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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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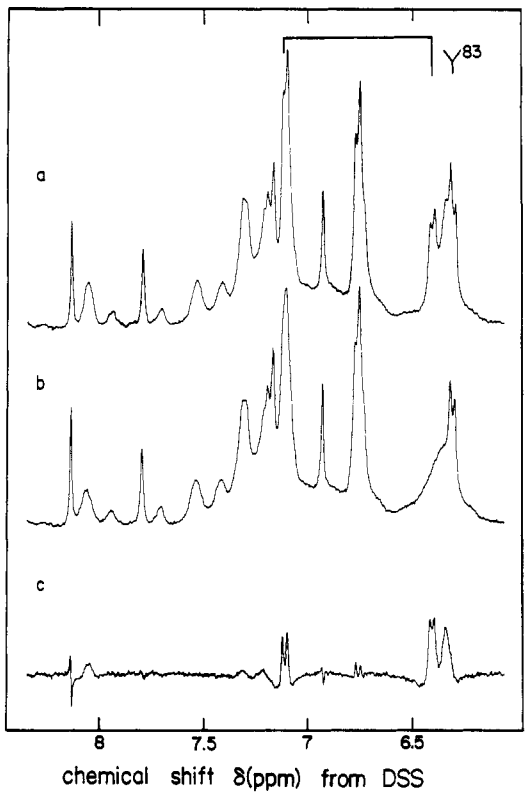

FIGURE 1: 360-MHz ^1H NMR study of the interaction of oxidized *Anabaena variabilis* ferredoxin with the redox inhibitor $Cr(NH_3)_6^{3+}$. (a) Aromatic spectral region of the free ferredoxin; (b) after the addition of a 1:30 molar equivalent of $Cr(NH_3)_6^{3+}$; (c) difference spectrum [(a) − (b)]. The protein concentration was 3.3 mM in 0.05 M deuterated phosphate buffer, pH* 7.19 at 20 °C. Each spectrum was the sum of 512 acquisitions. The pair of coupled doublets broadened by $Cr(NH_3)_6^{3+}$ binding is assigned to the ring protons of tyrosine-83.

