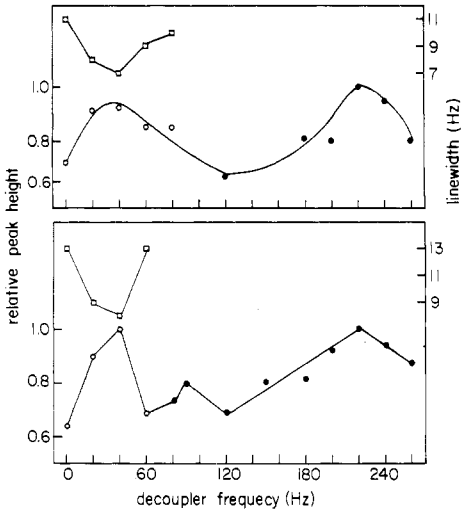

FIGURE 1: Model study of the effect of selective proton decoupling of tyrosine C_δ-H and C_ϵ-H groups on the C_ζ resonance. The experiments were run on the NT-470 (^{13}C frequency, 118.2 MHz) with a saturated solution of tyrosine in 80% 1 M NaO^2H and 20% ^2H_2O. The ^{1}H decoupler frequency was changed by 20-Hz intervals around the region of the C_δ-H and the C_ϵ-H of tyrosine. Two levels of decoupler power were used in separate experiments: $\gamma H_2/2\pi$ = 31 Hz for the curves in the lower box and 77 Hz for curves in the upper box. The C_ζ resonance appeared as a singlet (open circles) when the C_δ-H (40 Hz on the arbitrary scale) was irradiated and as a triplet (closed circles) when the C_ϵ-H (220 Hz) was irradiated. The peak heights (open and closed circles) and singlet line widths (squares) of the C_ζ resonances are plotted against the decoupler frequency.

Table I: Summary of the Heteronuclear Two-Dimensional Chemical Shift Correlated NMR Results for the Aromatic Region of Oxidized *Anabaena variabilis* Ferredoxin II[a]

cross-peak no. from Figure 2a	chemical shift[b] ^{13}C δ	^{1}H δ	assignment[c]
1	136.5	7.80	H_ϵ^{93}
2	135.7	8.16	H_ϵ^{16}
3	132.3	7.11	$Y_{\delta 1,\delta 2}^{83}$
4	131.6	7.11	$Y_{\delta 1,\delta 2}^{99}$
5	131.5	7.30	$Y_{\delta 1,\delta 2}^{76}$
6	130.8	6.77	$Y_{\delta 1,\delta 2}^{35}$
7	130.9	7.10	
8	130.6	6.35	
9	130.4	7.09	
10	130.4	7.55	Phe/$Y_{\delta 1,\delta 2}^{35}$
11	130.1	7.11	
12	129.5	7.25	
13	129.3	7.20	
14	128.3	7.10	
15	119.1	6.94	H_δ^{16}
16	116.6	7.17	H_δ^{83}
17	116.9	6.76	$Y_{\epsilon 1,\epsilon 2}^{76}$
18	116.3	6.76	$Y_{\epsilon 1,\epsilon 2}^{99}$
19	116.7	6.41	$Y_{\epsilon 1,\epsilon 2}^{83}$
20	116.2	6.31	$Y_{\epsilon 1,\epsilon 2}^{35}$

[a] The pH of the sample was 7.06. [b] The error in the chemical shift was ±0.02 ppm in the ^{1}H domain and ±0.1 ppm in the ^{13}C domain. [c] Based on the ^{1}H NMR assignments (Chan & Markley, 1983; Chan et al., 1983a).

data set the proton frequency was set at 6 ppm from DSS, and a 700-Hz proton window was covered giving 5.5-Hz resolution in the proton frequency domain. For the high-field data set the proton frequency was set at 5.8 ppm from DSS, and a 1200-Hz proton window was covered giving 9.3-Hz resolution. In presenting the contour data, it is convenient to refer to the one-dimensional spectra. For the low-field region, the normal decoupled ^{13}C spectrum is plotted on one side, and the subspectrum (Chan et al., 1982) of ^{13}C peaks that are coupled to protons is plotted on the other side. The one-dimensional ^{1}H 470-MHz NMR spectrum is plotted at the bottom. For the low-field region, the normal decoupled ^{13}C spectrum is plotted on the bottom, and the 470-MHz ^{1}H spectrum is plotted on the side. Since we have determined that the ^{1}H spectra obtained at 200 and 470 MHz are similar, we have used the better resolved spectra obtained at the higher frequency.

Assignment of Tyrosine C_ζ Resonances by Coherent Selective Proton Decoupling. A trial experiment was carried out with tyrosine by using the NT-470 spectrometer (^{13}C frequency, 118.22 MHz). Two different power levels ($\gamma H_2/2\pi$ = 77 Hz and 31 Hz) were used. In each case, the decoupler was stepped through the proton frequency range in intervals of 20 Hz. The results are summarized in Figure 1. All intensities were normalized to that of the carbonyl carbon resonance. At either level of decoupling power, the plot of the relative peak height vs. decoupler frequency showed two maxima: a sharper one around the frequency of the C_δ-H and a broader one around the frequency of the C_ϵ-H. When the C_ϵ-H resonance was irradiated, the C_ζ resonance appeared as a triplet; when the C_δ-H resonance was irradiated, the C_ζ resonance became a singlet (using a line-broadening factor of 3 Hz). It was found that the maximum at the C_δ-H frequency is sharp at the lower decoupling power; hence, a decoupling power of around $\gamma H_2/2\pi$ = 30–40 Hz yields optimal selectivity. In protein spectra, the maxima at C_ϵ-H frequencies were found to be smaller, as may be explained by the larger line widths of protein peaks in general and the triplet nature of this particular peak.

