Nuclear Magnetic Resonance Studies of Two-Iron-Two-Sulfur Ferredoxins. 2. Determination of the Sequence of *Anabaena variabilis* Ferredoxin II, Assignment of Aromatic Resonances in Proton Spectra, and Effects of Chemical Modifications†

Tze-Ming Chan,‡ Mark A. Hermodson, Eldon L. Ulrich, and John L. Markley*

ABSTRACT: The primary amino acid sequence of ferredoxin II from *Anabaena variabilis* was determined. Comparison of ^{1}H NMR spectra (360 MHz or 470 MHz) of six plant-type ferredoxins whose primary sequences are known had led to assignments of all the resolved tyrosine resonances of ferredoxins from three cyanobacteria (*Anabaena variabilis*, *Anacystis nidulans*, and *Spirulina maxima*). Several assignments were corroborated by chemical modification experiments and by a $Cr(NH_3)_6^{3+}$ binding experiment. Two-dimensional Fourier transform (^{1}H,^{1}H) chemical shift correlated spectroscopy was used to investigate structural differences between the oxidized and reduced ferredoxins. *A. variabilis* ferredoxin was modified by two methods: limited proteolysis by carboxypeptidase A resulted in complete removal of tyrosine-99 and partial removal (35%) of leucine-98; reaction with 2,4,6-trinitrobenzenesulfonic acid was specific for lysine-15.

In the preceding paper (Chan & Markley, 1983a) the titration properties of the histidine residues in two cyanobacterial ferredoxins were reported. In this paper, assignments of aromatic amino acid side chain resonances, especially the tyrosines, are discussed. It was necessary to determine the amino acid sequence of *Anabaena variabilis* ferredoxin II which was not available in the literature. We have employed several experimental techniques to make first-order peak assignments (assignments to a particular kind of amino acid): measurement of peak intensities and chemical shifts, spin decoupling to determine coupling patterns, and *J*-modulated, spin-echo spectroscopy (Campbell et al., 1975). Two-dimensional Fourier transform correlated spectroscopy (COSY)[1] (Bax & Freeman, 1981; Nagayama & Wüthrich, 1981) was used to confirm the results of the decoupling experiments and to compare the properties of oxidized and reduced proteins. Second-order assignments (assignments to particular residue positions) were made mainly by correlating NMR spectra with the amino acid sequences of the ferredoxins from different species. A number of assignments made by homology were corroborated by well-defined and specific chemical modifications, and the effects of these modifications on the structure of ferredoxin were investigated.

Experimental Procedures

Materials. Ferredoxins from spinach (*Spinacia oleracea*) and koa (*Leucaena glauca*) leaves and from three cyanobacteria, *Spirulina maxima*, *Anabaena variabilis*, and *Anacystis nidulans*, were isolated in our laboratory. *Phytolacca americana* ferredoxin I was a gift from Dr. H. Matsubara. Crude *A. nidulans* ferredoxin was a gift from Dr. M. Ludwig. Koa leaves were collected in Hawaii through the assistance of Dr. K. T. Yasunobu; the leaves were frozen before use. Fresh spinach was used within one day of purchase.

Carboxypeptidase A (DFP treated) was purchased from Sigma Chemical Co. as an aqueous suspension and used without further purification. Trinitrobenzenesulfonic acid (TNBS) was purchased from Sigma Chemical Co. and was purified by the procedure suggested by Glazer et al. (1975). Sources of other chemicals used are listed in Chan & Markley (1983a).

Isolation of Ferredoxins. The isolation of *A. variabilis* and *S. maxima* ferredoxins have been described (Chan & Markley, 1983a). *A. nidulans* ferredoxin was purified by the same procedure used for *A. variabilis* Fd. Fractions with A_{423}/A_{275} ratios higher than 0.58 were considered to be pure. Koa ferredoxin was isolated from frozen koa leaves according to the procedure of Benson & Yasunobu (1969), except that the ferredoxin was purified further on a DEAE-Sephadex column (NaCl linear gradient, 0.4–0.7 M). Koa ferredoxin fractions considered to be pure had A_{420}/A_{277} ratios higher than 0.50. Spinach ferredoxin was isolated according to the procedure of Ellefson & Krogmann (1978) except that the protein was purified further on a DEAE-Sephadex column. Spinach ferredoxin fractions considered to be pure had A_{420}/A_{280} ratios higher than 0.46.

Enzymatic Removal of the C-Terminal Tyrosine. A. variabilis Fd (10 mg) was dissolved in 4 mL of 10 mM phosphate buffer, pH 7.5, containing 0.1 M NaCl; 24 μL of a carboxypeptidase A suspension containing 0.5 mg of the enzyme was added. Aliquots (40 μL) used for amino acid analysis were withdrawn 30 and 60 min after mixing. Each aliquot was added to 0.2 mL of a 10% Cl_3CCOOH solution to precipitate the protein, which was then removed by centrifugation. The

† From the Departments of Chemistry (T.-M.C., E.L.U., and J.L.M.) and Biochemistry (M.A.H.), Purdue University, West Lafayette, Indiana 47907. *Received March 9, 1983; revised manuscript received July 21, 1983.* This work was supported partially by grants from the U.S. Department of Agriculture Competitive Research Grants Office, Cooperative State Research Service, Science and Education, to J.L.M. and E.L.U. and to M.A.H. The Purdue University Biochemical Magnetic Resonance Laboratory has financial support from Grant RR 01077 from the Biotechnology Resources Program of the Division of Research Resources, National Institutes of Health. Much of the work described was carried out in partial fulfillment of the requirements for the Ph.D. degree by T.-M.C. (Chan, 1982).

‡ Present address: Schering-Plough, Bloomfield, NJ 07003.

[1] Abbreviations: COSY, two-dimensional Fourier transform chemical shift correlation; TNBS, 2,4,6-trinitrobenzenesulfonic acid; Fd, ferredoxin; DEAE, diethylaminoethane; A_{423}/A_{275}, ratio of the absorbance at 423 nm to the absorbance at 275 nm; Cl_3CCOOH, trichloroacetic acid; PTH, phenylthiohydantoin; FID, free induction decay; pH*, pH meter reading of a solution in ^2H_2O uncorrected for the deuterium isotope effect; DFP, diisopropyl phosphorofluoridate; ferredoxin-Tyr^{99}, ferredoxin treated with carboxypeptidase to remove the C-terminal tyrosine-99; TNP-ferredoxin, the trinitrophenyl derivative of ferredoxin; FNR, ferredoxin-NADP$^+$ oxidoreductase.

0006-2960/83/0422-5988$01.50/0 © 1983 American Chemical Society

Table I: Time Course of the Carboxypeptidase A Modification of Ferredoxin

ferredoxin	time (h)	relative amount of each residue released[a] residue 99 (Tyr)	residue 98 (Leu)	residue 97
Anabaena variabilis	0.5	1.13	0.23	0 (Asp)
	1.0	1.15	0.41	0 (Asp)
Spirulina maxima	0.5	1.15	0.80	0 (Gly)
	1.0	1.18	1.04	0 (Gly)
	2.0	1.14	1.11	0 (Gly)
	4.0	1.15	1.12	0 (Gly)

[a] Determined by amino acid analysis and reported relative to an internal sample of norleucine present in the 10% Cl_3CCOOH solution. The resulting concentration of norleucine was slightly lower than the concentration of ferredoxin.

supernatant was extracted with ether to remove the Cl_3CCOOH. The resulting aqueous solution was then evaporated to dryness. Amino acid analysis (Table I) indicated that the C-terminal tyrosine (Tyr^{99}) was removed completely and that the next residue (Leu^{98}) was removed from 35% of the protein molecules after 1 h of reaction at room temperature. The reaction mixture was loaded on a Sephadex G-75 column after 1 h of reaction. The brown ferredoxin fractions were pooled for NMR studies. Part of the sample was treated again with carboxypeptidase A after the NMR experiments were completed, and leucine was the only amino acid released.