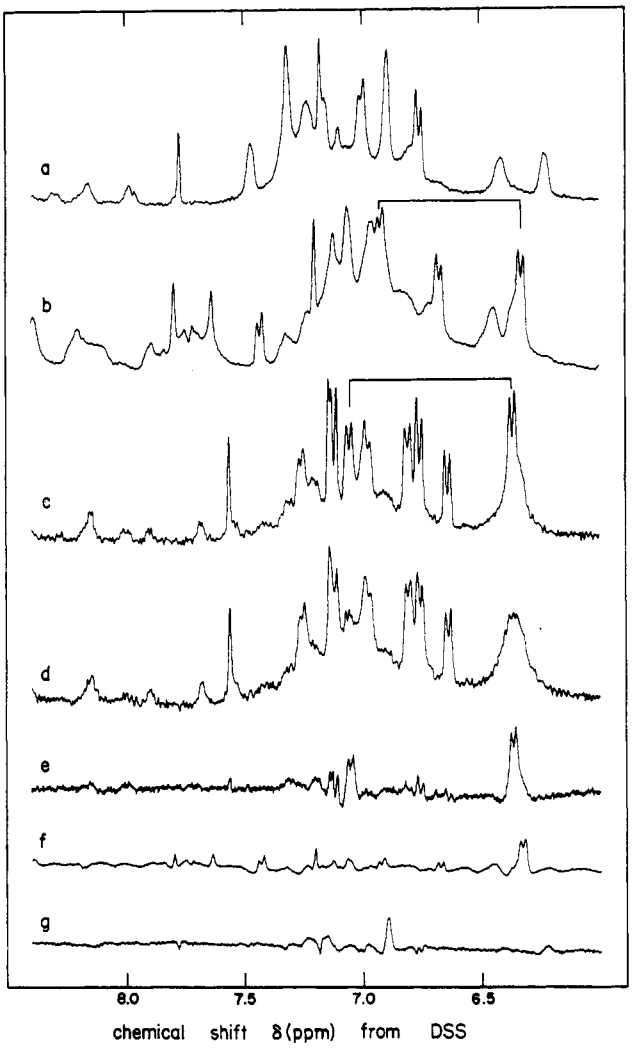

FIGURE 2: Effect of $Cr(NH_3)_6^{3+}$ binding on the aromatic region of 360-MHz ^1H NMR spectra of three plant-type ferredoxins. Spectra of the free ferredoxins in 0.05 M deuterated phosphate buffer: (a) *Phytolacca americana* ferredoxin I, 1.5 mM, pH* 7.33, 1400 repetitions; (b) spinach ferredoxin, 3.3 mM, pH* 7.24, 512 repetitions; (c) *Spirulina maxima* ferredoxin, 3.3 mM, pH* 7.22, 256 repetitions. (d) The spectrum of *S. maxima* ferredoxin under similar conditions after addition of a 1:30 molar equivalent of $Cr(NH_3)_6^{3+}$; (e) the difference spectrum [(c) − (d)]. Analogous difference spectra obtained after addition of a 1:30 molar equivalent of the chromium complex to (f) spinach and (g) *P. americana* ferredoxins. The spectra of spinach and *P. americana* ferredoxins after addition of $Cr(NH_3)_6^{3+}$ are not shown.

protein dissolved in 50 mM deuterated phosphate buffer solution, pH* around 7.2. $Cr(NH_3)_6^{3+}$ was dissolved in the same buffer used for the protein, and the pH* of this solution was adjusted to be the same as that of the ferredoxin solution. A calculated volume of the $Cr(NH_3)_6^{3+}$ solution (typically about 5 μL) was added to the ferredoxin solution to achieve a $Cr(NH_3)_6^{3+}$:ferredoxin ratio of 1:30. After mixing, the pH* of the solution was measured and readjusted to the original value if necessary. For *A. variabilis* Fd and *S. maxima* Fd, several aliquots of the $Cr(NH_3)_6^{3+}$ solution were added in order to study the effects of the chromium complex at higher concentrations.

Interaction between Anabaena variabilis Fd and Spinach FNR. FNR (natural abundance) was dialyzed extensively against deionized water and lyophilized. A ^{13}C spectrum of ^{13}C-enriched *A. variabilis* Fd was obtained in 10 mM deuterated phosphate buffer containing 0.1 M NaCl at pH 7.5. Lyophilized FNR was added to the solution to give a FNR:Fd ratio of 1:4. The pH* of the solution was readjusted, and a ^{13}C spectrum was taken. Three more portions of FNR were added to achieve a final FNR:Fd ratio of 1:1.

Results

Binding of $Cr(NH_3)_6^{3+}$ to Ferredoxin. The ^1H NMR spectrum of oxidized *A. variabilis* Fd (aromatic region) is shown in Figure 1a. The addition of a 1:30 molar equivalent of $Cr(NH_3)_6^{3+}$ selectively broadens (Figure 1b) the pair of doublets assigned to Tyr^{83} (Chan et al., 1983). The result can be seen best in the difference spectrum (Figure 1c). At higher concentrations of $Cr(NH_3)_6^{3+}$, other resonances in the aromatic region begin to broaden nonselectively; the extent of broadening increases with increasing concentration of the chromium complex.

These results were confirmed by studies of ferredoxins from other species. ^1H NMR spectra (aromatic region) of *Phytolacca americana* Fd I, spinach Fd, and *Spirulina maxima* Fd are shown in Figure 2a–c. Figure 2d is the spectrum (aromatic region) of *S. maxima* Fd in the presence of a 1:30 molar equivalent of $Cr(NH_3)_6^{3+}$. The pair of coupled doublets assigned to Tyr^{83} (Chan et al., 1983) exhibits selective broadening. Difference spectra illustrate the effect of $Cr(NH_3)_6^{3+}$ binding to ferredoxins from these additional species: Figure 2e [(c) − (d)], *S. maxima* Fd; Figure 2f, spinach Fd; Figure 2g, *P. americana* Fd I. In the case of *S. maxima* Fd, other resonances in the spectrum begin to broaden nonselectively at higher concentrations of the chromium complex. $Cr(CN)_6^{3-}$, at similar concentrations (1:30 to 1:5 molar equivalent), has no effect on the spectrum (aromatic region) of oxidized *A. variabilis* Fd (spectra not shown).