A proton spectrum was obtained by using the decoupler coil of the ^{13}C probe as the transmitter/observer coil, and the proton resonance frequencies for the tyrosine doublets were determined directly. Then the decoupler frequency was stepped through the proton frequency range in such a way that ^{13}C spectra were obtained with the decoupler on-resonance and 20 and 40 Hz off-resonance in each direction from each tyrosine proton resonance. A decoupling power level corresponding to a $\gamma H_2/2\pi$ value of 37 Hz was used (approximately 5 J; Grutzner, 1972). The free induction decays were then Fourier transformed with a line-broadening factor of 5 Hz (the natural line width of the resonance) to optimize the signal-to-noise ratio.

T_1 Relaxation Measurements. Spin–lattice relaxation times (T_1) of the resolved single-carbon resonances were determined for both oxidized and reduced ferredoxin. The inversion–recovery pulse sequence (delay–180°–pulse–τ–90°–pulse–acquisition) was used. High decoupling power (10 W) was applied during acquisition, and low decoupling power (2 W) was used the rest of the time.

Results

Heteronuclear Two-Dimensional NMR. A total of 20 contour peaks was resolved in the aromatic region of oxidized *Anabaena variabilis* ferredoxin II (Figure 2a). These are listed in Table I along with the assignments of ^{13}C peaks that could be made on the basis of ^{1}H NMR assignments (Chan & Markley, 1983; Chan et al., 1983). The contour plot allows the assignment of the well-resolved C_ϵ resonances of His^{16} and His^{93} and locates the C_δ resonance of His^{93} and the C_δ and C_ϵ resonances of four tyrosine residues which are not well resolved in the one-dimensional ^{13}C NMR spectrum. The heteronuclear two-dimensional NMR spectrum of reduced

Table II: Summary of the Heteronuclear Two-Dimensional Chemical Shift Correlated NMR Results for the Aromatic Region of Reduced *Anabaena variabilis* Ferredoxin II[a]

chemical shift[b]		
^{13}C δ	^{1}H δ	assignment[c]
136.1	8.12	H^{16}_{ϵ}
136.6	7.88	H^{93}_{ϵ}
132.3	7.10	$Y^{83}_{\delta 1,\delta 2}$
131.6	7.30	$Y^{76}_{\delta 1,\delta 2}$
131.3	7.06	$Y^{99}_{\delta 1,\delta 2}$
130.8	6.75	$Y^{35}_{\delta 1,\delta 2}$
134.4	7.25	
130.6	6.44	
130.4	7.56	Phe/$Y^{25}_{\delta 1,\delta 2}$
130.3	7.25	
130.0	6.92	
128.0	7.10	
129.4	7.14	
119.3	6.92	H^{16}_{δ}
116.7	7.25	H^{93}_{δ}
116.4	6.45	$Y^{83}_{\epsilon 1,\epsilon 2}$
116.3	6.33	$Y^{35}_{\epsilon 1,\epsilon 2}$
116.7	6.75	$Y^{99}_{\epsilon 1,\epsilon 2}$
116.7	6.75	$Y^{76}_{\epsilon 1,\epsilon 2}$

[a] The pH* of the sample was 7.2. [b] The error in the chemical shift was ±0.02 ppm in the ^{1}H domain and 0.1 ppm in the ^{13}C domain. [c] Based on the ^{1}H NMR assignments (Chan & Markley, 1983; Chan et al., 1983a).

Table III: Assignments of the Protonated Carbon Resonances in the Aromatic Region of Oxidized and Reduced *Anabaena variabilis* Ferredoxin II

chemical shift δ from Me_4Si			
oxidized	reduced	remarks	assignment
136.49	136.54	*a*	H^{93}_{ϵ}
135.99	136.15	*a*	H^{16}_{ϵ}
116.6	116.7	*b*	H^{93}_{δ}
119.14	119.27	*a*	H^{16}_{δ}
131.40	131.50	*a*	$Y^{76}_{\delta 1,\delta 2}$
132.12/131.62	132.19	*a*	$Y^{83}_{\delta 1,\delta 2}$
132.12/131.62	131.25	*a*	$Y^{99}_{\delta 1,\delta 2}$
130.70	130.71	*a*	$Y^{35}_{\delta 1,\delta 2}$
116.7	116.4	*b*	$Y^{83}_{\epsilon 1,\epsilon 2}$
116.3	116.6	*b*	$Y^{76}_{\epsilon 1,\epsilon 2}$
116.3	116.6	*b*	$Y^{99}_{\epsilon 1,\epsilon 2}$
116.2	116.3	*b*	$Y^{35}_{\epsilon 1,\epsilon 2}$

[a] The chemical shifts of these resonances were obtained from one-dimensional ^{13}C NMR spectra. The pH* of the ferredoxin sample was 7.32 (oxidized) and 7.31 (reduced). The temperature was 24 °C. [b] These resonances were not resolved in the one-dimensional NMR spectrum; therefore, their chemical shifts had to be obtained from the heteronuclear two-dimensional chemical shift correlation spectrum, and the values are less precise. The pH* of the ferredoxin solution was 7.06 (oxidized) and 7.20 (reduced).

ferredoxin (not shown) was used to generate the assignments shown in Table II.