Enzymatic removal of the C-terminal tyrosine from *S. maxima* Fd was accomplished by a similar procedure. After 1 h of reaction, the C-terminal residue (Tyr^{99}) had been removed from essentially all the protein molecules. The adjacent residue (Leu^{98}) had been removed from 88% of the molecules, while the next residue (Gly^{97}) was not affected even after 4 h of reaction (Table I). A reaction time of 1 h was used in preparing the sample studied by NMR spectroscopy.

Chemical Modification of Lysine-15. *A. variabilis* ferredoxin (17.2 mg) was dissolved in 20 mL of 50 mM borate buffer, pH 9.0, containing 0.1 M NaCl. TNBS was also dissolved in the same buffer (2.73 mg/mL; 100 times the molarity of ferredoxin). A small-scale reaction was monitored by visible spectroscopy at 367 nm: 1 mL of borate buffer was placed in the reference cell and 1 mL of the protein solution in the sample cell. An aliquot (100 μL) of the TNBS solution (10-fold molar excess) was added to each cuvette, and the change in absorbance at 367 nm was monitored (Glazer et al., 1975). The results are plotted in Figure 1. About 1.1 mol of the trinitrophenyl derivative was formed per mol of ferredoxin after 1 h of reaction at room temperature ($\epsilon_{367} = 1.1 \times 10^{-4}$ M^{-1} cm^{-1} for TNP derivative). Another aliquot (1.9 mL) of the TNBS solution was added to the remaining 19 mL of ferredoxin solution, and the reaction was stopped after 1 h. The solution was dialyzed overnight against 10 mM phosphate buffer, pH 7.5, containing 0.1 M NaCl. The solution was then loaded on a DEAE-Sephadex column equilibrated with the same buffer. A linear gradient of NaCl (0.55–0.65 M) was applied, and three distinct bands were obtained. The first one eluted was the major band; it was studied by NMR, and its N-terminal amino acid sequence was determined. The other two bands were not characterized further because of insufficient material.

Amino Acid Sequence Determination of Anabaena variabilis Ferredoxin. The protein was dialyzed against 40% acetic acid to remove the iron-sulfur cluster and lyophilized. The denatured protein was then reduced in a buffer containing

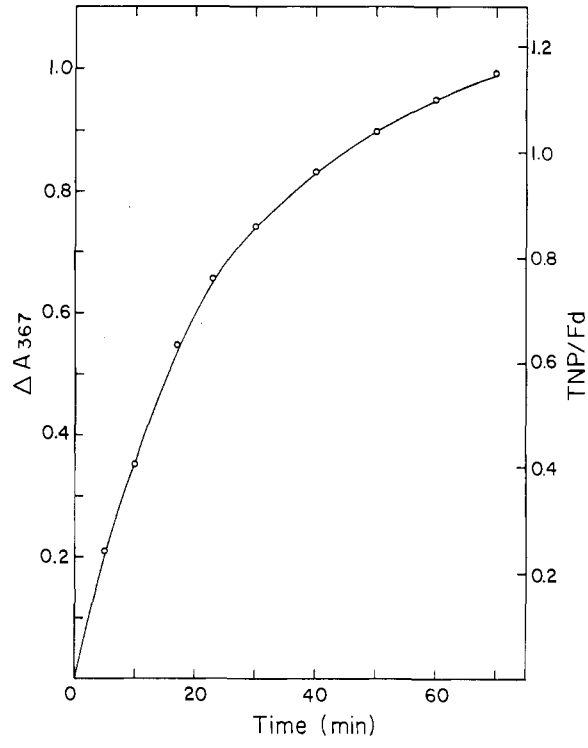

FIGURE 1: Time course of the reaction between *A. variabilis* ferredoxin and trinitrobenzenesulfonic acid. The reaction was followed by monitoring the absorbance of the solution at 367 nm. The change in absorbance (ΔA_{367}) is plotted against time.

dithioerythritol and alkylated with 4-vinylpyridine. The N-terminal amino acid sequence was obtained with a Beckman 890C automatic amino acid sequencer (Hogg & Hermodson, 1977). The sequence was obtained to Ala^{45}, but residues 40, 41, and 42 could not be identified with certainty. Another portion of the alkylated protein was modified with citraconic anhydride and digested with trypsin in 100 mM NH_4HCO_3 buffer, pH 8.0, to cleave the protein into two halves at the invariant Arg^{42} (Hase et al., 1978). The products were not completely soluble in 9% formic acid solution. However, the C-terminal fragment was found to be the insoluble fraction, and the N-terminal fragment was found to be soluble. The C-terminal fragment was collected by centrifugation and sequenced. The C-terminal sequence Leu^{98}-Tyr^{99} was obtained by carboxypeptidase A digestion as described above.

^1H NMR Spectroscopy. Procedures for preparation of samples and details of the NMR instrumentation and methods have been described in the preceding paper (Chan & Markley, 1983a). The two-dimensional Fourier transform chemical shift (^1H,^1H) correlated spectra were obtained with the Jeener (1971) pulse sequence; 512 sets of FIDs each of 2K data points were obtained with quadrature detection. The spin coupling connectivities reported are based on one-dimensional decoupling experiments as confirmed by 2D FT COSY spectra. The spin multiplicities of the resolved resonances in spectra of the cyanobacterial ferredoxins studied were established by *J*-modulated spin-echo spectroscopy. The multiplicity of the Phe^{26} system of *P. americana* was not established. Standard conditions were used to accumulate and process the COSY spectra (Nagayama et al., 1980). COSY data collection normally required 16 h; the free induction decays were resolution enhanced by a sine-bell function.

Results

Amino Acid Sequence. The amino acid sequence determined for *Anabaena variabilis* ferredoxin II is shown in Figure