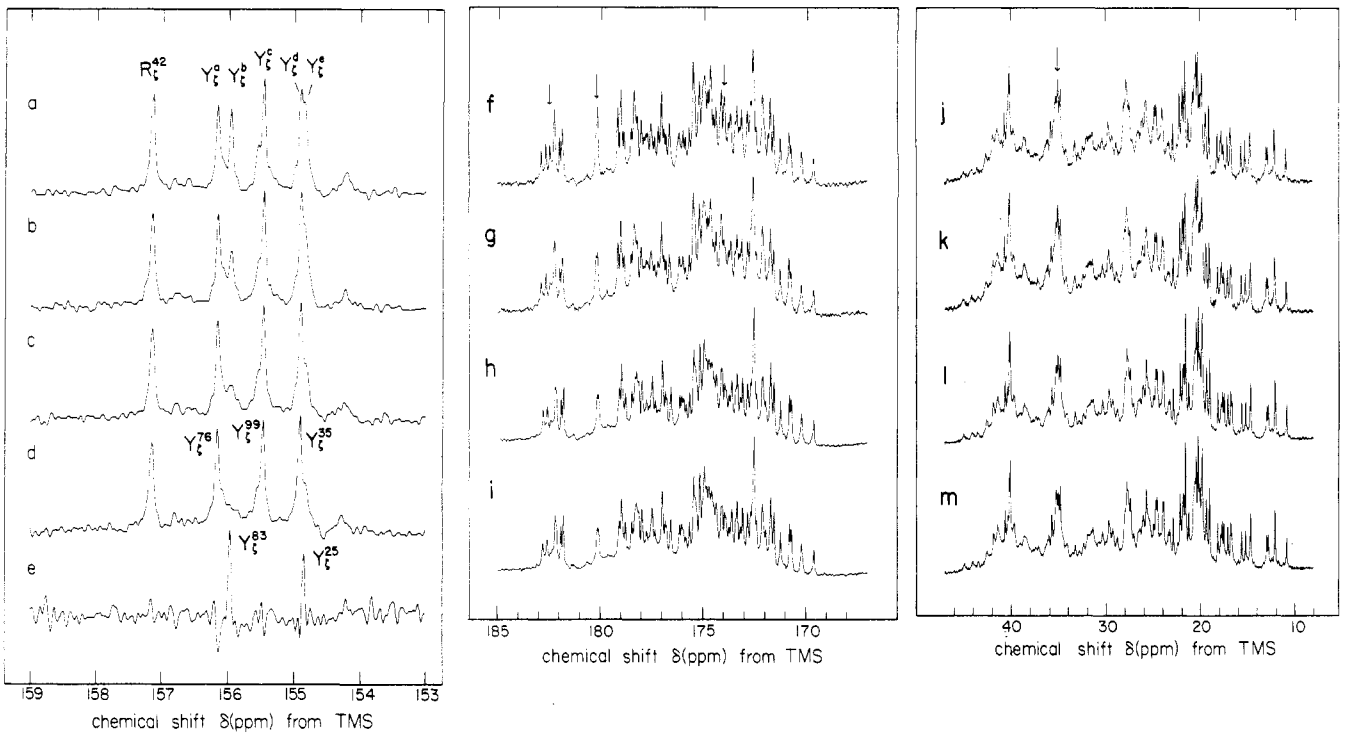

FIGURE 3: Effect of the addition of $Cr(NH_3)_6^{3+}$ on the 50.3-MHz ^{13}C NMR spectrum of oxidized *Anabaena variabilis* ferredoxin. The [ul 20% ^{13}C]ferredoxin concentration was 4.5 mM in 2.5 mL of 0.05 M deuterated phosphate buffer, pH* 7.07. The spectra are the sum of 4600 repetitions. Spectral region from 154 to 158 ppm: (a) free ferredoxin; after addition of $Cr(NH_3)_6^{3+}$ at a molar ratio of (b) 1:30, (c) 1:10, and (d) 1:6; (e) difference spectrum [(a) − (c)]. Carbonyl spectral region: The arrows indicate the resonances that are selectively broadened. (f) Free ferredoxin; after addition of $Cr(NH_3)_6^{3+}$ at a molar ratio of (g) 1:30 , (h) 1:10, and (i) 1:6. Aliphatic spectral region: The arrows indicate the resonances that are selectively broadened. (j) Free ferredoxin; after addition of $Cr(NH_3)_6^{3+}$ at a molar ratio of (k) 1:30, (l) 1:10, and (m) 1:6.