Contour plots of similar quality were obtained for the aliphatic region (Figure 2b,c). Detailed assignments in this region must await the ^{1}H NMR assignments. It is clear that the contour plots will permit the cross assignment of a great number of the aliphatic carbon resonances.

Carbon Subspectra. The aromatic region of the ^{13}C spectrum of oxidized and reduced ferredoxin was decomposed into two subspectra (Chan et al., 1982; Patt & Schoolery, 1982), one containing resonances of only the protonated carbons (Figure 3, Table III) and the other containing resonances of only the nonprotonated carbons (Figure 4). A similar decomposition is not possible with the aliphatic region because it contains carbons with one, two, and three attached protons.

Table IV: Assignments of the Nonprotonated Carbon Resonances in the Aromatic Region of Oxidized and Reduced *Anabaena variabilis* Ferredoxin II[a]

chemical shift δ from Me_4Si		
oxidized	reduced	assignment[c]
157.13	157.35	R^{42}_{ζ}
156.15	156.24	Y^{76}_{ζ}
155.95	155.92	Y^{83}_{ζ}
155.46	155.27	Y^{99}_{ζ}
154.88	154.90	Y^{35}_{ζ}
154.82	154.82	Y^{25}_{ζ}
132.12	132.26	H^{16}_{γ}
136.22	136.19	H^{93}_{γ}
138.36	138.34	F^{66}_{γ}
138.01	137.94	F^{3}_{γ}
137.22	137.64	F^{39}_{γ}
130.69[b]	130.71	Y_{γ}
	130.17	
129.37	129.45	
128.37	128.48[b]	
128.02		

[a] The pH* of the ferredoxin solution was 7.32 (oxidized) and 7.31 (reduced). The temperature of the samples was 24 °C. [b] Two-carbon peak intensity. [c] The C_{ζ} resonances of Tyr^{25} and Tyr^{83} were misassigned (reversed assignment) in the preliminary communication of this work (Chan et al., 1981).

Assignments of Nonprotonated Carbons. Sixteen resonances are expected from nonprotonated carbons in the 125–160 ppm region: C_{ζ} of Arg^{42}, C_{ζ} and C_{γ} of five tyrosines, C_{γ} of three phenylalanines, and C_{γ} of two histidines. Fifteen resolved resonances account for the 16 carbons (Figure 4a). The peak at 130.7 ppm in the spectrum of oxidized ferredoxin and the peak at 128.5 ppm for the reduced protein have two-carbon intensities. The Tyr C_{ζ} peaks were assigned by selective proton decoupling by using the procedure worked out for tyrosine (Figure 1). Three such spectra are shown in Figure 5b–d. The peak heights of the resonances were measured for each spectrum and plotted against the decoupler frequency (Figure 6). The resonances at 154.9 and 154.8 ppm were not resolved separately, and their combined peak height was measured and plotted. The resulting assignments are summarized in Table IV.

T_1 Relaxation Measurements. The spin–lattice relaxation times (T_1) of the resolved single-carbon resonances were determined for both oxidized and reduced ferredoxin. The T_1 values of nine, nonprotonated, single-carbon resonances in the region 110–160 ppm are tabulated in Table V.

Discussion

Assignments of Tyrosine C_{ζ} Resonances by Coherent Selective Decoupling. The 154–158 ppm region in the ^{13}C spectrum of oxidized *A. variabilis* ferredoxin II contains six resonances which are due to the ζ-carbons of the single arginine and five tyrosine residues. The C_{ζ} of arginine is coupled to the two C_{δ}-H's through three bonds, and the coupling constant ($^{3}J_{C\text{-}H}$) is dependent on the dihedral angle. In this case, an average dihedral angle of 60° is expected because of the free rotation of the C_{δ}–N_{ϵ} single bond. A coupling constant of 1 Hz is obtained for a fixed dihedral angle of 60° in model compounds (Delbaere et al., 1973). In the case of a tyrosine side chain, the three bond coupling between C_{ζ} and C_{δ}-H is 8 Hz, and the two bond coupling between C_{ζ} and C_{ϵ}-H is estimated to be between 1 and 3 Hz (T.-M. Chan and J. L.