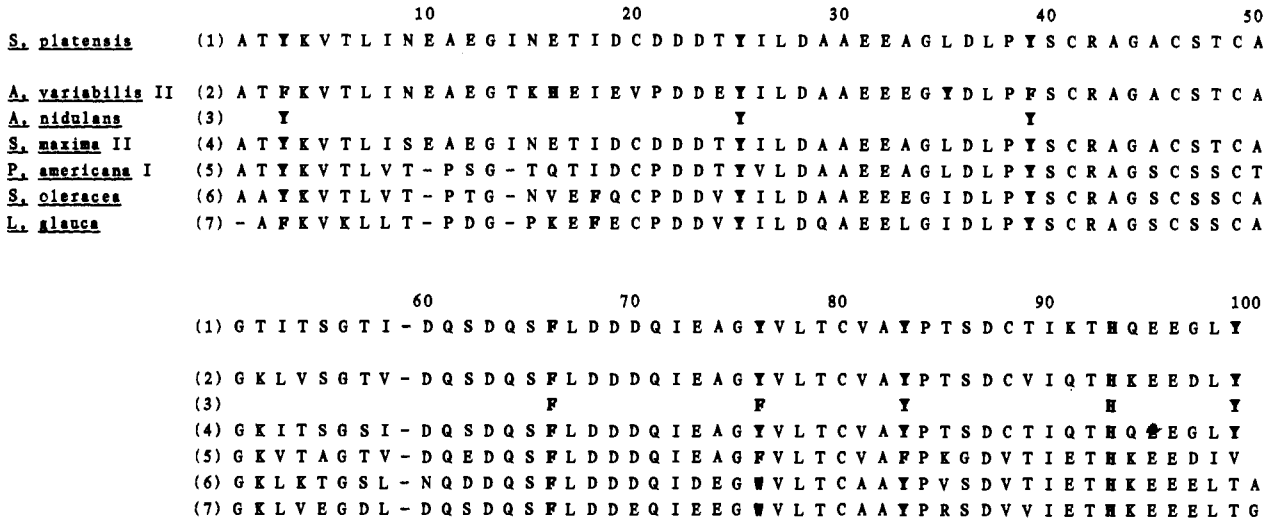

FIGURE 2: Amino acid sequences of the seven ferredoxins pertinent to this work. The amino acid sequence of (1) *Spirulina platensis* ferredoxin II (Wada et al., 1975; Tanaka et al., 1976) is listed along with those of (2) *Anabaena variabilis* ferredoxin II (determined in the present work), (4) *Spirulina maxima* ferredoxin II (Tanaka et al., 1975), (5) *Phytolacca americana* ferredoxin I (Wakabayashi et al., 1978), (6) *Spinacia oleracea* (spinach) ferredoxin (Matsubara & Sasaki, 1968), and (7) *Leucaena glauca* (koa) ferredoxin (Benson & Yasunobu, 1969). The numbering system adopted is from Hase and co-workers (1978). *Anacystis nidulans* ferredoxin II (3) is being sequenced by S. Buckel and M. A. Hermodson; only the positions of the aromatic residues are shown.

Table II: Assignments of ^{1}H NMR Peaks to the Aromatic Residues of Ferredoxins

	chemical shifts (ppm from DSS)					
	Tyr^3	Tyr^{35}	Tyr^{76}[a]	Phe^{76}[a]	Tyr^{83}	Tyr^{99}
A. variabilis: ox		6.31, 6.77	6.74, 7.30		6.41, 7.11	6.76, 7.11
red		6.32, 6.78	6.77, 7.35		6.44, 7.13	6.75, 7.08
A. nidulans: ox	6.64, 6.92			7.18, 7.28, 7.42	6.41, 7.08	6.77, 7.13
red	6.63, 6.91			7.24, 7.30, 7.47	6.49, 7.09	6.79, 7.10
S. maxima: ox	6.64, 6.97		6.80, 7.26		6.37, 7.06	6.76, 7.12
red	6.64, 6.97		6.84, 7.32		6.42, 7.08	6.78, 7.14
P. americana I: ox	6.76, 6.99					
spinach: ox	6.78, 6.92				6.32, 6.92	
koa: ox					6.33, 6.99	

[a] Residue 76 is phenylalanine in *A. nidulans* ferredoxin and tyrosine in all the other ferredoxins studied (see Figure 2).

2. Arg^{42} and His^{93} were not observed in the sequence analysis, but Arg^{42} was placed by the specificity of the tryptic digestion, and His^{93} was placed by homology (Hase et al., 1978) and the presence of two histidine residues per molecule of protein. The sequence determined is identical with that of *Nostoc muscorum* ferredoxin I (Hase et al., 1976). The TNBS-modified ferredoxin was denatured, reduced, and alkylated as above. The N-terminal sequence was determined to be the same as that of the unmodified protein to residue 20, except that no PTH-amino acid was found in cycle 15. The *A. variabilis* sequence is compared with those of the other ferredoxins studied (Figure 2): *Spirulina maxima* ferredoxin II, *Phytolacca americana* ferredoxin I, spinach, and koa ferredoxins. The sequence of *Anacystis nidulans* ferredoxin II is being determined by S. Buckel and M. Hermodson (unpublished results); for the purpose of this paper, only the positions of its aromatic residues are indicated in Figure 2. The sequence of *Spirulina platensis* ferredoxin is listed as a reference; no NMR studies of it were undertaken, but its X-ray structure has been determined (Fukuyama et al., 1980; Tsukihara et al., 1981) and will be used as a model in discussions of the other 2Fe·2S* ferredoxins.

NMR Spectra. The aromatic region of the ^{1}H NMR spectra of six oxidized ferredoxins (*A. variabilis*, *A. nidulans*, *S. maxima*, *P. americana*, spinach, and koa) are shown in Figure 3. The assignments are summarized in Table II.

Carboxypeptidase A Modification. The aromatic region of the ^{1}H NMR spectrum of *A. variabilis* Fd modified by carboxypeptidase A is shown (Figure 4b) along with that of the unmodified ferredoxin (Figure 4a). To facilitate comparison, the chemical shifts of the resonances for both proteins are listed in Table III. The coupled doublets around 6.8 and 7.1 ppm are not present in spectra of the modified protein and are thus assigned to Tyr^{99}.

Table III: Chemical Shifts of the Tyrosine and Histidine Resonances of Native and Modified *Anabaena variabilis* Ferredoxins[a]

residue	native ferredoxin	ferredoxin-Tyr^{99}[b]	TNP-ferredoxin[c]
Tyr^{35}	6.77, 6.31	6.75, 6.36	6.79, 6.28
Tyr^{76}	7.30, 6.74	7.32, 6.82	7.30, 6.74
Tyr^{83}	7.11, 6.41	7.12, 6.41	7.11, 6.40
Tyr^{99}	7.11, 6.76	*d*	7.11, 6.76
His^{16}	8.11, 6.94[e]	8.12, 6.92	*f*
His^{93}	7.78, 7.16[e]	7.89, 7.14	*f*

[a] Chemical shifts are reported in ppm from DSS; the pH* of the sample solutions was 7.26, and the temperature was 20 ± 2 °C. [b] Ferredoxin modified by treatment with carboxypeptidase A. [c] Ferredoxin modified by reaction with trinitrobenzenesulfonic acid. [d] Resonances were not observed near those assigned to this residue in native ferredoxin. [e] Chemical shifts obtained from ^{1}H NMR pH titration curves (Chan & Markley, 1983a). [f] See Figure 6 for the effect of this modification on the histidine residues.