^{13}C NMR spectra were obtained of *A. variabilis* Fd, free and in the presence of three concentrations of $Cr(NH_3)_6^{3+}$ (1:30, 1:10, and 1:6 molar equivalent). Figure 3 shows the spectral region from 150 to 160 ppm, which includes the C_ζ resonances of Arg^{42} and the five tyrosines (positions 25, 35, 76, 83, and 99). Two tyrosine C_ζ resonances are broadened by addition of a 1:10 molar equivalent of $Cr(NH_3)_6^{3+}$; the effect is seen clearly in the difference spectrum (Figure 3e). At higher concentrations of $Cr(NH_3)_6^{3+}$ (1:6 molar equivalent), the resonances at 157.1 and 155.5 ppm start to lose intensity (Figure 3d) apparently as the result of nonspecific binding of the chromium complex. The spectral region from 165 to 185 ppm, which contains the resonances of the carbonyl carbons, is shown in Figure 3f–i, and the aliphatic spectral region from 10 to 45 ppm is shown in Figure 3j–m.

Interaction between Anabaena variabilis Ferredoxin and Spinach FNR. The complete ^{13}C NMR spectrum of ^{13}C-enriched *A. variabilis* ferredoxin is shown in Figure 4a (low-field region) and Figure 4d (high-field region). In general, all of the ferredoxin peaks broadened as the sample was titrated with FNR because of the longer correlation time of the complex (M_r ~46 000). For example, consider the four resonances farthest upfield (10.5–12.5 ppm; Figure 4d) which can be assigned to the C_δ resonances of the five isoleucine residues. Their chemical shifts do not change with increasing FNR concentration, but their line widths (corrected for the line broadening factor used in processing the data) increase from about 3 Hz in the spectrum of free Fd to 6 Hz in the spectrum of the 1:1 complex. As another example, the line width of the carbonyl carbon resonance at 169.6 ppm increases from 4 Hz to 10 Hz in the 1:1 complex.

Beyond the general broadening effects, additional broad peaks appear at higher FNR concentrations, which may be attributed to the resonances of FNR in the complex. Therefore, it becomes difficult to assign peaks in spectra taken at higher FNR concentrations. The optimal ratio for detecting changes in the ferredoxin spectrum attributable to the binding process appears to be around an Fd:FNR ratio of 4:1 (Figure 4b,e). The difference spectrum (Figure 4c,f) was obtained by first broadening the spectrum of ferredoxin through the use of a larger line broadening factor in the exponential multiplication so as to increase the line widths of all peaks by about 1 Hz, to the level attributed to the average increased molecular weight (increased rotational correlation time) of the mixture. The most obvious peaks appearing in the difference spectrum, at 34.8, 35.0, and 35.3 ppm, result from specific broadening.

The rate of dissociation of the complex has been estimated from the line width of the ferredoxin carbonyl ^{13}C resonance at 182.9 ppm which broadens on complex formation. In free ferredoxin, the natural line width (w_0) of this resonance is about 3 Hz; in the ferredoxin–FNR mixture (4:1), the line width increases to about 13 Hz. Under these conditions the increase in the ferredoxin correlation time as a result of complex formation contributes about 1 Hz to the line broadening; thus, an experimental line width (w_{exp}) of 12 Hz was used in the following calculations:

$$w_{exp} = w_0 + (\pi\rho)^{-1}$$

where ρ is the probability per unit time of the ferredoxin nucleus moving to a site where it has a different Larmor precession frequency. When the values above are used, ρ for Fd is estimated to be 0.035; and since the ferredoxin:FNR molar concentration ratio is 4:1, ρ for FNR is 0.009. This gives a dissociation rate of 110 s^{-1} for the ferredoxin–FNR complex.