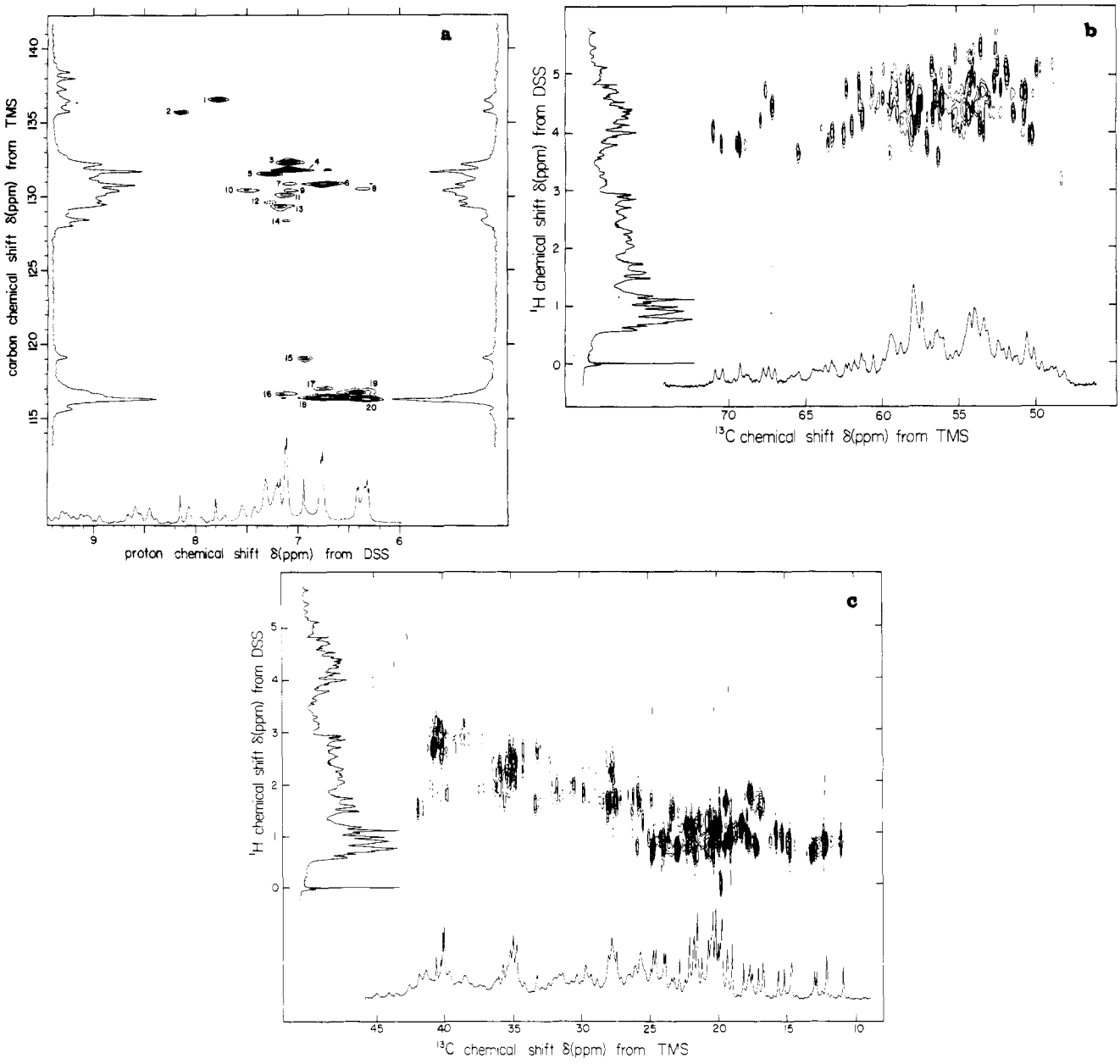

FIGURE 2: Contour plots of two-dimensional, heteronuclear (^{1}H, ^{13}C), chemical shift correlated spectra of oxidized *A. variabilis* ferredoxin II. The [ul 20% ^{13}C]ferredoxin concentration was 5.5 mM in 2.5 mL of 0.05 M deuterated phosphate buffer, pH* 7.06 at 24 °C. The experiment was performed on the NT-200 spectrometer (200 MHz for ^{1}H, and 50.3 MHz for ^{13}C). The data were collected in two separate experiments: one for the low-field region (a) and one for the high-field region, presented here as two separate contour plots, (b) and (c), for clarity. The spectrometer settings used are described in the text. Corresponding ^{1}H NMR spectra obtained at 470 MHz are plotted on one axis of each contour map. Two ^{13}C spectra are plotted on the sides of the contour map of the low-field region (a): the one on the left is the normal ^{1}H decoupled spectrum, the one on the right side is a subspectrum that contains only the protonated carbons (Chan et al., 1982). Only the normal proton decoupled one-dimensional ^{13}C NMR spectrum is plotted at the bottom of the contour maps of the high-field region b and c. [Spectrum a is reproduced from Chan & Markley (1982).]

Markley, unpublished results). Hence in a coupled spectrum of a protein where the line widths of the resonances are around 5 Hz, the tyrosine C_ζ is a triplet and is much broader than the arginine C_ζ resonance. When the decoupled and coupled spectra of oxidized *A. variabilis* ferredoxin II (Figure 5a,e) are compared, the resonance at 157.13 ppm which remains sharp is assigned to the C_ζ of Arg^{42}, and the other five resonances at 156.2, 156.0, 155.5, 154.9, and 154.8 ppm which broaden are assigned to the C_ζ of the five tyrosine residues.