TNBS-Modified Ferredoxin. The aromatic region of the spectrum of *A. variabilis* Fd is shown (Figure 5a) along with the spectrum of the TNBS derivative (Figure 5b) and the

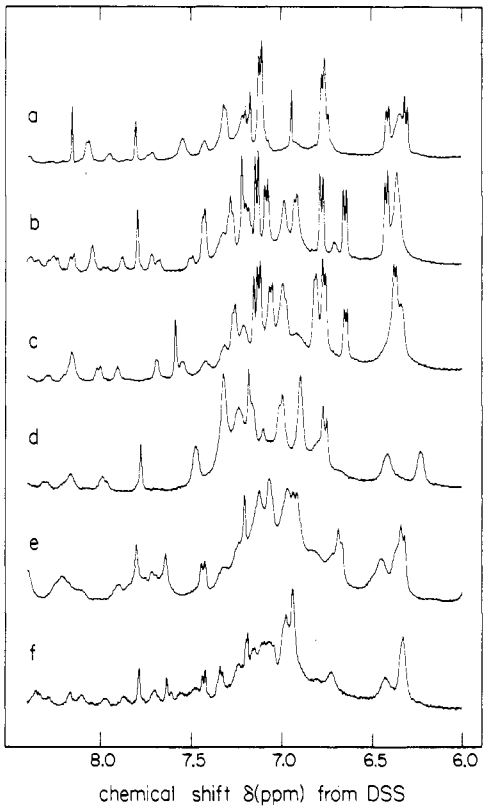

FIGURE 3: Aromatic region of ^1H NMR spectra of the oxidized forms of the six ferredoxins studied: (a) *Anabaena variabilis* ferredoxin II, 3.3 mM, pH* 7.22, 256 repetitions, at 470 MHz; (b) *Anacystis nidulans* ferredoxin II, 3.3 mM, pH* 7.20, 256 repetitions, at 470 MHz; (c) *Spirulina maxima* ferredoxin II, 3.3 mM, pH* 7.17, 256 repetitions, at 470 MHz; (d) *Phytolacca americana* ferredoxin I, 1.5 mM, pH* 7.33, 1400 repetitions, at 360 MHz; (e) spinach ferredoxin, 3.3 mM, pH* 7.25, 512 repetitions, at 360 MHz; (f) koa ferredoxin, 3.3 mM, pH* 7.70, 512 repetitions, at 470 MHz.

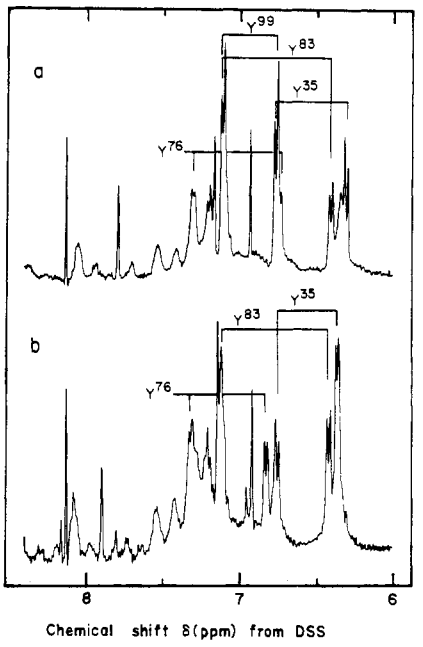

FIGURE 4: Experimental basis for the assignment of the tyrosine-99 peaks in the ^1H NMR spectrum of oxidized *A. variabilis* ferredoxin: (a) native, oxidized *Anabaena variabilis* ferredoxin II, 3.3 mM in 0.05 M deuterated phosphate buffer, pH* 7.19, 20 °C; (b) carboxypeptidase A modified, oxidized *A. variabilis* ferredoxin II from which the C-terminal tyrosine-99 has been removed, about 1.5 mM in 0.05 M deuterated phosphate buffer, pH* 7.26, 19 °C. Both spectra were obtained at 360 MHz and represent the sum of 256 repetitions; only the aromatic region is shown. Because of scaling differences, spectrum b is reproduced at a higher gain than spectrum a. Decoupling and COSY 2D FT experiments showed that the peaks near δ 6.8 in (a) contain three doublets. On removal of Tyr^{99} by limited proteolysis, the His_δ^{93}-H peak moves upfield into the position of the Tyr^{83} doublet leading to increased intensity at δ = 7.1 in (b).

(native-modified) difference spectrum (Figure 5c). The chemical shifts of the resonances due to the four resolved tyrosine residues are listed in Table III. The pH* dependence of the chemical shifts of the histidine resonances of the modified protein is shown in Figure 6.

Comparison of Oxidized and Reduced Ferredoxins. Two-dimensional Fourier transform homonuclear (^1H, ^1H) chemical shift correlated spectra were obtained of both oxidized and reduced *A. variabilis* Fd. The aromatic regions of these COSY spectra are shown in Figure 7A along with the corresponding one-dimensional spectra. Similar spectra were obtained of oxidized and reduced *A. nidulans* Fd (Figure 7B) and *S. maxima* Fd (Figure 7C).

Discussion

^1H NMR spectra of the oxidized forms of the three cyanobacterial ferredoxins studied (*A. variabilis*, *A. nidulans*, and *S. maxima*) are well resolved (aromatic spectral regions are shown in Figure 3a–c). Assignments of these resonances will be discussed below. Spectra of *Phytolacca americana* (I), spinach, and koa ferredoxins (Figure 3d–f) are not as well resolved; therefore, fewer assignments have been made in their spectra. The spectra of higher plant ferredoxins are used here only to help assign resonances in spectra of the cyanobacterial ferredoxins by sequence homology. The poorer quality of the spectra of the higher plant ferredoxins may stem from thermal instability of these proteins. Denaturation of the protein may release paramagnetic Fe(III) into the solution, which would broaden the NMR peaks. Spectra of the reduced forms of the three cyanobacterial ferredoxins are well resolved (Figure 7), but well-resolved spectra were not obtained of the reduced ferredoxins from the other three species.

Assignment of Tyrosine-83 Peaks. Coupled doublets around 6.4 and 7.0 ppm were observed for all ferredoxins studied except *P. americana* (Table II). Comparison of these results with the amino acid sequences of the ferredoxins (Figure 2) leads to the assignment of these peaks to Tyr^{83}. Residue 83 is tyrosine in all the ferredoxins studied except *P. americana* in which it is a phenylalanine. This assignment is corroborated by $Cr(NH_3)_6^{3+}$ binding experiments (Chan et al., 1983).

Assignment of Tyrosine-99 Peaks. The ^1H NMR spectrum of carboxypeptidase A modified *A. variabilis* Fd in which the C-terminal Tyr^{99} has been removed is well resolved (Figure 4b). The coupled doublets at 6.76 and 7.11 ppm present in the spectrum of the unmodified protein (Figure 4a) are absent, but the other tyrosine peaks appear largely unaffected. Therefore, the missing doublets are assigned to Tyr^{99}.

S. maxima Fd modified in a similar manner is not thermally stable, and the spectrum obtained (not shown) was not as well resolved. the coupled doublets around 6.8 and 7.1 ppm were not present in the spectrum of the modified protein, which is consistent with their assignment to Tyr^{99}. The higher plant ferredoxins that do not have a C-terminal tyrosine do not exhibit coupled doublets in these positions (Figure 3; Table II).