Discussion

The lifetimes of both complexes studied are relatively short on the NMR time scale; that is the spectrum of a partial

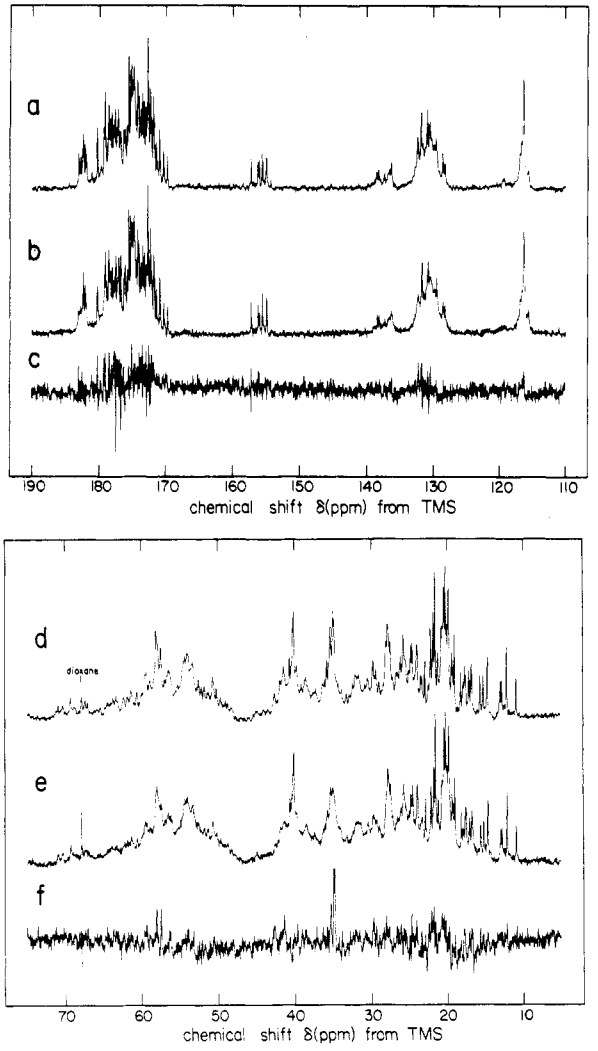

FIGURE 4: 50.3-MHz ^{13}C NMR study of the interaction between oxidized spinach ferredoxin-NADP$^+$ oxidoreductase (FNR) and oxidized *Anabaena variabilis* ferredoxin. The [ul 20% ^{13}C]ferredoxin concentration was 1 mM in 10 mM deuterated phosphate buffer, 0.1 M NaCl, pH* 7.5, 16 636 repetitions. Aliphatic spectral region: (a) free ferredoxin; (b) after addition of a 1:4 molar equivalent of FNR (natural abundance); (c) difference spectrum [(a) − (b)]. Aromatic and carbonyl spectral region: (d) free ferredoxin; (e) after addition of a 1:4 molar equivalent of FNR (natural abundance); (f) difference spectrum [(d) − (e)].

complex is the average of that of free ferredoxin and the 1 to 1 complex except for exchange broadening of a few resonances as noted above. This property has enabled us to identify groups on ferredoxin involved in complex formation or near the binding site. $Cr(NH_3)_6^{3+}$ is paramagnetic and causes severe broadening of a large number of ferredoxin peaks at higher (saturating) concentrations. When a small amount of the chromium complex is added, only those protein groups very near the specific binding site are affected. This experimental approach was developed by Campbell et al. (1973) in their pioneering studies of Gd(III) binding to lysozyme.