Coherent selective decoupling was used to assign these resonances to specific tyrosine residues in the sequence. Since four pairs of ^{1}H NMR doublets have been assigned to four tyrosine side chains, four of the five C_ζ resonances should be assignable by selective decoupling and the fifth by elimination. In the plots of peak height vs. decoupler frequency (Figure 6), a maximum for each C_ζ resonance occurs at the corresponding C_δ-H frequency. The maximum for resonances Y^d_ζ and Y^e_ζ (at 154.9 and 154.8 ppm) is less distinct than the others, because the combined peak height is plotted while only one of the C_ζ resonances is decoupled from its C_δ-H. The C_ζ resonance at 156.2 ppm is assigned to Tyr^{76}. Those at (156.0, 155.5 ppm) are assigned to (Tyr^{83}, Tyr^{99}). One of the remaining resonances (154.9 and 154.8 ppm) must correspond to Tyr^{35}. The resonances at 156.0 and 154.8 ppm are broadened by $Cr(NH_3)_6^{3+}$ (Chan et al., 1983b). Therefore, the resonance at 156.0 ppm has to be assigned to Tyr^{83} and the resonance at 155.5 ppm to Tyr^{99}. The resonance at 154.8 ppm must be assigned to Tyr^{25} by its proximity to Tyr^{83} in the

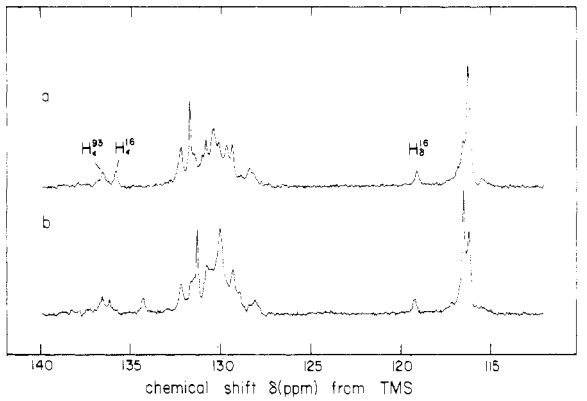

FIGURE 3: ^{13}C NMR subspectra (Chan et al., 1982) of (a) oxidized and (b) reduced *A. variabilis* ferredoxin II containing the protonated carbons in the aromatic region. The pH* of the oxidized protein sample was 7.09, and that of the reduced protein sample was 7.31. The sample temperature was 24 °C. The subspectra were generated by taking the difference between a normal ^1H broad-band decoupled spectrum and an APT spectrum (Patt & Schoolery, 1982). Differences in the chemical shifts of the H_ϵ^{16} and H_δ^{16} peaks in the two spectra result from a difference in the pH* of the oxidized and reduced samples.

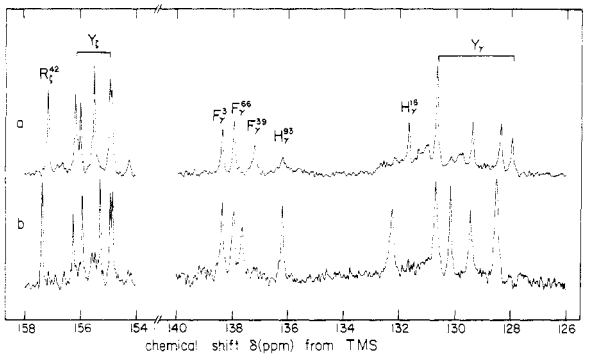

FIGURE 4: ^{13}C NMR subspectra containing the nonprotonated carbon resonances in the aromatic region of (a) oxidized and (b) reduced *A. variabilis* ferredoxin II. The [ul 20% ^{13}C]ferredoxin concentration was 6.5 mM in 2.5 mL of 0.05 M deuterated phosphate buffer. The experimental conditions are described in the legend to Figure 3. The difference in pH* between the oxidized sample (7.09) and the reduced sample (7.31) is responsible for the difference in chemical shift of the H_γ^{16} resonances.

X-ray structure (Fukuyama et al., 1980). The resonance at 154.9 ppm is assigned to Tyr^{35} by elimination. These assignments are consistent with the decoupling experiments, the T_1 values, and the distances of the C_ζ atoms from the iron-sulfur center tabulated in Table V.

Assignments of Other Nonprotonated Carbon Resonances. Sixteen resonances are expected in the 125–160 ppm region (Figure 4). In addition to C_ζ of Arg^{42} and C_ζ of the five tyrosines discussed above, the region should contain resonances from C_γ of five tyrosines, C_γ of three phenylalanines, and C_γ of two histidines. The resonances at 132.1 and 136.2 ppm were assigned previously to C_γ of His^{16} and His^{93} (Chan & Markley, 1983).