Assignment of Tyrosine-76 Peaks. Residue 76 is one of the more variable positions. It is a tyrosine in *A. variabilis* and *S. maxima* ferredoxins, a phenylalanine in *A. nidulans* and *P. americana* ferredoxins, and a tryptophan in spinach and koa ferredoxins (Figure 2). Oxidized *A. variabilis* Fd and *S. maxima* Fd exhibit coupled doublets at (6.74, 7.30 ppm) and

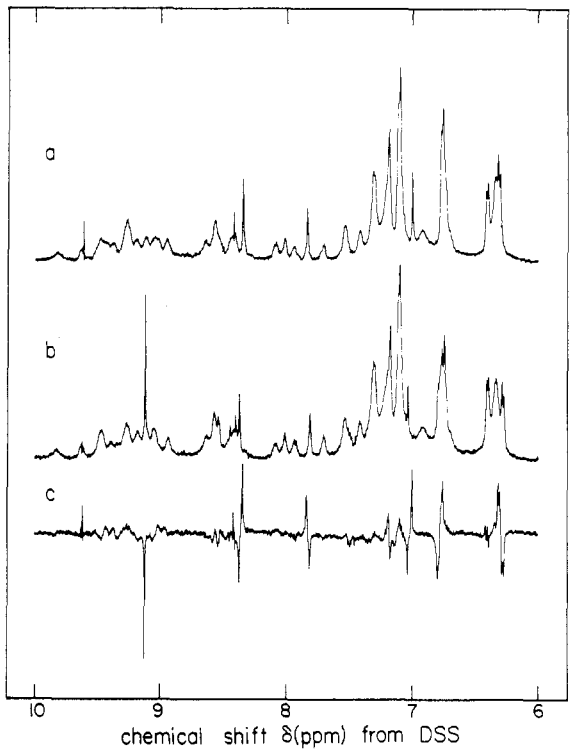

FIGURE 5: Aromatic region of the ^1H NMR spectrum (470 MHz) of (a) oxidized, native *Anabaena variabilis* ferredoxin II, (b) the oxidized form of the ferredoxin modified at lysine-15 with trinitrobenzenesulfonic acid, and (c) the difference spectrum: (a) − (b). The concentration of the native ferredoxin was 3.3 mM in 0.05 M deuterated phosphate buffer, pH* 6.80 at 22 °C, and the spectrum was the result of 256 repetitions. The exact concentration of the modified ferredoxin was not known; it was estimated to be about 2 mM, pH* 6.82 at 22 °C. The spectrum was the result of 1000 repetitions.

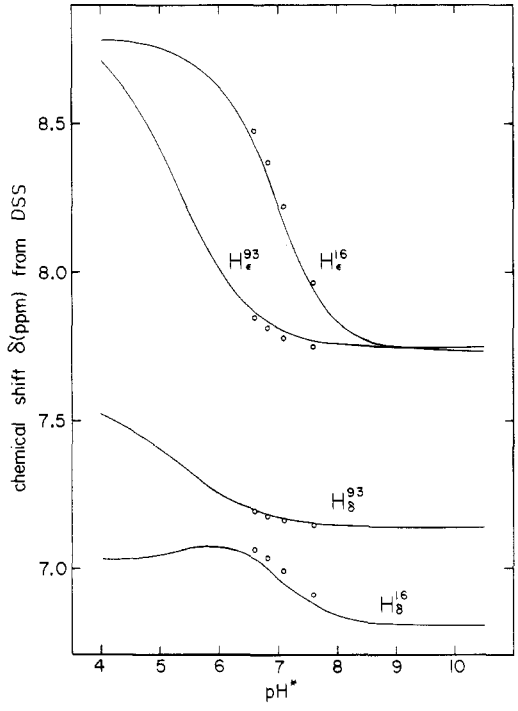

FIGURE 6: pH* dependence of the chemical shifts of the histidine resonances of *Anabaena variabilis* ferredoxin modified at lysine-15 with trinitrobenzenesulfonic acid. The circles are the chemical shifts of the histidine resonances of the modified ferredoxin at 4 pH* values. The solid curves are the titration curves of the histidine resonances of the native ferredoxin at 22 °C (Chan & Markley, 1983a).

(6.80, 7.26 ppm), respectively. Upon reduction of the proteins, these doublets shift slightly downfield to (6.77, 7.35 ppm) and (6.84, 7.32 ppm), respectively. This pair of doublets is absent in the spectrum of oxidized *A. nidulans* Fd, and a doublet–triplet–triplet system (at 7.42, 7.28, and 7.18 ppm) is present instead. This partial spin system shifts slightly upon reduction of the protein (to 7.47, 7.30, and 7.24 ppm). A similar spin system appears to be present in the spectrum of oxidized *P. americana* Fd I (Figure 3d). Spinach and koa ferredoxins show no coupled doublets at 6.8 and 7.3 ppm. By homology, the doublets at these positions in *A. variabilis* and *S. maxima* ferredoxins are assigned to Tyr^{76}. The doublet–triplet–triplet system in the spectrum of *A. nidulans* Fd is assigned to Phe^{76}. No attempt has been made to assign the resonances of Trp^{76} in spinach and koa ferredoxins because the spectra are not very well resolved.

Assignment of Tyrosine-3 Peaks. Tyr^3 is assigned by comparing the spectra of koa and spinach ferredoxins (Figure 3). The amino acid sequences of the two proteins only have one difference in the aromatic residues (Figure 2): position 3 is occupied by phenylalanine in koa ferredoxin, while it is a tyrosine in spinach ferredoxin. Oxidized spinach ferredoxin (Figure 3e) shows coupled doublets at 6.68 and 6.92 ppm, but these resonances are not present in the spectrum of koa ferredoxin (Figure 3f). Therefore, they are assigned to Tyr^3. Coupled doublets in similar chemical shift positions in the spectra of *P. americana* Fd I, *A. nidulans* Fd, and *S. maxima* Fd are assigned to Tyr^3 by homology. The reduced forms of the latter two proteins have been studied by ^1H NMR; the chemical shifts of the resonances assigned to Tyr^3 are not affected by reduction of the proteins. Tyr^3 is located far from the 2Fe·2S* center in the X-ray structure (Figure 8). The assignment is also strengthened by the absence of corresponding doublets in the spectrum of *A. variabilis* Fd (Figure 3a), in which phenylalanine replaces tyrosine in position 3.

Assignment of Tyrosine-35 Peaks. A pair of doublets at 6.31 and 6.77 ppm in the spectrum of oxidized *A. variabilis* ferredoxin (Figure 3a) is not present in spectra of any other ferredoxin studied. These doublets are assigned to Tyr^{35} which is the only tyrosine present in *A. variabilis* ferredoxin that is not present in any of the other ferredoxins studied (Figure 2).

Effect of Removing Tyrosine-99. In the spectrum of carboxypeptidase A modified *A. variabilis* ferredoxin (Figure 4b), besides the obvious absence of the resonances due to Tyr^{99} (doublets at 6.76 and 7.11 ppm), the resonances of Tyr^{35}, Tyr^{76}, and His^{93} are shifted. The resonances of Tyr^{83} and His^{16} are not affected (Table III). In the X-ray structure (Figure 8), Tyr^{76} is close to Tyr^{99}, which may explain the change in chemical shift observed for the resonances assigned to Tyr^{76} when Tyr^{99} is removed. The changes for His^{93} and Tyr^{35} must be explained by a disruption of the backbone structure of the protein upon removal of the C-terminal residue(s). The resonance at 7.80 ppm is probably due to the C_ϵ-H of His^{93} of the minor ferredoxin species (35%) lacking both Leu^{98} and Tyr^{99}. A minor set of resonances located downfield (at 8.16 and 6.95 ppm) from the major peaks of His^{16} corresponds to His^{16} in the same minor ferredoxin species. This result indicates that a further change of the backbone structure of the protein occurs when Leu^{98} is removed.