Location of the Binding Site for $Cr(NH_3)_6^{3+}$. A small amount of $Cr(NH_3)_6^{3+}$ (1 molecule to 30 molecules of ferredoxin) broadens the pair of doublets (6.41, 7.11 ppm) in the aromatic region of the spectrum of oxidized *A. variabilis* Fd assigned to Tyr^{83}. The other resonances are not perturbed (Figure 1b), and this pair of doublets is the only prominent feature in the difference spectrum (Figure 1c). This result indicates that $Cr(NH_3)_6^{3+}$ interacts with *A. variabilis* Fd at a specific site, in agreement with the conclusions of Armstrong and co-workers with parsley ferredoxin (1978, 1979). A similar pair of coupled doublets in spectra of *Spirulina maxima* (6.37 and 7.05 ppm) and spinach ferredoxins (6.32 and 6.92 ppm) is broadened on addition of a 1:30 molar equivalent of $Cr(NH_3)_6^{3+}$ (Figure 2). These are peaks that were assigned to Tyr^{83} by homology (Chan et al., 1983). On the other hand, *P. americana* Fd does not have a coupled doublet at this position, nor does it show a tyrosine pattern in the difference spectrum on binding the chromium complex (Figure 2). When the amino acid sequences are compared (Chan & Markley, 1983a,b) only one position (residue 83) is found to be occupied by a tyrosine in *A. variabilis*, *S. maxima*, and spinach ferredoxins, but not in *P. americana* Fd I, where phenylalanine replaces tyrosine. If one assumes that the binding site for $Cr(NH_3)_6^{3+}$ is similar in the four proteins, the selective broadening of the coupled doublets around 6.4 and 7.0 ppm assigned to Tyr^{83} confirms this assignment which was based on homology arguments. Homologous residues in homologous proteins generally give rise to similar NMR spectra, but exceptions are known [for example, see Ogino et al. (1982)]. The resonance at 6.89 ppm in the spectrum of *P. americana* Fd I which is broadened is tentatively assigned to Phe^{83}.

The analogous ^{13}C NMR experiment was performed with *A. variabilis* Fd (ul, 20% ^{13}C). The experiment is more difficult than the ^1H experiment for several reasons. First, for a nucleus interacting with an electron spin, the T_2^{-1} of the resonance is proportional to the square of the nuclear magnetogyric ratio. Therefore, the paramagnetic broadening of ^{13}C resonances is 16 times smaller than that of ^1H resonances, and a larger amount of the chromium complex has to be added. This in turn increases the amount of free chromium complex in solution which may cause nonselective broadening. Second, even with 20% enrichment, a ^{13}C spectrum with good signal-to-noise ratio required about 3 h of accumulation time because of the intrinsic insensitivity of ^{13}C NMR spectroscopy and the small amount of labeled protein available; several ^1H spectra could be obtained in the same period of time. Third, $Cr(NH_3)_6^{3+}$ is not stable in water at room temperature, it slowly hydrolyzes in a period of about 24 h (Oppegard, 1950), which further complicates the situation.

On addition of $Cr(NH_3)_6^{3+}$, the most obvious changes in the spectrum are in the region from 154 to 158 ppm, which contains resonances from the C_ζ of Arg^{42} and the five tyrosines. As the concentration of $Cr(NH_3)_6^{3+}$ increases, the heights of the resonances at 156.0 and 154.8 ppm decrease, while the other four resonances are not affected (Figure 3a–d). This effect can be observed better in the difference spectrum (Figure 3e), which shows only two peaks at 156.0 and 154.8 ppm. These two resonances are assigned to the C_ζ of two tyrosine residues. The ^1H NMR studies above establish that the $Cr(NH_3)_6^{3+}$ binding site is near Tyr^{83}. Therefore, one of these two resonances should correspond to Y_ζ^{83}. The peak at 156.0 ppm has been assigned to Y_ζ^{83} by a separate selective decoupling experiment (Chan & Markley, 1983b). In the X-ray structure of *S. platensis* ferredoxin, the aromatic rings of Tyr^{83} and Tyr^{25} are stacked against each other. A "structure" of *A. variabilis* ferredoxin (Figure 5) was constructed on the basis of its sequence (Chan et al., 1983) and the *S. platensis* X-ray coordinates (Tsukihara et al., 1981; Brookhaven Data Bank) by using a computer program developed by R. Feldman (R. Feldmann and J. L. Markley, unpublished data). The structure reveals that Tyr^{25} and Tyr^{83} are the only two tyrosine residues close enough to one another to be affected by a single bound $Cr(NH_3)_6^{3+}$. Therefore, the resonance at 154.8 ppm is assigned to Y_ζ^{25}. ^1H NMR peaks from Tyr^{25} were not