The three resonances between 137 and 138.5 ppm are due to the C_γ of the three phenylalanine residues. The two (138.4 and 138 ppm) that are not affected by reduction are assigned to the phenylalanines that are farthest from the iron-sulfur center, Phe_γ^{66} and Phe_γ^3. The ^{13}C spectrum of oxidized *S. maxima* ferredoxin II, which has only one phenylalanine (Phe^{66}), has a single sharp resonance at 138.53 ppm. Therefore, the resonance at 138.4 ppm is tentatively assigned to Phe_γ^{66} by homology. The resonance at 137.2 ppm in the spectrum of the oxidized protein shifts downfield to 137.6 ppm upon reduction. It is assigned to Phe_γ^{39} by its proximity (about 7 Å) to the 2Fe-2S* center. These assignments are consistent

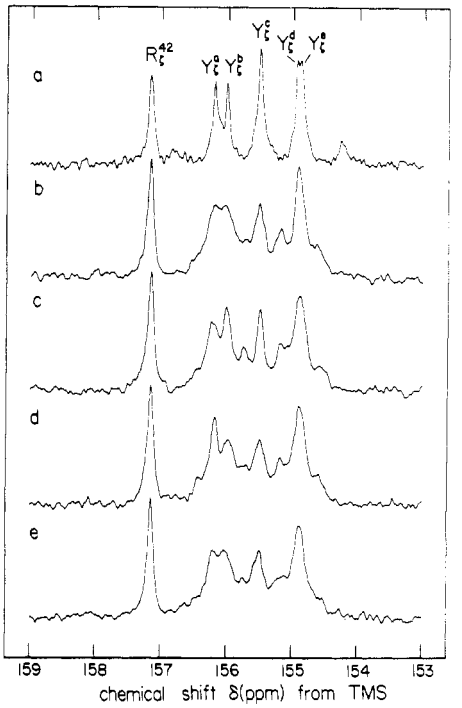

FIGURE 5: Use of selective proton decoupling to assign the C_ζ resonances from Arg^{42} and five tyrosines in the ^{13}C spectrum of oxidized *A. variabilis* ferredoxin II. (a) ^1H broad-band decoupled ^{13}C NMR spectrum at 50.3 MHz (NT-200). (b–d) Selectively decoupled ^{13}C NMR spectra at 118.2 MHz (NT-470), with the ^1H decoupler set at 6.77, 7.11, and 7.30 ppm, respectively. $\gamma H_2/2\pi$ was 37 Hz. (e) ^1H coupled ^{13}C NMR spectrum at 118.2 MHz. For the spectrum obtained at 50.3 MHz, the concentration of [ul 20% ^{13}C]ferredoxin was 3.6 mM in 2.5 mL of 0.05 M deuterated phosphate buffer, pH* 7.14; sample temperature 24 °C. For the spectra obtained at 118.2 MHz, the [ul 20% ^{13}C]ferredoxin concentration was 9 mM in 1 mL of 0.05 M deuterated phosphate buffer, and other sample conditions were 23 °C, pH* 7.15. All spectra are the result of 3000 averaged repetitions with a recycle time of 3 s.

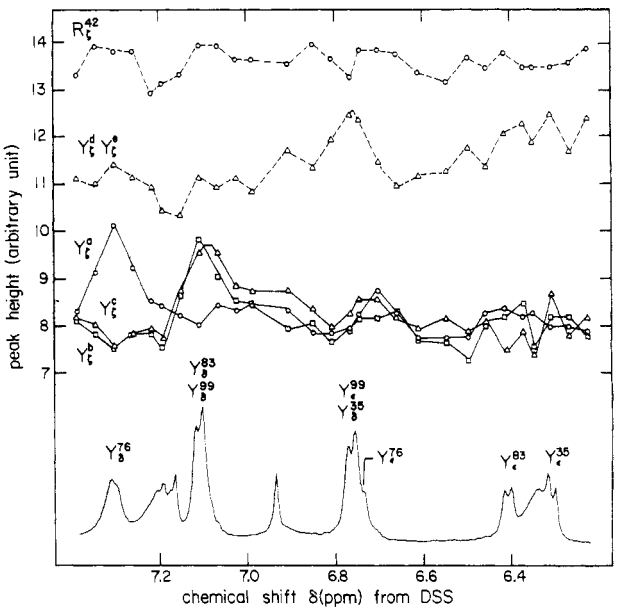

FIGURE 6: Peak heights of selectively decoupled ^{13}C resonances of *A. variabilis* ferredoxin II (Figure 5) plotted as a function of the proton decoupler frequency. The aromatic region of the ^1H NMR spectrum at 470 MHz of oxidized *A. variabilis* ferredoxin II is plotted at the bottom to indicate where the proton resonances occur. The cross assignments shown in Figure 5 were obtained by comparing the maximum for each curve with the proton spectrum.

Table V: T_1 Values of Several Nonprotonated Carbon Resonances of *Anabaena variabilis* Ferredoxin and the Distances of the Carbons from the Iron Atoms[a]

T_1 (s)		distance[b] (Å) from		assignment
oxidized	reduced	Fe^1	Fe^2	
0.74 ± 0.02	1.16 ± 0.02	14.4	12.7	$Y\zeta^{16}$
0.89 ± 0.03	1.03 ± 0.05	15.2	13.5	$Y\zeta^{83}$
0.84 ± 0.03	1.17 ± 0.01	13.2	13.0	$Y\zeta^{89}$
0.94 ± 0.04	0.88 ± 0.04	~18[c]		$Y\zeta^{35}$
0.63 ± 0.01	0.86 ± 0.05	11.4	10.0	$Y\zeta^{25}$
0.22 ± 0.01	0.63 ± 0.02	7.6	8.2	$R\zeta^{42}$
0.64 ± 0.02	0.64 ± 0.02	10.8	8.7	$F\gamma^{66}$
0.71 ± 0.03	0.73 ± 0.02	~23.7	~21.3[d]	$F\gamma^{3}$
0.10 ± 0.01	0.25 ± 0.02	~7.2	~7.4[d]	$F\gamma^{39}$