Carboxypeptidase A modified *S. maxima* Fd shows less thermal stability than the native protein or carboxypeptidase A modified *A. variabilis* Fd. Since Leu^{98} was removed from 88% of the protein molecules after 1 h of reaction, more disrupture of the tertiary structure is expected for the modified *S. maxima* Fd, which may explain part of the difference in stability. Native higher plant ferredoxins, which do not have a tyrosine at the C-terminus, show intrinsic thermal instability

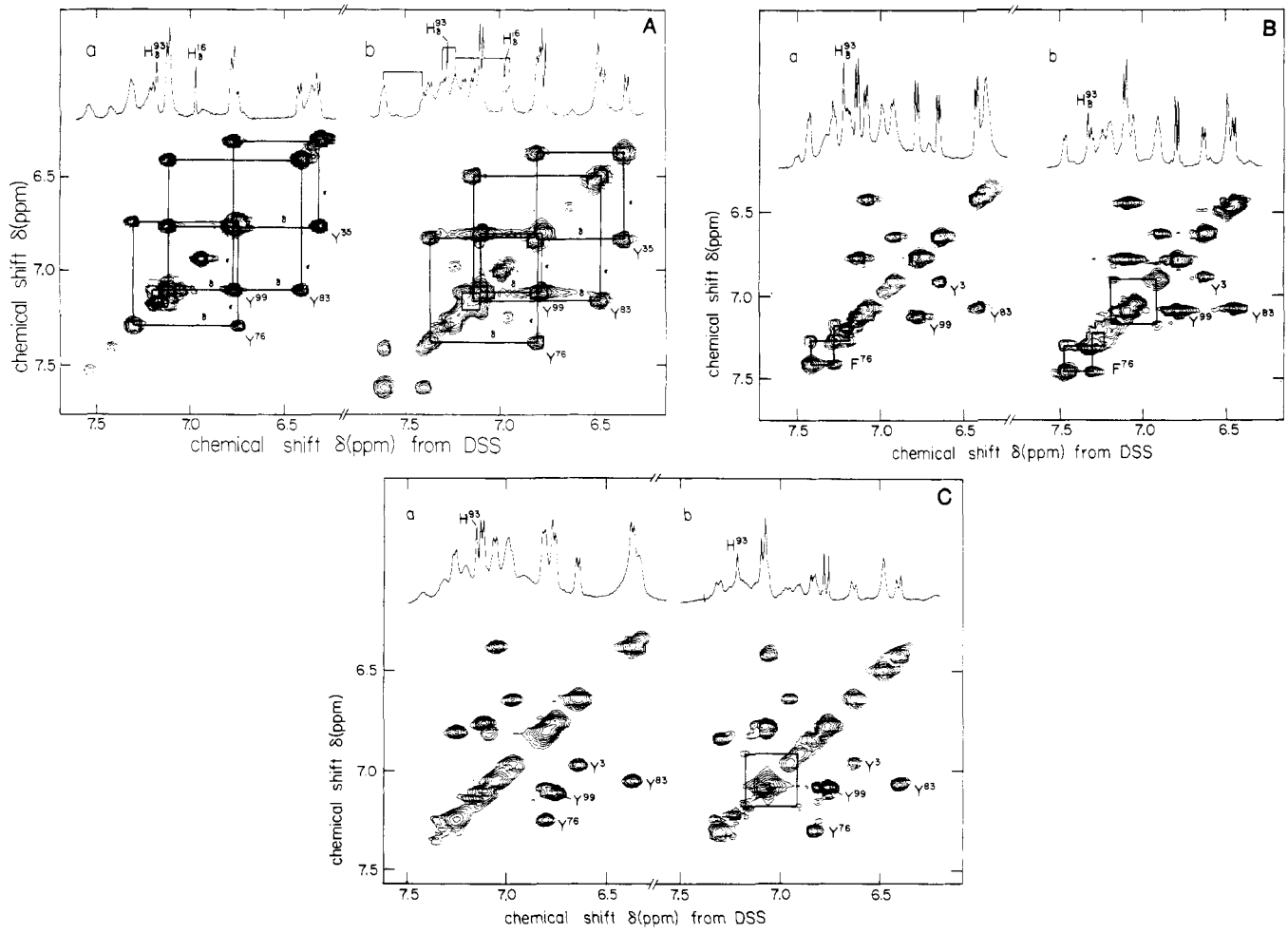

FIGURE 7: Contour plots (aromatic region) of two-dimensional Fourier transform (^1H, ^1H) chemical shift correlated (COSY) spectra of ferredoxins. (A) (a) Oxidized and (b) reduced *A. variabilis* ferredoxin II. The ferredoxin concentration in both experiments was 6.5 mM in 0.05 M deuterated phosphate buffer. The data were collected with the NT-470 spectrometer (470 MHz) at 22 °C. The pH* of the sample of oxidized ferredoxin was 7.06, and the pH* of the reduced ferredoxin was 6.95. For the spectrum of the oxidized protein, 512 sets of FIDs, each of 2K data points, were taken; for the reduced protein, 512 sets of 1K data points were taken. Each FID was the sum of 32 accumulations. The corresponding one-dimensional spectra are plotted for comparison. Cross peaks for tyrosine-35, -76, -83, and -99 and another pair of coupled resonances are connected to demonstrate their similar positions in the spectra of the oxidized and reduced protein. Additional cross peaks appear in the spectrum of the reduced protein. They are not connected in the two-dimensional spectrum for clarity of display, but the corresponding coupled resonances in the one-dimensional spectrum are connected. The Roman letters indicate amino acid assignments in the one-letter code, the Arabic numbers refer to residue assignments, and the Greek letters specify assignments to particular C-H groups within the residue as verified by heteronuclear (^{13}C, ^1H) two-dimensional Fourier transform chemical shift correlation spectroscopy (Chan & Markley, 1982). (B) (a) Oxidized and (b) reduced *Anacystis nidulans* ferredoxin II. The ferredoxin concentration was 6.5 mM in 0.05 M deuterated phosphate buffer. The data were collected with the NT-470 spectrometer at 470 MHz and 22 °C. The pH* was 7.26 for the oxidized protein sample and 7.25 for the reduced protein. In both cases, 512 FIDs each of 2K data points were taken. Each FID was the sum of 32 repetitions. The off-diagonal cross peaks for tyrosine-3, -83, and -99 are not connected. Those of phenylalanine-76 are connected to demonstrate the pattern. The additional pair of cross peaks in the spectrum of the reduced ferredoxin is also connected. (C) (a) Oxidized and (b) reduced *Spirulina maxima* ferredoxin II. The ferredoxin concentration was 6.5 mM in 0.05 M deuterated phosphate buffer. The data were collected with the NT-470 spectrometer at 470 MHz and 22 °C. The pH* was 7.26 for the oxidized protein sample and 7.24 for the reduced protein. The reduced ferredoxin sample became partially oxidized during the course of the experiment; hence extra cross peaks due to the oxidized protein appear in the spectrum. For both spectra, 512 sets of FIDs were collected, each of 32 repetitions and 2K data points.

(Hasumi et al., 1979). These observations strongly suggest that the C-terminal tyrosine is important for the stability of cyanobacterial ferredoxins. The relatively bulky side chain of tyrosine exerts a strong influence on the packing of atoms around it (Gelin & Karplus, 1975). Removal of additional residues from the carboxyl terminus further destabilizes the protein. It has been observed that wheat ferredoxin (which does not have a tyrosine at the C-terminus) modified by reaction with carboxypeptidase A is less stable than the native protein (Yokoyama et al., 1978). The packing of atoms in this part of the molecule apparently is very important in maintaining the integrity of the protein.