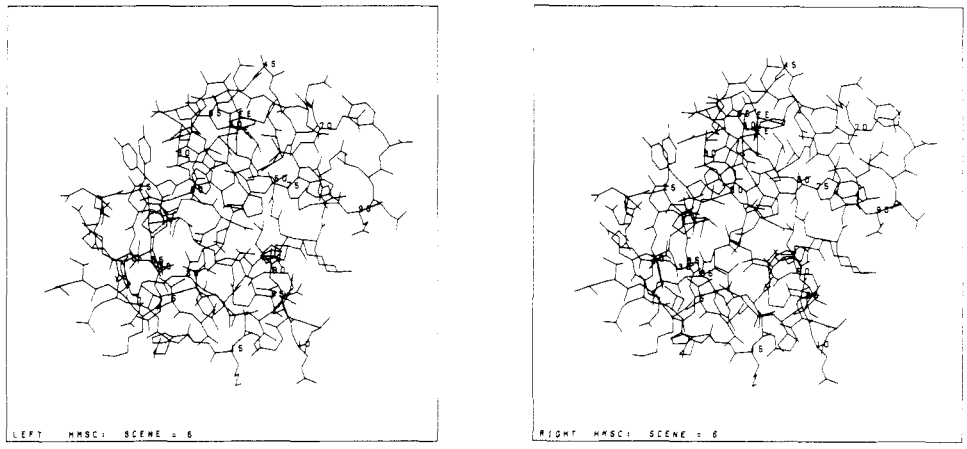

FIGURE 5: Model of the structure of *Anabaena variabilis* ferredoxin based on that of *S. platensis* ferredoxin (Tsukihara et al., 1981). It was calculated by a computer program that performs amino acid replacements and reorients the replaced side chains as necessary to avoid van der Waals overlaps (Berzofsky et al., 1982). Note that the numbering system used in the structure is continuous and does not account for the deletion at position 59 (compared at higher plant ferredoxins). Hence, to convert to the numbering system used in the text, all residue numbers in the figure from 59 to the carboxyl terminus should be incremented by one.

observed, apparently because the residue is too near the iron-sulfur center (Chan et al., 1983). Small decreases in peak height were observed for several other resonances in the 110–132 ppm region. These peaks probably correspond to C_ϵ and C_δ of Tyr^{83} and Tyr^{25}. Since this region of the spectrum is not as well resolved as the 150–160 ppm region and the intensity changes are smaller, interpretation is more difficult and will not be discussed further.

Other resonances that are selectively broadened occur at 182.4, 180.1, 173.9, and 35.0 ppm (Figure 3). The peak at 35.0 ppm correlates with a proton resonance at ~2.2 ppm in the heteronuclear (^{13}C, ^1H) two-dimensional Fourier transform chemical shift correlation spectrum (Chan & Markley, 1983b); hence, it is assigned to a glutamate C_γ. The peak at 182.4 ppm can be assigned to a glutamate C_δ on the basis of its chemical shift and selective proton decoupling pattern (maximum near 2.2 ppm which is characteristic of glutamate; a typical aspartate would have a maximum near 2.8 ppm). In the X-ray structure, Glu^{24} is located near Tyr^{25} and Tyr^{83}. Therefore, the resonances at 182.4 and 35.0 ppm are tentatively assigned to Glu^{24}. The remaining two peaks in the carbonyl region that are broadened by $Cr(NH_3)_6^{3+}$ are not assigned. Glu^{24} is not a conserved residue and is not required apparently for the binding of $Cr(NH_3)_6^{3+}$, since the chromium complex was found to bind to other ferredoxins that lack Glu^{24}. The nearby conserved aspartates, Asp^{22}, Asp^{23}, and Asp^{63}, could be the groups responsible for providing negative charges to interact with the positively charged chromium complex. The presence of Glu^{24} is not essential but probably enhances the binding affinity. Since the chromium complex binds to *P. americana* Fd in which residue 83 is a phenylalanine, and to the other ferredoxins in which it is a tyrosine, the identity of residue 83 apparently is not critical for binding, and its NMR peaks are broadened only because the residue is adjacent to the $Cr(NH_3)_6^{3+}$ binding site.