[a] Measurements were made at 50.3 MHz and 24 °C; the pH* of the sample was 7.3. [b] The distances were calculated from the coordinates of *Spirulina platensis* ferredoxin (Fukuyama et al., 1980). Fe^1 is the iron atom that is directly bonded to Cys-41 and -46, and Fe^2 is the other iron atom which is bonded to Cys-49 and -80. Coordinates of conserved residues were assumed to be the same for both proteins. [c] Residue 35 in *S. platensis* ferredoxin is leucine; the distance listed is an estimate. [d] Residues 3 and 39 in *S. platensis* ferredoxin are tyrosines, which are replaced by phenylalanines in *A. variabilis* ferredoxin II. The distances of the γ-carbons from the iron atoms are assumed to be the same in both cases.

with the T_1 values obtained for these resonances (Table V).

The four peaks in the region from 128 to 131 ppm are assigned to C_γ of the five tyrosine residues (two carbon resonances overlap). From the chemical shift values, it appears that two C_γ resonances are shifted upon reduction. Assignment of these resonances to individual tyrosines is not possible with the present experimental data.

Assignments of Protonated Carbons. Heteronuclear two-dimensional NMR spectroscopy provides an efficient method of cross-assigning proton and carbon resonances. It can assist as well in the assignment of ^{1}H NMR spectra, for example, by permitting one to distinguish readily between resonances from N-H and C-H groups (Chan & Markley, 1982). The protonated carbons in the 110–140 ppm region are due to the ring carbons of the aromatic residues: histidines, tyrosines, and phenylalanines. They are broader than the resonances of the nonprotonated carbons because of dipolar relaxation by the attached protons (Oldfield et al., 1975). The chemical shifts of the C_ϵ and C_δ resonances of His^{16} are pH* and temperature sensitive near the pK_a' of the residue (7.0), in the pH* range of 7, while those of His^{93} are not (Chan & Markley, 1983). The His^{93}_δ resonance was not resolved from the Tyr_ϵ resonances; therefore, its temperature dependence was not obtained.

The C_δ and C_ϵ resonances of Tyr^{99} are shifted upon reduction. An additional resonance is found at 134 ppm in the spectrum of the reduced protein. This resonance, which apparently shifts downfield from its position in the spectrum of the oxidized protein, may be attributed to either Tyr^{25} or Phe^{39}. More intensity due to protonated carbons appears in the spectrum of the reduced protein than in the spectrum of the oxidized protein (Figure 3). This observation parallels the ^{1}H NMR results and fits the conclusion from T_1 measurements (see next section) that reduced ferredoxin has a shorter electron relaxation time than oxidized ferredoxin.

T_1 Relaxation Results. Ferredoxins are paramagnetic in both the oxidized and reduced states at room temperature; the reduced protein has a higher magnetic susceptibility than the oxidized protein (Palmer, 1973). Carbon nuclei that are close to the 2Fe·2S* are expected to have short T_1 values resulting from their interaction with the electron spin. Carbon nuclei that are farther away are expected to have T_1 values typical of diamagnetic proteins of similar molecular weight. The T_1 of the carbon resonances of oxidized ferredoxin were found to be shorter than those of reduced protein (Table V). This result was surprising at first, since reduced ferredoxin is more paramagnetic. The experimental results can be reconciled if the electron spin relaxation time (T_e) of the iron-sulfur center is shorter in reduced ferredoxin than in oxidized ferredoxin. In the oxidized state, both iron atoms are high spin Fe(III)·, one of them becomes high-spin Fe(II) upon reduction. Fe(II) having an electronic ground state of ^{5}E has the shorter T_e. Through the antiferromagnetic coupling with Fe(II), the Fe(III) in reduced ferredoxin also has a shorter T_e when compared with Fe(III) in the oxidized protein (Bertini, 1979). This may explain the longer T_1 values for the nonprotonated carbon resonances observed in reduced ferredoxin, especially for Arg^{42}_γ and Phe^{39}_γ. The relaxation data for the ζ-carbons of the tyrosines have to be interpreted more cautiously, since the reduced ferredoxin samples were anaerobic and the oxidized samples were not; the presence of oxygen in the oxidized samples may have contributed to the differences in T_1 values observed.