Effect of Chemical Modification of Lysine-15. TNBS can react with either the ϵ-NH_2 group of lysine residues or the terminal α-NH_2 of the protein (Glazer et al., 1975). In the aromatic region of the ^1H NMR spectrum of TNBS-modified *A. variabilis* Fd (Figure 5b), the resonance at 9.12 ppm can be assigned to the two protons on the trinitrophenyl group introduced into the protein. It is not possible to measure the exact intensity of the peak, since it overlaps with an N-H resonance. However, the peak has about twice the intensity of the resonance at 7.8 ppm, which has been assigned to the C_ϵ-H of His^{93}. This implies that this modified protein sample is a mono-TNP derivative. The NMR spectrum is also indicative of a single species, since none of the peaks in the aromatic region is doubled. As described in the experimental section, amino acid sequencing showed that the site of modification is Lys^{15}.

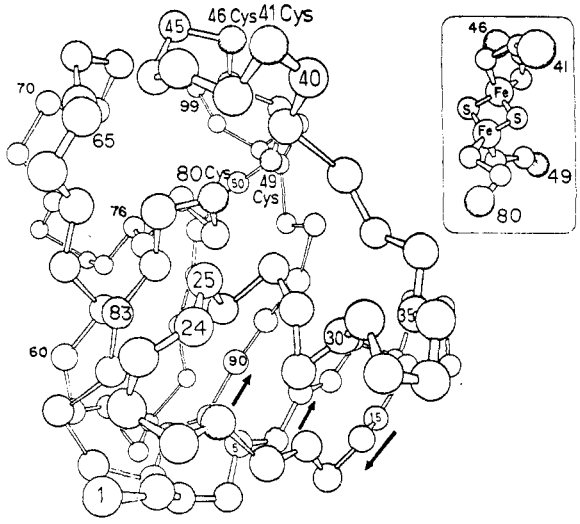

FIGURE 8: Amino acid backbone structure of a plant-type ferredoxin. The drawing adapted from Fukuyama et al. (1980) is of *Spirulina platensis* ferredoxin based on the X-ray crystallographic structure. The numbering system is that used in Figure 2. The circles represent the α-carbon positions. The 2Fe·2S* cluster, which is omitted in the diagram of the protein for clarity, is drawn in the box. Residues 41, 46, 49, and 80 are the cysteine ligands to the 2Fe·2S* cluster.

^{1}H NMR spectra of the modified protein were taken at four pH* values. The resonances of His^{93} are shifted upfield, while those of His^{16} are shifted downfield. Resonances due to other aromatic residues are not affected except those of Tyr^{35}. These effects can be seen best in the difference spectrum (Figure 5c). Only the resonances of Tyr^{35}, His^{16}, and His^{93} are shifted. The site of modification, Lys^{15}, is next to His^{16} and close to Tyr^{35} in the X-ray structure (Tsukihara et al., 1981; Figure 8). The region around Tyr^{35} has several glutamic acid residues (at positions 10, 12, 31, 32, and 33) and glycine residues (at positions 13 and 34). The glycines should give flexibility to the region. The removal of a positive charge on Lys^{15} by this modification alters the electrostatic interaction between the negative charges and appears to change the local environment of this region of the molecule. His^{93} is farther from position 15, and an indirect mechanism appears required to explain the change in its chemical shift.

Masaki and co-workers (1977) modified four amino groups on spinach ferredoxin by acetic anhydride. At low ionic strength, the modified ferredoxin was very unstable; its inactivity in the FNR–diaphorase assay system was attributed to its inability to bind FNR. In the presence of high salt (1 M Na^+ or Mg^{2+}), the acetylated ferredoxin was active in the assay system, and its absorption spectrum, CD spectrum, and the fluorescence of the sole tryptophan residue were identical with those of the native ferredoxin. Masaki et al. (1977) suggested that these positively charged amino groups are important in minimizing the repulsion between the negatively charged groups on the protein and maintaining the protein conformation. The specific and well-defined modification at Lys^{15}, which alters the charge distribution, may be useful in studying the interaction between ferredoxin and FNR.

Comparison of Oxidized and Reduced Cyanobacterial Ferredoxins. Spectra (aromatic region) of oxidized and reduced *A. variabilis* Fd are shown in Figure 7A. The eight resolved doublets in the oxidized protein (Figure 7A, part a) were assigned to four tyrosine residues by decoupling experiments or by reference to the COSY spectrum [6.31, 6.77 (Tyr^{35}); 6.74, 7.30 (Tyr^{76}); 6.41, 7.11 (Tyr^{83}); 6.76, 7.11 ppm (Tyr^{99})]. Eight doublets are also observed in the same general positions in the spectrum of the reduced protein shown in Figure 7A (part b) (6.32, 6.78; 6.77, 7.35; 6.44, 7.13; and 6.75, 7.08 ppm). The protein contains a fifth tyrosine (Tyr^{25}) in addition to those whose ^{1}H NMR peaks have been resolved and assigned. Apparently Tyr^{25} has abnormal chemical shifts or resonances that are too broad to be resolved. This may be because of its proximity to the paramagnetic 2Fe·2S* center or because of restricted rotation of the aromatic ring about the C_β–C_γ bond. From the coordinates obtained from the 2.5-Å resolution X-ray structure of *S. platensis* Fd (Tsukihara et al., 1981), Tyr^{25} is about 9 Å from the 2Fe·2S* center, while the other four tyrosines are farther away. These distances suggest that hyperfine effects are responsible for the abnormal positions or widths of the Tyr^{25} resonances. The ^{1}H NMR results indicate that Tyr^{35}, Tyr^{76}, Tyr^{83}, and Tyr^{99} are affected very little by reduction of the protein as can be seen from the COSY spectra (Figure 7A) and that the assignments made for the oxidized protein can be carried over to the reduced protein (Table II). The other pair of coupled resonances (7.11, 7.18 ppm) not affected by reduction is assigned to Phe^3 by the same reasoning. In the COSY spectrum of the reduced ferredoxin (Figure 7A, part b), additional coupled resonances, which are not present in the spectrum of the oxidized protein, appear at (6.94, 7.22), (722, 7.28), and (7.39, 7.60) ppm. These may correspond to Phe^{39} and Tyr^{25}.

From measurements of T_1 values of several single-carbon resonances in ^{13}C NMR spectra, the electron spin relaxation time, T_e, of the paramagnetic 2Fe·2S* cluster was found to be shorter in the reduced protein than in the oxidized protein (Chan & Markley, 1983b). The shorter T_e implies that resonances of nuclei that are near the paramagnetic 2Fe·2S* center will be broadened to a smaller extent in the case of the reduced protein than in the oxidized protein. This may be the reason why more resonances can be observed in the aromatic region of the ^{1}H NMR spectrum of reduced ferredoxin than of oxidized ferredoxin. Another possible explanation is that local conformational changes around these aromatic residues upon reduction of the protein increase the flipping rates of the aromatic rings. Although our present data cannot rule out this possibility, the former explanation appears to be more straightforward and more probable.