Complex between Ferredoxin and FNR. We believe that the present investigation of the ferredoxin–FNR complex represents the first use of uniform ^{13}C enrichment of a protein to study a protein–protein interaction. A salt concentration corresponding to an ionic strength of about 0.12 M (10 mM phosphate, 0.1 M NaCl, pH* 7.5) was used because ferredoxin was found to be most active in the diaphorase activity of FNR at an ionic strength of about 0.1 M (Nakamura & Kimura, 1971). At this ionic strength, the dissociation constant for the spinach ferredoxin–spinach FNR complex is about 2.5×10^{-5} M (Foust et al., 1969). A dissociation constant of the same order of magnitude was assumed for the *A. variabilis* Fd–spinach FNR complex.

On complexation with FNR, several specific changes appear in the ferredoxin spectrum, in addition to the general broadening of ferredoxin resonances attributed to an increase in the average rotational correlation time (Figure 4c,f). The most obvious changes involve three peaks at 34.8, 35.0, and 35.3 ppm that are broadened in the spectrum of the mixture. The chemical shifts of these resonances suggest that they are due to C_γ of glutamate residues. This assignment is supported by the presence of cross peaks in the heteronuclear (^{13}C, ^1H) two-dimensional chemical shift correlated spectrum (Chan & Markley, 1983b) that connect the three carbon resonances to proton resonances around 2.2 ppm from DSS (the expected chemical shift for C_γ-H of Glu).

The analysis of the carbonyl carbon region is complicated by the crowding of a large number of resonances into a small chemical shift range; therefore, the difference spectrum must be interpreted with extreme care. Three resonances around 176.7 ppm in the ferredoxin spectrum shift into one peak in the spectrum of the mixture; two resonances around 177.5 ppm also merge. Resonances at 182.9, 182.6, 182.4, 180.1, 179.0, 178.9, and 178.2 ppm are broadened. The three peaks around 182 ppm can be attributed to C_δ of glutamate residues by their chemical shift and by the coherent proton decoupling pattern (maxima near 2.2 ppm). The other four resonances may be due to the C_δ of glutamate residues or C_γ of aspartate residues by their chemical shifts (Wüthrich, 1976).

These results indicate that the resonances of at least three glutamate residues of ferredoxin are affected upon FNR binding. These residues may interact directly (electrostatically) with positively charged groups on the surface of FNR or may be otherwise located in the contact region of the complex so that their chemical shifts are altered. Of the three glutamate C_γ and C_δ resonances that are broadened by the addition of 1:4 molar equivalent of spinach FNR, one of each group (at 35.0 and 182.4 ppm) is broadened by $Cr(NH_3)_6^{3+}$. The tentative assignments of these peaks to Glu^{24} made above suggest that this glutamate may be involved in binding FNR and that the chromium complex and FNR bind to the same general region of the ferredoxin molecule. Verification of this and the assignments of the other two glutamate residues probably will have to be accomplished by selective chemical modification of the acidic residues of the ferredoxin molecule.

The resonances at 57.3 and 58.0 ppm are also broadened in the spectrum of the mixture (FNR:Fd = 1:4). There are some minor changes in the aromatic region, the resonance at 132.2 ppm is broadened, and the resonance at 157.13 ppm, which has been assigned to Arg^{42} C_ζ is shifted downfield. This peak is shifted by 6 Hz in the 1:1 complex from its position in free ferredoxin, but its line width has increased only from 4 to 7 Hz; i.e., the resonance is shifted without additional broadening.

These NMR results are consistent with other experimental findings. The interaction between ferredoxin and FNR was found to be mainly electrostatic in nature (Zanetti et al., 1979; Davis & San Pietro, 1977; Masaki et al., 1977). In this respect the interaction may resemble that between cytochrome peroxidase and cytochrome *c*, where three aspartate side chains on cytochrome *c* peroxidase appear to form a triangle of negative charges that are in the right positions to interact with the positive charges of lysine side chains around the exposed heme edge of cytochrome *c* (Kraut, 1981). Model building experiments have to await the sequencing of FNR and the refinement of its X-ray structure (Sheriff & Herriott, 1981). Because of the great similarity between the spectrum of free ferredoxin and that of the 4:1 ferredoxin–FNR mixture, it appears unlikely that ferredoxin undergoes a large conformational change upon complexation; however, small changes in local conformation may account for the spectral differences around 58 ppm.

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Registry No. $Cr(NH_3)_6^{3+}$, 14695-96-6; FNR, 9029-33-8; glutamic acid, 56-86-0.

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