The ζ-carbon of Tyr^{25} has the shortest T_1 in the group of Tyr_ζ resonances. This reflects the fact that Tyr^{25} is closest to the 2Fe·2S* cluster. Rapid relaxation of Tyr^{25}_ζ by the 2Fe·2S* center is consistent with the ^{1}H NMR result that the C_δ-H and C_ϵ-H of Tyr^{25} are not observed. Phe^{39} is also very strongly relaxed by the paramagnetic 2Fe·2S* center, since a very short T_1 is obtained for Phe^{39}_γ, when compared with those for the other two Phe_γ resonances of ferredoxin or to the values obtained for BPTI (Levy et al., 1982). This may explain the inability to observe the resonances due to Tyr^{39} in ^{1}H NMR spectra of *S. maxima* ferredoxin II and *A. nidulans* ferredoxin (Chan et al., 1983a). Since the reduced ferredoxins have a shorter electron spin relaxation time, ^{1}H resonances from nuclei near the 2Fe·2S* center should be sharper in spectra of the reduced protein than the oxidized protein as was observed.

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Nuclear Magnetic Resonance Studies of Two-Iron-Two-Sulfur Ferredoxins. 4. Interactions with Redox Partners†

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ABSTRACT: Binding of the redox inhibitor $Cr(NH_3)_6^{3+}$ or the protein ferredoxin-$NADP^+$ oxidoreductase to ferredoxin was studied by ^1H and ^{13}C NMR spectroscopy. Selective paramagnetic broadening results indicate that $Cr(NH_3)_6^{3+}$ binds near ferredoxin residues 25 and 83. *Anabaena variabilis* ferredoxin (^{13}C enriched to 20% isotope) was titrated with unlabeled (natural abundance ^{13}C) spinach oxidoreductase. The studies indicate that at least three glutamate residues of ferredoxin are at or near the contact region between ferredoxin and ferredoxin-$NADP^+$ oxidoreductase. Ferredoxin does not undergo a major conformational change when it binds to the oxidoreductase. The oxidoreductase and the chromium complex appear to bind to the same region of the ferredoxin molecule.

Ferredoxin has been found to reduce several inorganic complexes, and the kinetics of the electron transfer reactions between ferredoxins isolated from spinach and parsley and small inorganic complexes have been studied in detail (Armstrong et al., 1978, 1979; Armstrong & Sykes, 1978). From these kinetic data, Armstrong and co-workers concluded that a single $Cr(NH_3)_6^{3+}$, which itself is redox inactive, completely blocks the reaction between parsley ferredoxin and cobalt complexes. They inferred that ferredoxin has a single $Cr(NH_3)_6^{3+}$ binding site with an association constant of about 470 M^{-1}. Cr(III) has an electron spin relaxation time around 10^{-9}–10^{-10} s; this and the unique binding site make $Cr(NH_3)_6^{3+}$ an ideal relaxation probe for structural studies of ferredoxin. We have used high-resolution ^1H and ^{13}C NMR to locate the site at which $Cr(NH_3)_6^{3+}$ binds to ferredoxin.

Ferredoxin-$NADP^+$ oxidoreductase (FNR)[1] contains a noncovalently bound FAD prosthetic group and has a molecular weight of about 34000. Foust and co-workers (1969) have shown that FNR forms a tight 1:1 complex with ferredoxin and $NADP^+$. Ferredoxin was found to be essential for the photoreduction of $NADP^+$ in fragmented chloroplasts (Arnon & Buchanan, 1974). The complex between FNR and ferredoxin was found to persist even under partial or complete reduction of the two proteins (Zanetti & Curti, 1981). Ferredoxins from higher plants and cyanobacteria are interchangeable in the reaction with FNR (Hall & Rao, 1977). From chemical modification studies, FNR appears to have one arginine residue at or near the ferredoxin binding site (Zanetti et al., 1979). The modification of a single amino group in spinach ferredoxin (specific site unknown) inhibited the formation of the FNR–ferredoxin complex (Davis & San Pietro, 1977). In another study, Masaki and co-workers (1977) suggested that amino groups on ferredoxin are important in maintaining the protein conformation but may not be involved in binding FNR. In the present work, ^{13}C NMR was used to study the interaction between *A. variabilis* Fd and spinach FNR.

Experimental Procedures

Materials. Ferredoxins from *Spirulina maxima*, *Anabaena variabilis* (natural ^{13}C abundance and ^{13}C enriched to 20% isotope), and spinach (*Spinacia oleracea*) were obtained as described previously (Chan & Markley, 1983a; Chan et al., 1983). *Phytolacca americana* Fd I was a gift from Dr. H. Matsubara. FNR was isolated in the laboratory from fresh spinach leaves according to the procedure of Ellefson & Krogman (1979). $Cr(NH_3)_6^{3+}$ was prepared as the nitrate salt according to a published procedure (Oppegard, 1950) by students in an inorganic preparation laboratory course at Purdue. $Cr(CN)_6^{3-}$ was a gift from Dr. D. C. McCain, University of Southern Mississippi. Sources of all other chemicals have been listed in the first paper of this series (Chan & Markley, 1983a).

Interaction between $Cr(NH_3)_6^{3+}$ and Ferredoxins. Similar procedures were used in both the ^1H NMR and ^{13}C NMR studies. Typically, a spectrum was taken of the oxidized

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[1] Abbreviations: FNR, ferredoxin-$NADP^+$ oxidoreductase; 2Fe·2S*, the iron-sulfur center consisting of two iron atoms and two inorganic sulfur atoms; Fd, ferredoxin; pH*, pH meter reading of a solution in ^2H_2O uncorrected for the deuterium isotope effect; ul, uniformly labeled.

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