Spectra of oxidized and reduced *A. nidulans* Fd are shown in Figure 7B. Six doublets in the spectrum of the oxidized ferredoxin (Figure 7B, part a) can be assigned to three tyrosines by simple spin decoupling experiments or by reference to the COSY spectrum [6.64, 6.92 (Tyr^3); 6.41, 7.08 (Tyr^{83}); 6.77, 7.13 ppm (Tyr^{99})]. Since the protein contains five tyrosines, resonances from two have not been resolved. Three pairs of coupled doublets are also observed in the same general positions (6.63, 6.91; 6.49, 7.09; 6.79, 7.10 ppm) in the spectrum of the reduced protein (Figure 7B, part b). It seems reasonable that these would correspond to the two tyrosine residues closest to the iron-sulfur center, Tyr^{25} ($\sim$9 Å) and Tyr^{39} ($\sim$10 Å). A doublet–triplet–triplet system, which is not affected by reduction of the protein, has been assigned to Phe^{76}. An extra pair of coupled resonances (6.91 and 7.20 ppm) present only in the spectrum of the reduced protein may be assigned to either Tyr^{25} and Tyr^{39}. Tyr^{25} appears to be the more likely assignment because resonances in the same positions are observed in reduced *A. variabilis* Fd that has a Phe^{39}.

The eight resolved doublets in the one-dimensional ^{1}H NMR spectrum (Figure 7C, part a) of oxidized *S. maxima* ferredoxin are assigned to four tyrosines by simple decoupling experiments [6.64, 6.97 (Tyr^3); 6.80, 7.26 (Tyr^{76}); 6.37, 7.06 (Tyr^{83}); 6.76, 7.12 ppm (Tyr^{99})]. Two of the six tyrosines of the protein, Tyr^{25} and Tyr^{39}, apparently are not observed. Four pairs of

doublets are also observed in the same general positions (Figure 7C, part b) for the reduced proteins (6.64, 6.97; 6.84, 7.32; 6.42, 7.08; 6.78, 7.14 ppm). In the COSY spectrum of the oxidized protein, the assigned coupling patterns of the doublet pairs are confirmed. The additional doublet cross peaks at 6.8 and 7.1 ppm are broader than the other eight doublets leading to less intense contour peaks.

Attempts to obtain a COSY spectrum of the reduced protein were less successful. The sample became partially oxidized in the course of the experiments; hence, there are extra cross peaks due to the oxidized protein in the spectrum (Figure 7C, part b). However, comparison of the COSY spectra can proceed with consideration of this fact. The pair of cross peaks at 6.8 and 7.15 ppm can be assigned to the same residue that gives rise to the pair of resonances at similar positions in the oxidized protein. Another pair of coupled resonances at 6.9 and 7.18 ppm occupies positions similar to the extra peaks observed in the spectra of reduced *A. variabilis* and *A. nidulans* ferredoxins and probably corresponds to Tyr^{25}. However, assignment of resonances to residues close to the paramagnetic 2Fe·2S* center by homology arguments may be risky since a subtle difference in conformation may give rise to a large change in the line widths and/or chemical shifts of the NMR resonances.

Acknowledgments

We thank Dr. H. Matsubara, Osaka University, Japan, for the gift of *P. americana* ferredoxin I, Dr. D. W. Krogmann, Purdue University, for the *S. maxima* cell paste and *A. variabilis* cell culture, Dr. K. T. Yasunobu, University of Hawaii, Manoa, for the koa leaves, Dr. M. Ludwig, University of Michigan, Ann Arbor, for the crude *A. nidulans* ferredoxin, and Drs. R. E. Santini and W. M. Westler, Purdue University, for assistance with NMR instrumentation and helpful discussions.

Registry No. Tyrosine, 60-18-4.

References

Bax, A., & Freeman, R. (1981) *J. Magn. Reson. 44*, 542–561.

Benson, A. M., & Yasunobu, K. T. (1969) *J. Biol. Chem. 244*, 955–963.

Campbell, I. D., Dobson, C. M., Williams, R. J. P., & Wright, P. E. (1975) *FEBS Lett. 57*, 96–99.

Chan, T.-M. (1982) Ph.D. Thesis, Purdue University.

Chan, T.-M., & Markley, J. L. (1982) *J. Am. Chem. Soc. 104*, 4010–4011.

Chan, T.-M., & Markley, J. L. (1983a) *Biochemistry* (first paper of five in this issue).

Chan, T.-M., & Markley, J. L. (1983b) *Biochemistry* (third paper of five in this issue).

Chan, T.-M., Ulrich, E. L., & Markley, J. L. (1983) *Biochemistry* (fourth paper of five in this issue).

Ellefson, W. L. (1978) Ph.D. Thesis, Purdue University.

Ellefson, W. L., & Krogmann, D. W. (1979) *Arch. Biochem. Biophys. 194*, 593–599.

Fukuyama, K., Hase, T., Matsumoto, S., Tsukihara, T., Katsube, Y., Tanaka, N., Kakudo, M., Wada, K., & Matsubara, H. (1980) *Nature (London) 286*, 522–524.

Gelin, B. R., & Karplus, M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2002–2006.

Glazer, A. N., Delange, R. J., & Sigman, D. S. (1975) *Chemical Modification of Proteins*, p 76, Elsevier, New York.

Hase, T., Wada, K., Ohmiya, M., & Matsubara, H. (1976) *J. Biochem. (Tokyo) 80*, 993–999.

Hase, T., Wakabayaski, S., Matsubara, H., Roa, K. K., Hall, D. O., Widmer, H., Gysi, J., & Zuber, H. (1978) *Phytochemistry 17*, 1863–1867.

Hasumi, H., Nakamura, S., Koga, K., & Yoshizumi, H. (1979) *Biochem. Biophys. Res. Commun. 87*, 1095–1101.

Hogg, R. W., & Hermodson, M. A. (1977) *J. Biol. Chem. 252*, 5135–5141.

Jeener, J. (1971) Ampère International Summer School, Basko Polje, Yugoslavia.

Masaki, R., Wada, K., & Matsubara, H. (1977) *J. Biochem. (Tokyo) 81*, 1–9.

Matsubara, H., & Sasaki, R. M. (1968) *J. Biol. Chem. 243*, 1732–1757.

Nagayama, K., & Wüthrich, K. (1981) *Eur. J. Biochem. 114*, 365–374.

Nagayama, K., Kumar, A., Wüthrich, K., & Ernst, R. R. (1980) *J. Magn. Reson. 40*, 321–334.

Tanaka, M., Haniu, M., Yasunobu, K. T., Roa, K. K., & Hall, D. O. (1975) *Biochemistry 14*, 5535–5540.

Tanaka, M., Haniu, M., Yasunobu, K. T., Roa, K. K., & Hall, D. O. (1976) *Biochem. Biophys. Res. Commun. 69*, 759–765.

Tsukihara, T., Fukuyama, K., Nakmura, M., Katsube, Y., Tanaka, N., Kakudo, M., Wada, K., Hase, T., & Matsubara, H. (1981) *J. Biochem. (Tokyo) 90*, 1763–1773.

Wada, K., Hase, T., Tokunaga, H., & Matsubara, H. (1975) *FEBS Lett. 55*, 102–104.

Wakabayashi, S., Hase, T., Wada, K., Matsubara, H., Suzuki, K., & Takaichi, S. (1978) *J. Biochem. (Tokyo) 83*, 1305–1319

Yokoyama, Z.-I., Abe, A., & Shin, M. (1978) *Agric. Biol. Chem. 42*, 2169–2171.